Molecular Genetic Analysis of Chemical-Induced Sporulation of Myxococcus xanthus

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March 1994

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ACKNOWLEDGEMENTS

Firstly I would like to Dr. David A. Hodgson for his excellent supervision and wealth of information. I would also like to thank my laboratory colleagues for their help and support.

Secondly I would like to thank Dr. D. Kaiser's laboratory and Dr D.R. Zusman's laboratory for providing *M. xanthus* strains and plasmid clones.

Finally I would like to thank my family and friends for their patience and support over the last three years. Particular thanks should go to Howard, John, Tim and Martin.

DECLARATION

I declare that all the work reported in this thesis is my own. Where help or information was obtained from other sources, the source has been referenced. No work within this thesis has been submitted previously for any other degree.

SUMMARY

Mutants resistant to glycerol-induction of sporulation were isolated from wild-type *M. xanthus*. The glycerol-resistant *glrA* and *glrB* loci, previously mapped by Mx8 transductions, were analysed by restriction mapping of clones and by complementation analysis. The location of the *glrA* gene(s) was mapped to within a 2.2kb region, whilst the *glrB* region proved very complex. The *glrA* and *glrB* gene products were required early in chemical-induced sporulation since two chemical-inducible *lacZ* fusions were not expressed in either *glrA* or *glrB* mutants during chemical-induction.

Only a minority of the glycerol-resistant mutants were unable to undergo fruiting body sporulation. Complementation studies of the *glrA* and *glrB* regions confirmed that mutations in chemical-induced and fruiting body sporulation were not linked. This suggests that the induction pathways of chemical-induced sporulation and fruiting body sporulation share few common genes.

Glycerol-resistant mutants were isolated from a non-motile strain, which is unable to form fruiting bodies. The majority of these mutants were able to form spores in the absence of fruiting bodies. Two mutants were unable to form spores. Isolating such mutants may provide a means of identifying truly sporulation-deficient mutants.

Expression from the chemical-inducible *isgB*>*lacZ* fusion, identified previously in a promoter probe vector, was blocked in 24 different glycerol-resistant mutants. Hence, the gene product was required late in the chemical-induced sporulation pathway. The complete transcription unit was cloned and disruption of the region by the insertion of a tetracycline cassette demonstrated that the gene is not essential for chemical-induced or starvation-induced sporulation.

Expression from a second chemical-inducible *lacZ* fusion, ΩDK4530, identified previously by random *Tn5 lac* insertion, was suppressed by amino acids in the growth media during chemical-induced sporulation and was blocked in both *glrA* and *glrB* mutants. Hence, expression of the gene product is dependent on both the $glrA^+$ and $glrB^+$ genes.

ABBREVIATIONS

Ap^r = Ampicillin resistance determinant

Cm^r = Chloramphenicol resistance determinant

DMSO = Dimethylsulphoxide

DNA = Deoxyribonucleic acid

EMS = Ethyl Methyl Sulphonate

fd term. = major transcription terminator of coliphage *fd*

fis = uncharacterized mutation in starvation-induced, fruiting-independent sporulation

fru = uncharacterized mutation in fruiting body sporulation

Fru^- = unable to undergo fruiting body sporulation

Fru^+ = undergoes fruiting body sporulation normally

frz = gene involved in chemotaxis and the frequency of reversal of *M. xanthus* cells

glr = uncharacterized mutation in glycerol-induced sporulation which results in glycerol-resistant phenotype

glrA = gene involved in glycerol-induced sporulation i.e. mutation in this gene results in glycerol-resistant phenotype

glrB = gene involved in glycerol-induced sporulation i.e. mutation in this gene results in glycerol-resistant phenotype

Gly^r = Glycerol-Resistant i.e. not induced to sporulate by the addition of glycerol

Gly^s = Glycerol sensitive i.e. induced to sporulate by the addition of glycerol

isgB = glycerol-inducible *lacZ* fusion

Km^{r*} = Kanamycin resistance determinant derived from Tn*903*

Km^r = Kanamycin resistance determinant derived from Tn*5*

lacZ = promoterless *E. coli lacZ* gene

mgl = motility gene, a mutation in which blocks both adventurous and social motility systems

4-MUG = 4-Methyl umbelliferyl galactoside

Mx DNA = *M. xanthus* chromosomal DNA; the loci is indicated in parentheses.

NTG = N-Methyl-N'-Nitro-N-Nitrosoguanidine

ONP = ortho-nitrophenol

ONPG = ortho-nitrophenyl-ß-D-galactoside

P1*inc* = Coliphage P1 plasmid incompatibility region.

P1*inc*▲ = P1*inc* region with a deletion between the *Hpa*I and *Kpn*I sites, which removes a head gene.

PEA = Phenylethyl alcohol

PEG = Polyethylene glycol

RNA = Ribonucleic acid

sglA1 = partial motility block in social motility locus

Tc^r = Tetracycline resistance determinant.

UV = Ultraviolet

X-gal = 5-Bromo-4-chloro-3-indolyl-ß-D-galactoside

DEDICATION

I dedicate this thesis in memory of my grandfather John Edward Chatwin who passed away on the 17th November 1992.

Chapter 1

Introduction

1.1 The Myxobacteria

Myxobacteria are Gram-negative, pigmented bacteria which were first described by Thaxter (1892). They possess gliding motility and all are obligate aerobes. In nature they are usually found in soil, dung and other rotting organic material. They are found as either swarms of vegetative cells or as fruiting bodies filled with spores. The degree of cellular cooperation required to form fruiting bodies is remarkable for a prokaryote. The cooperative swarming of the vegetative cells is also remarkable, and increases their ability to digest complex organic substrates (Rosenberg et al., 1977). With the exception of one genus, Sorangium, which can digest cellulose, the myxobacteria digest proteins and cannot use sugars as a sole carbon and energy source (Reichenbach and Dworkin, 1981).

The myxobacteria seem to be a genuine taxonomic group based on 16S ribosomal RNA homology (Ludwig et al., 1983; Shimkets and Woese, 1992) and DNA homology (Johnson and Ordal, 1969). Their raised multicellular fruiting bodies are also unique amongst prokaryotes and they have similar DNA base ratios (G+C approximately 70%). A classification of myxobacteria is given in Table 1.1.

Myxobacterial cells are usually embedded in an extracellular matrix that is often referred to as slime. This extracellular matrix is a complex of proteins, polysaccharides and lipids. The principal monosaccharides contained in polysaccharide secreted during vegetative growth are mannose, D-glucose, D-

Order:	**Myxobacterales**
Suborder:	Cystobacterineae
Families and Genera:	Myxococcaceae
	Myxococcus
	Corallococcus
	Angiococcus
	Archangiaceae
	Archangium
	Cystobacteraceae
	Cystobacter
	Mellittangium
	Stigmatella
Suborder:	Soragineae
Families and Genera:	Sorangiaceae
	Sorangium
	Polyangium
	Haploangium
	Chondromyces
	Nannocystis

Table 1.1 Taxonomic Survey of the Myxobacteria. From Reichenbach and Dworkin. (1981).

galactose and hexosamines, and these are also present at approximately the same ratio in developmental exopolysaccharide (Sutherland and Thomson, 1975). While it has been tacitly assumed that the slime exists in an amorphous form, the recent discovery of fibrils (Arnold and Shimkets, 1988a,b; Behmlander and Dworkin, 1991), which consist largely of polysaccharide, suggests that this may not be the case.

1.2 Developmental Biology of Myxococcus xanthus

1.2.1 Description of M. xanthus

Myxococcus xanthus is the best studied myxobacterium. The vegetative cells are rod-shaped, up to 7μm long and 0.7μm wide. Under starvation conditions fruiting bodies are formed which are approximately 100μm in diameter. These fruiting bodies are filled with spores of 2μm diameter. These spores can withstand UV irradiation, desiccation and temperatures of up to 60^{o}C for at least twenty minutes (Sudo and Dworkin, 1969).

M. xanthus degrades and feeds on insoluble macromolecules in its environment and is bacteriolytic and mycolytic. A variety of extracellular enzymes are secreted such as proteases, amidases and glucamidases which enable them to break down the cell walls and proteins of the bacteria and fungi on which they feed. The breakage of the cell walls is enhanced by contact with M. xanthus or with its secreted slime layer which entraps secreted enzymes. Besides lytic enzymes, antibiotics and bacteriocins are also produced (Rosenberg

and Varon, 1984).

The cooperative swarming behaviour of M. xanthus is important for efficient proteolysis. For example, the growth rate of M. xanthus on casein increased with cell density in the range of 10^4 to 10^7 cells/ml. There was no growth at all at cell densities below 10^3 cell/ml. On hydrolysed casein (hydrolysed with the myxobacterial enzymes) the growth rate was independent of cell density (Rosenberg et al., 1977). Sugars cannot be used as a sole carbon source but can be co-metabolized with proteins (Bretscher and Kaiser, 1978).

M. xanthus moves by gliding motility, a form of translocation on a surface in which a rod progresses smoothly in the direction of its long axis without the aid of flagella (Henrichsen, 1972). Several mechanisms have been suggested for gliding motility including surface tension phenomena (Keller et al., 1983; Dworkin et al., 1983), directed slime secretion (Burchard, 1984) and cellular contractile waves.

The cell envelope of M. xanthus is typical of Gram-negative bacteria in that it consists of inner and outer membranes separated by a layer of peptidoglycan. Unlike the peptidoglycan of other bacteria, however, it exists not as a continuous layer but in patches connected by trypsin-SDS sensitive material (White et al., 1968). The patch-like, rather than continuous nature may be related to the flexibility and gliding motility of these cells and to the ability of these cells to convert to spherical myxospores.

The genome of M. xanthus has been measured by pulse-field gel electrophoresis of DNA digested with rare-cutting restriction endonucleases, and found to be 9,454kb (Chen et al., 1990). The organization of the genomic DNA was examined by hybridization of the sixteen AseI restriction fragments with a genomic library prepared in yeast artificial chromosomes (YAC) and SpeI digested genomic DNA (Chen et al., 1991). The results were consistent with M. xanthus containing a single circular chromosome.

1.2.2 Developmental Cycle of M. xanthus

1.2.2.1 Requirements for Development

Vegetative cells will only undergo development if three conditions prevail; (1) Starvation conditions, (2) the cells are on a solid surface where gliding can occur and (3) the cells are at a sufficiently high density. Starvation is a metabolic prerequisite for development. The cessation of growth for any other reason, such as the cells entering stationary phase, does not induce development. Starvation for any required amino acid (leucine, isoleucine, valine and phenylalanine) will initiate development. The link between nutrition and fruiting is not limited to amino acid metabolism, however, since starvation for carbon, energy and inorganic phosphate also induces fruiting. These starvation conditions were also found to increase the intracellular levels of guanosine tetraphosphate and guanosine pentaphosphate (Manoil and Kaiser, 1980a,b). A

role for these compounds in the initiation of fruiting is still unclear. However, in Bacillus subtilis the increase in guanosine tetraphosphate and guanosine pentaphosphate is accompanied by a decrease in the levels of guanosine triphosphate (GTP). It is this decrease in GTP which initiates sporulation events (Smith, 1989). A similar mechanism may operate in M. xanthus.

For coordinated development of fruiting bodies not only must the cells be able to respond to the changing environment, they must also be able to communicate with one another. Diffusible substances from fruiting bodies have been shown to cause development in vegetative cells. Fruiting bodies on one side of a permeable barrier induce vegetative cells on the other side of the barrier to form fruiting bodies and both sets of fruiting bodies are in alignment (Lev, 1954; McVittie and Zahler, 1962). Such diffusible molecules could be metabolic intermediates whose presence allows the bypass of the normal inhibition of development.

For sporulation to occur the cells must be above a critical density. The cells, therefore, must have a means of measuring their own cell density. There is some evidence that an adenine derivative is involved. If adenine or an adenine containing compound is added to cells at otherwise too low cell densities, aggregation and fruiting can occur (Shimkets and Dworkin, 1981). It is possible that these are non-specific effects brought about by the change in energy charge of the cell. However, other nucleotides do not have the same effect.

1.2.2.2 Chemotaxis and Directed Movement

The fruiting bodies of any one species of myxobacteria are characteristic of that species. The cells must, therefore, be directed towards a centre of aggregation in a controlled manner. Little is known of the mechanism of control of such directed movement. Chemotaxis has been suggested, however, attempts to demonstrate chemotaxis of myxobacteria and define the chemoeffectors have led to conflicting results (Lev, 1954; McVittie and Zahler, 1962; Ho and McCurdy, 1979; Shimkets et al., 1979; Dworkin and Eide, 1983). Observations of cells moving along artificial gradients or moving round objects could be explained as elasticotaxis, a process where cells follow lines of stress in the agar. More recently it has been demonstrated that M. xanthus does exhibit chemotaxis. Two-chambered petri dishes were employed to establish sharp chemical gradients and movement of M. xanthus cells towards yeast extract or casitone and away from dimethylsulphoxide or short chain alcohols was demonstrated (Shi et al., 1993). Positive and negative chemotactic movements were additive.

M. xanthus was also demonstrated to exploit the chemotactic behaviour of E. coli by releasing attractants. The E. coli cells respond rapidly to these attractants accumulating around the M. xanthus cells. The E. coli cells were later immobilized and digested by M. xanthus. E. coli mutants with known defects in

chemotaxis either no longer exhibited attraction to *M. xanthus* cells or showed a reduced attraction. Thus the chemotaxis signal-transduction machinery is required for the attraction behaviour. The attractants were detected in culture fluid after *M. xanthus* had been starved for three hours and increased gradually with longer periods of starvation. Whether the mechanism for the release of attractants is active or passive, *M. xanthus* has evolved a clever method to attract *E. coli* and probably other prey organisms to come to them during times of starvation (Shi and Zusman, 1993a).

The characteristic shape of fruiting bodies could be a product of the motility properties of the cells and the physical properties of the cells and their slime. Some mutants with altered motility produce aberrant fruiting bodies (Zusman, 1982; Blackhart and Zusman, 1985b). These *frz* mutants have an altered frequency of reversal of cell direction in comparison with the wild-type. The *frz* gene products have been sequenced (McBride *et al*., 1989; McCleary *et al*., 1990; McCleary and Zusman, 1990a) and shown to share significant homology with the chemotaxis genes of flagellated enteric bacteria. Enteric chemotaxis proteins become heavily methylated following binding of attractants and rapidly demethylated following binding of repellents (McBride *et al*., 1993). FrzCD is also reversibly methylated (McCleary *et al*., 1990). Yeast extract, casitone, lauric acid and lauryl alcohol resulted in increased methylation. In contrast several short chain alcohols and dimethylsulphoxide

caused demethylation of FrzCD (McBride et al., 1992). The chemotactic responses of M. xanthus towards yeast extract and casitone and away from dimethylsulphoxide or short chain alcohols were correlated with methylation and demethylation of FrzCD respectively (Shi et al., 1993). The frz mutants were found to be defective in chemotactic behaviour.

1.2.2.3 Developmental Lysis

Massive cell lysis has been reported to occur within fruiting bodies under a variety of nutrient conditions (Wireman and Dworkin, 1977). Lysis was thought to claim 80% of vegetative cells and appeared to accompany spore formation in the remaining 20% of cells. Lysis of some cells during fruiting could function to provide cells, destined to become spores, with a source of nutrients which enables them to complete the process of differentiation. Lysis may also release developmental signals. However, more recent data using improved techniques for harvesting and maintaining developing cells suggests that massive autolysis is not an obligate stage of development in M. xanthus (O'Connor and Zusman, 1988). They assessed the stability of developing cells in harvest buffers. Cells were removed from developing culture, suspended in several test buffers, and subsequently stored on ice. It was found that cell number decreased and the concentration of protein recovered in the test buffer increased over time. Including 1mM KH_2PO_4 and/or 10% Ficoll 400 in the

harvesting buffer decreased the amount of released protein and increased the final yield of cells obtained at various times during development. When cells were harvested into a buffer containing 1mM KH_2PO_4 and 10% Ficoll 400 up to 80% of cells which entered development survived through out fruiting body formation and the cell lysis that did occur appeared gradual. These results suggest both that developmental conditions can significantly alter the absolute level of developmental autolysis and that investigator-induced effects can alter the observed level of autolysis.

1.2.2.4 Peripheral Rods

When M. xanthus cells are starved some cells do not enter aggregates. Those cells which remain outside aggregates after aggregation has ceased in the rest of the population are described as peripheral rods. These cells are not vegetative cells as they express many developmental markers (O'Connor and Zusman, 1991a) and show a different pattern of protein expression from vegetative cells (O'Connor and Zusman, 1991b). Hence, peripheral rods should be considered a distinct, differentiated developmental cell type. Peripheral rods may play a significant role in the life cycle of M. xanthus by allowing the exploitation of low amounts of transient influxes of nutrients without the investment of energy in spore germination (O'Connor and Zusman, 1991c).

1.2.2.5 Fruiting Bodies

Fruiting bodies of M. xanthus consist of two domains; a hemispherical outer domain of densely packed and ordered cells and an inner domain of less ordered cells at three-fold lower cell density (Sager and Kaiser, 1993a). Single cells move in a bidirectional stream in the outer domain, orbiting the fruiting body throughout development, whereas in the inner domain cell movement ceases as the fruiting body matures. Eighty developmentally regulated transcriptional lacZ fusions were examined for expression restricted to one or other domain. Eight fusions were identified where expression was restricted to the inner domain (Sager and Kaiser, 1993b). One fusion, ΩDK7621, was identified which showed initial expression in the outer domain. This expression coincided with patches of spore precursors. Later in development, both ΩDK7621 expression and the patches expanded inward, eventually filling both the inner and outer domains. Hence, it is hypothesized that spore precursors are passively transported from the outer to the inner domain by the movements of undifferentiated rods (Sager and Kaiser, 1993b).

1.2.3 Spore Formation

During fruiting body formation spores are usually formed 40 to 50 hours after the onset of starvation. Commitment of the cells to sporulate does not occur until well after the initiation of development (Wireman, 1979). Cells can resume growth in nutrient (casitone) medium up

to 36 hours after the onset of starvation. After 36 hours the cells will form spores even when transferred to rich medium.

Considerable changes occur in the pattern of protein synthesis during fruiting body sporulation. At least 25% of proteins resolvable by polyacrylamide gel electrophoresis show significant changes in abundance during development (Inouye et al., 1979b). New mRNA species are also produced which have unusually long half lives (Nelson and Zusman, 1983a).

The most abundant protein during development is protein S which is first seen in the cytoplasmic fraction at about three to six hours after the initiation of development and increases dramatically to a maximum of 15% of total cell protein synthesis (Inouye et al., 1979a). It accumulates in the cytoplasm during aggregation and later forms the major spore coat protein (Inouye et al., 1979b, 1981; Nelson and Zusman, 1983b). Protein S is a 23 kDalton protein (Inouye et al., 1981) and appears to be translocated in the absence of a cleaved peptide signal (Nelson and Zusman, 1983b). Gene cloning and hybridization studies revealed the presence of two very similar genes separated by 1.4kb that could potentially encode protein S (Inouye et al., 1983a,b; Downard et al., 1984). Mutational and expression studies showed that the downstream gene, tps, is expressed early in development and encodes protein S, whereas the upstream gene, ops, is expressed much later, when spores form (Downard et al., 1984; Inouye, 1984; Komano et al.,

1984; Downard and Zusman, 1985; Teintze et al., 1985; Furuichi et al., 1985; Downard, 1987). Fruiting body formation was severely delayed when both the ops and the tps genes were deleted. However, this delay could be suppressed by either the ops or tps gene individually, although in the latter case a slight delay was still observed. Deletion of the 1.4kb spacer region had no effect on fruiting body sporulation.

Myxobacterial hemagglutinin (MBHA) also appears during the early stages of development (Cumsky and Zusman 1979, 1981a). This membrane-associated protein is a erythrocyte-lectin and makes up 1 to 2% of the total cell protein. MBHA appears to be localized at the cell poles, on the cell surface and in the periplasm, in developing cells and plays a subtle role in aggregation (Nelson et al., 1981; Romeo and Zusman, 1987). Strains containing null mutations in the mbhA gene are delayed in their development in the absence of exogenous Mg^{2+} (Romeo and Zusman, 1987). The gene for MBHA has been cloned and sequenced (Romeo et al., 1986). It contains four homologous domains and, hence, is physically multivalent in structure, as observed for all hemagglutinins. Studies into binding properties of MBHA show that MBHA binding sites for developmental cells differ clearly from vegetative binding sites (Cumsky and Zusman, 1981b).

Another developmentally regulated protein is protein U. Synthesis of protein U starts at a late stage of development when spore formation begins (Inouye et al., 1979b). Like protein S, protein U assembles on spore

surfaces and is considered a spore coat protein. The gene for protein U has been cloned and sequenced (Gollop et al., 1991). The protein product has a typical signal peptide of 25 amino acid residues at the amino terminal end. This indicates that protein U is produced as a secretory precursor pro-protein U, which is then secreted across the membrane to assemble on the spore surface. This is in sharp contrast to protein S which has no signal peptide.

Protein C is also a spore coat protein (McCleary et al., 1991). This 30 kDalton protein is not produced in vegetative cells but appears in extracts of developing cells by six hours. Its accumulation continues throughout development.

1.2.4 Chemical-Induction of Sporulation

Sporulation can be induced by adding high concentrations of low molecular weight compounds such as glycerol or dimethylsulphoxide (DMSO) to exponential phase cultures. The best inducers of sporulation are fully saturated aliphatic compounds containing two to four carbons with at least one primary or secondary alcohol group. High concentrations of non-penetrating solutes such as glucose do not induce spore formation, suggesting that induction is not the result of a simple osmotic effect (Sadler and Dworkin, 1966).

Glycerol-induced sporulation is very rapid and synchronous taking about two hours for the formation of spherical spores. Induction is independent of the cell

cycle and does not require starvation or a solid surface. However, aeration and either calcium or magnesium ions are essential for glycerol-induced sporulation. Although glycerol-induced spores are morphologically fully formed two to three hours after the addition of glycerol they are not yet as "mature" as fruiting body spores, but seem to acquire resistance comparable to that of fruiting body spores over a period of several hours. Resistance to all agents does not appear simultaneously. The cells first become resistant to sonic vibration, then to UV irradiation and finally to heat (Sudo and Dworkin, 1969). These changes probably reflect alterations within the cell which begin at different times and take different periods to be completed. Removal of the inducer at any stage prior to the appearance of non-refractile spores results in the sequence of morphological changes leading to spore formation being reversed, so that the cells revert to vegetative rods. It should be emphasized that this does not represent germination but rather an exact reversal of the stages leading to myxospore formation (Dworkin and Sadler, 1966).

Many studies of myxospore development have been of glycerol-induced spores because of the convenience of the system for experimental manipulation. During glycerol-induced myxospore morphogenesis, several biochemical changes occur, including the cessation of net synthesis of DNA, RNA, protein and phospholipid (Bacon and Rosenberg, 1967; Bacon et al., 1975; Rosenberg et al., 1967; Sadler and Dworkin, 1966). At the same time a

large increase in the activities of the glyoxylate cycle enzymes and a major shift towards gluconeogenesis occur (Bacon et al., 1975; Orlowski et al., 1972). The shift towards gluconeogenesis is accompanied by a decrease in respiration rate (Bacon et al., 1975).

Glycerol spores have a thin, single-layered spore coat, which does not contain protein S, and are less resistant to heat and sonication than are fruiting body spores (Sudo and Dworkin, 1969). They also have a relatively high respiration rate, up to 20% of that in vegetative cells (Bacon et al., 1975). In contrast, fruiting body spores have no detectable endogenous respiration. Glycerol spores contain unusual lamellar structures and ribosomes, which are not seen in fruiting body spores. Membranes and ribosomes would be broken down during fruiting body sporulation as part of the starvation response. It is tempting to speculate that the lamellar structures and ribosomes present in chemical-induced spores are present because of the lack of starvation during chemical-induced sporulation.

The mechanism of glycerol-induction is uncertain. Although glycerol was found to be incorporated by a cell suspension at a very low rate, other inducers such as ethylene glycol were not incorporated by the cells at all. These observations suggested that morphogenesis may be brought about by an interaction between the inducer and some peripheral structure of the vegetative cell. This is consistent with the observation that, after the addition of the inducer, there is a sharp decrease in the

optical density of the cells prior to any visible morphological change. Since the presence of glycerol is continuously required, it appears that it is not acting as a simple trigger but is required to maintain a continuous altered state of the cell structure or organisation (Sadler and Dworkin, 1966). DMSO appears to act in the same mechanism as glycerol while phenylethyl alcohol (PEA) appears to induce sporulation differently. Mutants isolated for their inability to sporulate in the presence of glycerol or DMSO can be induced to sporulate by phenylethyl alcohol (Burchard and Parish, 1975). However, the patterns of protein synthesis are almost identical for glycerol, DMSO and PEA-induced spores (Komano et al., 1980), although the final yield of spores is significantly different depending on the chemical used as an inducer.

1.2.5 Alternative Methods of Inducing Sporulation

1.2.5.1 Sporulation in Liquid Starvation Medium

When suspended in a liquid starvation medium, MCM (10mM MOPS [morpholinopropanesulfonic acid], 2mM $CaCl_2$, 4mM $MgSO_4$ [pH 7.2]), exponentially growing cells sporulated within three days. Approximately 15% of the initial number of wild-type cells had become mature, resistant spores and had aggregated into large, macroscopic clumps. The myxospores within these clumps were similar to spores developed within fruiting bodies as determined by electron microscopy and the production of spore-specific protein S (Rosenbluh and Rosenberg,

1989b). A similar effect was noted by Burchard (1975). Vegetative cells of a mutant formed clumps in growth medium. When transferred to starvation buffer, cells within these clumps differentiated into spores shaped like fruiting body spores.

1.2.5.2 AMI-Enhancement of Sporulation

AMI is an autocide, produced by M. xanthus, that induces lysis in vegetative or resting cells of the producing strain or related strains (Varon et al., 1984). It is composed of a mixture of free fatty acids containing almost equal concentrations of saturated and monounsaturated fatty acids with a small proportion of diunsaturates (Varon et al., 1986). All three fractions have lytic activity. Diunsaturates have the highest specific activity and the saturated fatty acids have the lowest specific activity. There was no synergistic effect for lytic activities in mixtures of various AMI component fatty acids.

Mutants resistant to lysis by AMI were isolated and all were defective in fruiting body formation to various degrees, whereas sporulation was generally not affected. These mutants could only be maintained under selective pressure and were highly unstable (Varon et al., 1986).

The addition of AMI (6 U/ml) to wild-type cells in developmental submerged cultures increased the final spore yield by 70% and accelerated the onset of aggregation and sporulation by six to eight hours (Rosenbluh and Rosenberg, 1989a). AMI also rescued both

aggregation and sporulation in dsg signalling mutants. This rescue was specific to dsg mutants and was not observed for the other classes of signalling mutants (Rosenbluh and Rosenberg, 1989a).

A transposon insertion mutant was identified that was incapable of aggregation or significant sporulation in developmental submerged culture. This mutant, ER304, could be rescued by AMI and was subsequently employed to characterize the AMI-induced rescue phenomenon. The three fatty acid fractions that constitute AMI each displayed developmental rescue activity. The rescue of development was found to be an early event (Rosenbluh and Rosenberg, 1990).

It has been suggested that development rescue is the result of increased permeabilization of the cell membrane rather than the result of lysis. This conclusion was based on three observations: (1) lower concentrations of autocide AMI were necessary for rescue activity than for lysis, (2) lytic and rescue activity could be uncoupled and (3) development rescue could be effected by AMI only at times earlier than those observed for developmental lysis. Increased permeabilization of the membrane of the developing cell would allow developmental signals to be exchanged. A permeabilizing agent might allow earlier and more efficient signalling in wild-type cultures, thus accelerating development and allowing more efficient sporulation. Lysis may be a secondary effect of AMI later in development (Rosenbluh and Rosenberg, 1990).

1.2.5.3 Glucosamine-Induction of Sporulation

When 15mM glucosamine was added to exponentially growing M. xanthus wild-type cells and the culture incubated with shaking at 32°C for 24 hours, lysis and sporulation of the vegetative cells occurred. Approximately 70 to 75% of the initial vegetative population lysed and 60 to 70% of the surviving cells sporulated. Glucosamine-induced lysis and sporulation was dependent on the relative concentrations of yellow- and tan-colony-phase-variants in the original vegetative population. Sporulation was also dependent on the initial cell density, as sporulation only occurred when the cell density was above 2×10^6 cells/ml and 50 to 75% of the cells had been lysed. The addition of glucosamine to vegetative cells resulted in increased phospholipase activity, increased production of the autocide AMI and also transiently stimulated glycerol production in the first two hours after addition of glucosamine. Glucosamine-induced spores, like fruiting body spores, are resistant to heat and sonication. However, electron microscopy revealed that glucosamine-induced spores were more like glycerol-induced spores in that they too, had a single, thin spore coat. Lysis and sporulation could also be induced by mannosamine, but no other carbohydrate tested induced either lysis or sporulation. It is proposed that glucosamine initiates lysis and sporulation by acting as an initial signal which stimulates one or more membrane phospholipases to hydrolyse membrane phospholipids. The hydrolysis products, AMI and glycerol

in turn act as second messengers and mediate the developmental events of lysis and sporulation respectively (Mueller and Dworkin, 1991).

The addition of 30 to 50mM glucosamine to wild-type developing cells inhibited fruiting body formation and sporulation. These concentrations of glucosamine stimulate a massive and premature lysis leaving the surviving cells at too low a cell density to form fruiting bodies or sporulate. Lower concentrations of glucosamine had different effects. Sporulation was enhanced 11-fold in the presence of 10mM glucosamine, while 20mM had an inhibitory effect on sporulation. The presence of both enhancement and inhibition of sporulation demonstrates a delicate balance between lysis and sporulation. The enhancement of sporulation could be due to the fact that glucosamine also causes the release of the myxobacterial autocide AMI (Mueller and Dworkin, 1991). The addition of AMI has been shown to enhance sporulation (Rosenbluh and Rosenberg, 1989a). Glucosamine and mannosamine were the only carbohydrates tested which inhibited fruiting body sporulation.

Glucosamine and mannosamine have also been shown to rescue fruiting body formation, lysis and sporulation of C signalling mutants (Janssen and Dworkin, 1985).

1.3 Genetic Systems in Myxobacteria

1.3.1 Myxophages

There are three main groups of generalized transducing myxophages, Mx4, Mx8 and Mx9. Mx8 and Mx4

are the most commonly used myxophage.

Mx4 was the first transducing myxophage isolated and came from a mixture of soil and manure (Campos et al., 1978). It has a genome size of approximately 62.0 kilobases. Mx41 and Mx43 are structurally and serologically related to Mx4 and are also transducing phages (Martin et al., 1978). Mx4 appears to be a temperate phage because it forms cloudy plaques. However, no free phage have yet been isolated from infected cells (Campos et al., 1978; Geisselsoder et al., 1978). The natural host range of Mx4, Mx41 and Mx43 appears to be limited to a set of non-fruiting mutants of M. xanthus but the limitation is at a stage beyond adsorption (Campos et al., 1978) and can be overcome by a phage mutation, hrm-1, isolated by Wolfner (unpublished). A mutation to high-frequency transduction has been combined with hrm-1 and another mutation to temperature sensitivity that reduces the killing of transductants to give a transducing strain of high efficiency (Geisselsoder et al., 1978).

Mx8 is a temperate phage with a genome size of 56 kilobases (Martin et al., 1978). Comparison of the restriction map of the prophage with that of DNA from phage has demonstrated: (1) the absence of unique end fragments, (2) the circular permutation of phage DNA and (3) unique prophage attachment sites in Mx8 and the M. xanthus chromosome (Orndorff et al., 1983; Stellwag et al., 1985). This allows Mx8 to integrate into the M. xanthus chromosome by site-specific recombination

between a site on the phage DNA, attP, and a corresponding site on the bacterial chromosome attB.

Mx9 is serologically distinct from both Mx4 and Mx8 (Martin et al., 1978). It was isolated from a carrier strain of M. xanthus and may also be a temperate phage, by analogy with Mx8. Its head diameter, 60nm, is similar to that of Mx4 and Mx8. Hence, a DNA content for Mx9 in the range of 50 - 65 kilobases seems likely.

1.3.2 Coliphage P1 and its Derivatives

Coliphage P1 can absorb to M. xanthus and cause its lysis or inhibit its growth (Kaiser and Dworkin, 1975). Lipopolysaccharide is the adsorption receptor for coliphage P1 in Escherichia coli (Lindberg, 1973) and M. xanthus possesses lipopolysaccharide resembling that of other Gram-negative bacteria. Although P1 can absorb to M. xanthus and inject its DNA it is not a productive infection as no infectious P1 particles issue from these tissues and no lysogenic bacteria are formed. P1 can, therefore, be used as a suicide vector for introducing DNA into cells.

If the P1 DNA carries a transposon, e.g. Tn5, once the DNA has been injected into M. xanthus, the transposon can transpose to any point on the host chromosome. Further transposition is very infrequent. Tn5 insertions have been used to characterise many mutant allelles and assign them to particular loci (Kuner and Kaiser, 1981).

The Tn5 transposase is encoded in IS50R. The IS50L transposase is defective and nearly all of the gene can

be deleted without affecting transposition. This offers great scope for inserting novel DNA sequences into *M. xanthus*. Tn*5* *lac* is a modified version of Tn*5* which is a promoter probe. A promoterless *lacZ* has been inserted such that it does not interfere with Tn*5* transposition nor the expression of the gene for kanamycin resistance. It is orientated so that transcription initiated from a promoter outside the Tn*5* *lac* DNA proceeds into the *lacZ* gene. There are translational stop signals ahead of the *lacZ* gene in all three reading frames. Consequently, transcriptional fusions, but not protein fusions are made (Kroos and Kaiser, 1984). Hence, Tn*5* *lac* insertions can detect activity from adjacent *M. xanthus* promoters.

1.3.3 Homology-Based Integration

There are no known self-replicating plasmids in *M. xanthus*. However, plasmids can be stably maintained if they are integrated into the host chromosome. Integration can occur if homologous recombination occurs between a plasmid, containing cloned *M. xanthus* sequences, and the chromosome. Coliphage P1 can package from 45 to 105 kilobases of DNA and will transduce whole plasmids into which a segment of P1 *inc* DNA has been incorporated (Shimkets *et al.*, 1983). This is a specialised transduction and results in merodiploids. This system for specialized P1 transduction takes advantage of the fact that bacteriophage P1, which lysogenizes *E. coli* as an extrachromosomal self-

replicating plasmid, exhibits the phenomenon of incompatibility (Sternberg *et al.*, 1981). It cannot be stably maintained in a cell containing a plasmid of the same incompatibility type. Therefore, cloning vectors modified by the addition of the *inc* region of P1, which is carried on a 6.7kb *Eco*RI fragment, can be stably maintained in a lysogenic cell only by formation of a cointegrate molecule by recombination between the shared P1 sequences. Upon induction of the cointegrate P1 lysogen to lytic growth, packaging of linear DNA into virions initiates from a unique *pac* site in the P1 portion of the cointegrate and proceeds by a sequence-independent mechanism which sequentially fills the virions with DNA. Analysis of the DNA contained in mature virions produced from such a strain showed that a very large proportion contain the entire vector plasmid flanked by P1 viral DNA (Gill *et al.*, 1988). Upon subsequent infection of *M. xanthus* by such a virus stock, these packaged linear DNA molecules are likely to undergo homologous recombination between the P1 *inc* sequences to regenerate the original circular plasmid, leading to the subsequent loss of all other P1-derived sequences. A cointegrate of phage and plasmid DNA is, therefore, transferred during transduction (Figure 1.1). The plasmid, once transduced to *M. xanthus*, can then integrate into the chromosome by homologous recombination. P1 can also transfer plasmids which do not contain P1 *inc* DNA from *E. coli* to *M. xanthus* (O'Connor and Zusman, 1983). However, concatomers can be

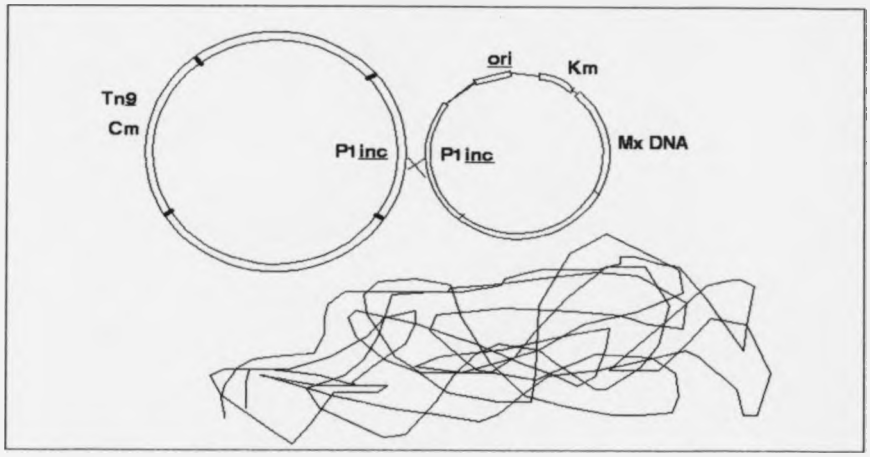
Fig. 1.1

Packaging of Plasmid by Coliphage P1 for Transduction into M. xanthus.

In E. coli a phage-plasmid cointegrate forms under combined selection for kanamycin and chloramphenicol resistance. The temperature is permissive for lysogenic growth.

At non-permissive temperatures, phage are induced. Replication and packaging of phage-plasmid cointegrates occurs and these phage can transduce M. xanthus. The plasmid then excises from the phage genome and can integrate into the M. xanthus chromosome by homologous recombination.

E. coli



Heat Induce P1 and Transduce M. xanthus

M. xanthus

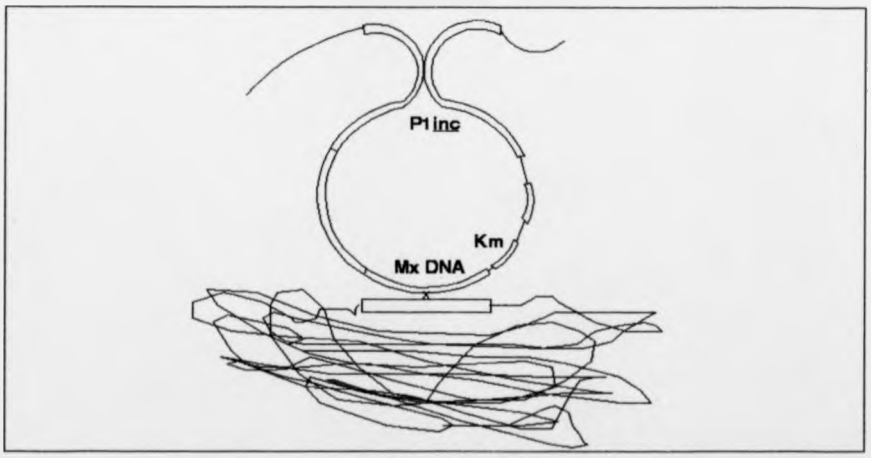


Fig. 1.1

formed and this can lead to multiple copies of the plasmid being inserted. For this reason integrative plasmids usually contain the cloned P1 *incB* region.

1.3.4 Site-Specific Integration

An alternative to integration of recombinant plasmids by homologous recombination makes use of the site-specific recombination system of the temperate myxophage Mx8. Mx8 integrates into the *M. xanthus* chromosome by site-specific recombination between a site on the phage DNA, *attP*, and a corresponding site on the bacterial chromosome, *attB* (Stellwag *et al.*, 1985). Plasmids containing the cloned Mx8 *attP* site and adjacent DNA have been shown to integrate into the chromosomal *attB* site (Stellwag *et al.*, 1985). It has not yet been determined whether site-specific recombination is mediated by recombination enzymes encoded for on the *attP* fragment or by cryptic bacteriophage in the *M. xanthus* strains. However, site-specific integration of plasmids is particularly efficient as a plasmid containing both an *attP* site and cloned *M. xanthus* DNA preferentially integrates through site-specific rather than homologous recombination (Shimkets and Asher, 1988). Once formed, the integrated plasmids are stably maintained. In sharp contrast to homologous recombination, gene conversion and marker rescue occur only rarely, if at all, upon integration via site-specific recombination (Shimkets and Asher, 1988; Stephens and Kaiser, 1987) presumably because the homologous regions of *M. xanthus* DNA are

separated by great distances.

1.3.5 Design of a Homology-Based Promoter Probe

An integrative-plasmid-based promoter probe contains a promoterless *lacZ*. Upstream of the *lacZ* gene the plasmid contains one or more unique restriction sites. A random library of chromosomal fragments can be cloned into the plasmid at this unique site (Figure 1.2)

The library can then be packaged, by infecting the host *E. coli* with P1 phage, and transduced into *M. xanthus*. There are two possible outcomes of integration of a plasmid containing a region of cloned *M. xanthus* DNA (Figure 1.3). If the cloned sequence contains a promoter this can direct transcription of *lacZ* and the existing copy of the gene remains intact. If there is no promoter in the cloned region a transcript fusion is still produced but the existing copy of the gene is inactivated because its promoter has been removed.

1.3.6 Advantages of a Homology-Based Promoter Probe

One advantage of the homology-based promoter probe is that if a promoter is cloned the locus that provides the promoter is not inactivated upon plasmid integration. This is important for investigation of essential genes whose inactivation would give rise to a lethal mutation.

A second advantage is the ease of cloning of promoters of interest. Recombination is reversible and excision of the plasmid occurs at a low frequency.

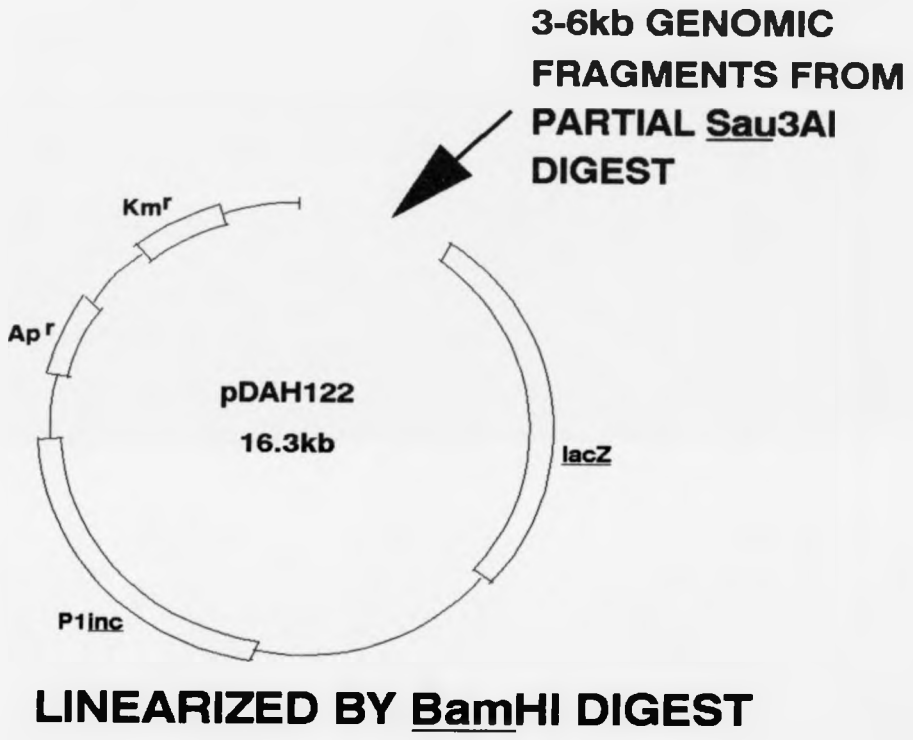


Fig. 1.2 Construction of a Gene Library in a Plasmid-Based Promoter Probe.

Fig 1.3

The Two Possible Outcomes of the Integration of a Plasmid-Based Promoter Probe Containing M. xanthus DNA.

A. There is a promoter in the cloned M. xanthus DNA. Once integrated into the chromosome, the promoter can direct transcription of the adjacent lacZ gene. The existing copy of the promoter and its corresponding gene/operon remain intact.

B. There is no promoter in the cloned region, as the cloned region is entirely within an gene/operon. In this case a transcript fusion with lacZ is formed. Furthermore, the existing copy of the gene/operon has now lost its promoter and so is inactive.

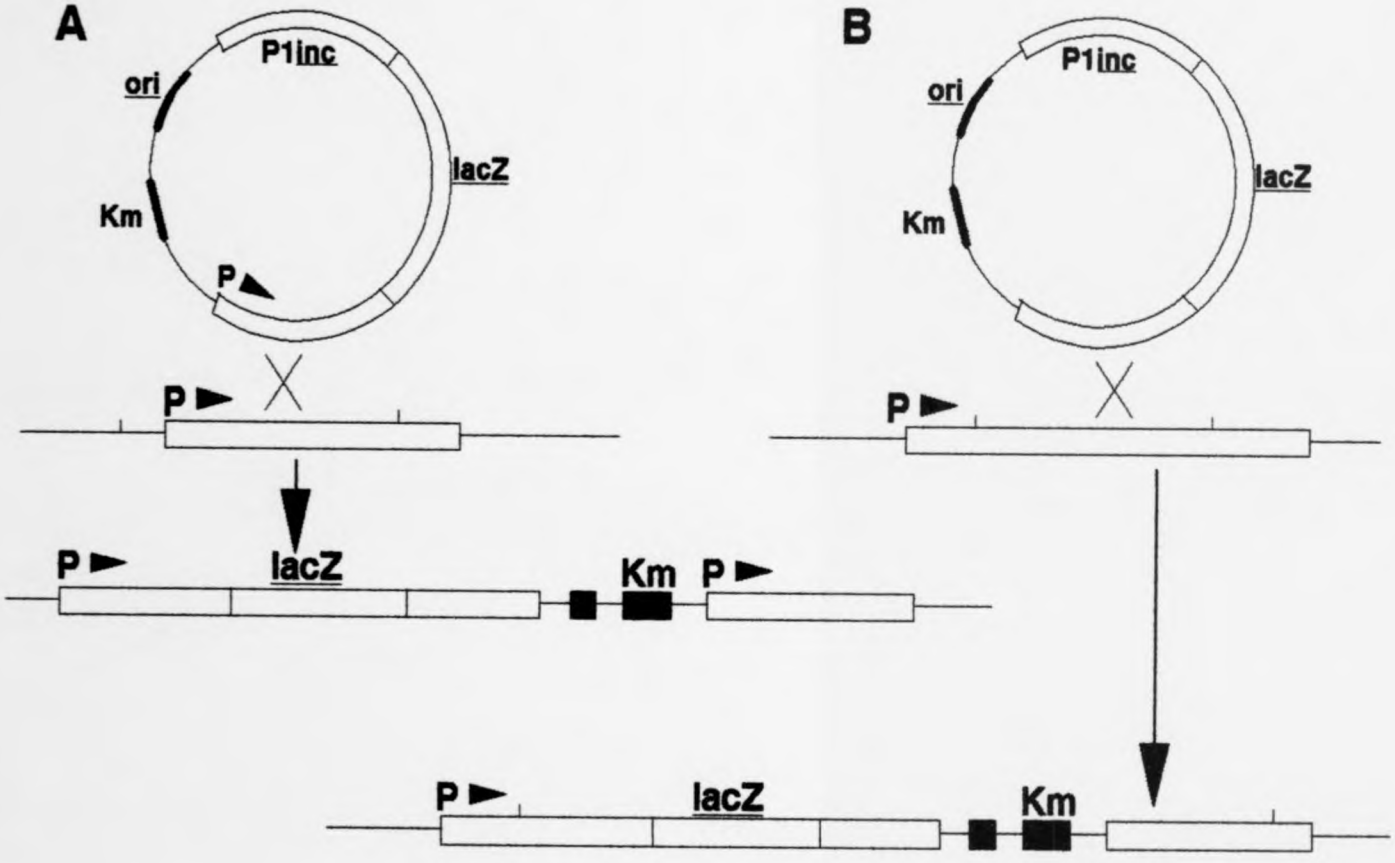


Fig. 1.3

Plasmid DNA can be isolated on a caesium chloride, ethidium bromide gradient and cloned and amplified by transformation of E. coli. Once a gene fusion of interest has been identified the intact copy of the gene can be cloned with relative ease. The region corresponding to the gene fusion and additional downstream sequences can be cloned as a plasmid using a restriction enzyme which cuts in the original plasmid but not in the cloned insert (Figure 1.4).

1.4 Genetics of M. xanthus Development

1.4.1 Introduction

A number of developmental mutants have been isolated. While some of these are specific to development, some mutants show visible defects in the vegetative cells, such as altered motility. Other mutants isolated include aggregation mutants and mutants defective in glycerol-induced sporulation.

1.4.2 Motility Mutants

Myxobacterial colonies on a nutrient medium are typically flat and spreading. At the edge of a colony a thin zone of cells forms and spreads progressively outwards. The process of multicellular spreading is called swarming. The ability to swarm clearly depends on cell motility, because mutants selected for alterations in the amount or pattern of swarming proved to have alterations in single cell motility (Burchard, 1970; MacRae and McCurdy, 1976; Hodgkin and Kaiser, 1977).

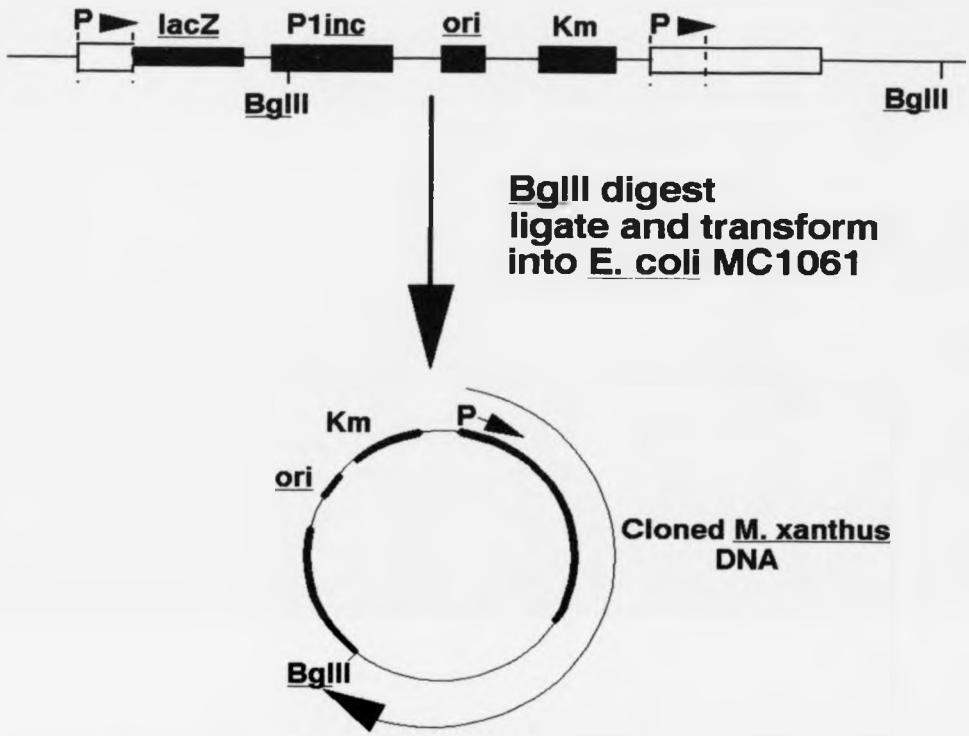


Fig. 1.4 Cloning the Gene for which a Gene Fusion has been Identified.

A restriction enzyme is chosen which cuts within the plasmid but not within the M. xanthus DNA already cloned. The enzyme shown here is BglII. The enzyme is used to cut chromosomal DNA obtained from a strain containing the integrated plasmid. A ligation reaction was performed and transformed into E. coli MC1061. A plasmid can be obtained, from the fragment containing the plasmid replication origin, which should contain additional DNA sequences to those cloned in the promoter probe. These sequences will be from downstream of the fusion point and so should include the gene(s) transcribed from the promoter.

Investigation of many motility mutants has revealed two independent multigene systems that control motility (Hodgkin and Kaiser, 1979b). System A, (for adventurous motility), is required for the movement of single cells or small groups of cells and has at least 23 genetic loci. System S, (for social motility), can cause cells to move in groups only and contains at least 10 genetic loci. Cells with a single mutation in the A system (A^-S^+) can move only when near another cell, as if contact is necessary to engage S motility (Kaiser and Crosby, 1983). Conversely, cells which have a single mutation in the S system (A^+S^-) glide primarily as individuals or long thin flares. Except for one locus (mgl), all non-motile mutants were found to be double mutants containing at least one mutation in system A and one in system S (A^-S^-). Since mutations in the mgl locus are the only mutations that can make a cell non-motile in a single step this must be required for both A and S motility.

Many non-motile mutants, although unable to move on their own, become transiently motile after contact with wild-type cells or mutant cells. This stimulation of motility is phenotypic and is not due to genetic alteration. Five stimulation groups have been identified in the A system and one in the S system. All mutations of a given stimulation group map at the same genetic loci (Hodgkin and Kaiser, 1979a,b; Sodergren and Kaiser, 1983).

The social motility system seems to be correlated with the presence of pili (Kaiser, 1979; Rosenbluh and

Eisenbach, 1992). Myxococcal pili are found only at the cell poles and are less than 10nm in diameter and 3-10μm in length. Pili are absent in mutants defective in any one of four different loci that affect the S motility system. One of these mutants, which can become transiently motile after contact with wild-type cells, can also transiently produce pili after being stimulated. Furthermore, the maximum distance from their neighbours that A^-S^+ can still move is 3.5μm which corresponds to the length of pili. However, dsp mutants are deficient in S motility but retain pili. Like the other S^- mutants, dsp mutants are less cohesive than wild-type cells, probably because of a failure of dsp mutants to form extracellular fibrils (Shimkets, 1986).

Recent evidence suggests that the A and S motility systems show selective advantages on various surfaces (Shi and Zusman, 1993b). A^+S^- cells (A-motile cells) swarmed better than A^-S^+ (S-motile cells) on relatively firm and dry surfaces. In contrast colonies of A^-S^+ cells swarmed much better than A^+S^- cells on soft and wet surfaces. Wild-type cells with both A and S motility systems were able to move well over a wide range of surfaces. These observations suggest that dual motility systems enable the myxobacteria to adapt to a variety of physiological and ecological environments.

The mgl locus is the only site of mutations that completely abolish motility. A 2.5kb fragment of M. xanthus DNA that complemented several mgl mutations was cloned (Stephens and Kaiser, 1987). The DNA sequence

revealed two open reading frames (ORFs) (Stephens et al., 1989). Most of the mutations that abolished gliding were in the downstream ORF, mglA. Antibodies raised against recombinant MglA were used to identify the 22 kDalton protein in extracts of M. xanthus (Hartzell and Kaiser, 1991a). MglA is primarily distributed in the cytoplasm. The predicted amino acid sequence of MglA indicates significant similarity to members of a class of GTP-binding proteins (Hartzell and Kaiser, 1991a). The region of similarity with MglA spans the consensus site for GTP-binding and hydrolysis. The similarities between MglA and this class of GTP-binding proteins suggests a role for MglA as a modulator of A and S components involved in gliding motility.

The upstream ORF, mglB, is cotranscribed with mglA. Mutants that carried deletions of portions of the mglB region exhibited reduced motility and colony spread (Hartzell and Kaiser, 1991b). Enzyme-linked immunosorbent assays indicated that these mglB mutants contained lower levels of MglA protein (11 to 17%) than did wild-type cells. The level of MglA protein corresponded roughly to the spreading rate of the mglB mutants. These observations indicate that mglB may affect the translation of mglA or that MglB may interact with and perhaps stabilize MglA. The predicted protein product of mglB shows similarity to one of the calcium-binding sites of yeast calmodulin. The significance of this finding is uncertain, but calcium is required for gliding of both M. xanthus and Stigmatella aurantiaca

(Burchard, 1984; Womack *et al.*, 1989).

The extent to which fruiting body sporulation is affected by mutations in motility has also been investigated. Non-motile cells (Mgl^-, A^-S^-) are unable to aggregate and are, therefore, unable to fruit. Mutants affected in the A system (A^-S^+) form quite normal, if undersized, fruiting bodies. Conversely, mutants affected in the S motility system (A^+S^-) are unable to move as aggregates and many are unable to form fruiting bodies. Mutants unable to form fruiting bodies are still able to form spores (Hodgkin and Kaiser, 1979b). This is dependent on cell alignment since aligned cells sporulate more than unaligned cells (Kim and Kaiser, 1990a). Hence, sporulation in non-motile strains increases with higher cell densities (Kroos *et al.*, 1988).

Another series of mutants which effect motility have been identified. These are known as frizzy (Frz) mutants (Morrison and Zusman, 1979; Zusman, 1982). The *frz* genes (*frzA*, *frzB*, *frzCD*, *frzE*, *frzF* and *frzG*) control the frequency of reversal of cell direction (Blackhart and Zusman, 1985b). Wild-type cells reverse their direction of movement every 6.8 $\pm$ 2.5 minutes but net movement is accomplished because of the large variation in the interval between switching. The majority of the Frz mutants reverse their direction of movement every two hours and as a result tend to glide in long streams. The *frzD* mutants reverse their direction of movement every 2.2 minutes but there is little variation in the interval

between reversals and as a result the cells show little net movement. Wild-type periodicity is essential for the formation of stable aggregates during fruiting body sporulation.

The *frz* genes have been cloned and mapped on a 7.5kb region of DNA (Blackhart and Zusman, 1985a). The nucleotide sequence of this region has been determined (McBride *et al.*, 1989; McCleary *et al.*, 1990; McCleary and Zusman, 1990a). The deduced gene products, with the exception of FrzB, show strong homology to the chemotaxis proteins of *Salmonella typhimurium* and *E. coli*. *frzA* is similar to *cheW*, *frzCD* (a single gene composed of the *frzC* and *frzD* loci) is similar to the genes for enteric methyl-accepting chemotaxis proteins (MCPs), *frzE* is similar to both *cheA* and *cheY*, *frzF* is similar to *cheR* and *frzG* (a region of DNA between *frzE* and *frzF* formerly referred to as the "gap" region) is similar to *cheB*. The deduced *frz* proteins exhibit 28 to 40% identity to their *che* counterparts at the amino acid level. Enteric chemotaxis proteins become heavily methylated following binding of attractants and rapidly demethylated following binding of repellents (McBride *et al.*, 1993). FrzCD is also reversibly methylated (McCleary *et al.*, 1990). Yeast extract, casitone, lauric acid and lauryl alcohol resulted in increased methylation of FrzCD while dimethylsulphoxide and short chain alcohols resulted in demethylation. Chemotactic responses have been demonstrated in *M. xanthus* towards yeast extract or casitone and away from dimethylsulphoxide or short chain

alcohols and these responses were correlated with methylation and demethylation of FrzCD (Shi *et al.*, 1993). The *frz* mutants were defective in chemotactic responses. Hence, the *frz* proteins are thought to be a sensory transduction pathway. FrzB and FrzF are thought to be required for methylation of FrzCD and FrzG is thought to be involved in demethylation of FrzCD. FrzE has been shown to have autophosphorylation activity (McCleary and Zusman, 1990b). It is hypothesized that FrzCD interacts with FrzA and FrzE altering the phosphorylation state of FrzE and that it is FrzE (or phospho-FrzE) that interacts with some component of the gliding motility machinery and determines whether cells reverse their direction. Recently a new gene *frzZ* has been identified in close proximity to the *frz* loci. It is located upstream and transcribed in a divergent manner. Its function is unknown but sequence data indicate that it is a response regulator protein, the second part of the two-component regulatory systems. FrzZ contains two of the response regulatory domains connected by a short alanine/proline rich region (Trudeau and Zusman, personal communication).

It should be noted that *M. xanthus* requires the *frz* sensory transduction system for aggregation as *frz* mutants are unable to aggregate. During aggregation of wild-type cells the FrzCD protein becomes heavily methylated (McBride and Zusman, 1993). This suggests that cells are sensing, responding and adapting to signals transmitted by other cells as part of the cell-

cell communication process. In addition, expression of some of the frz genes appears to be developmentally regulated (Weinberg and Zusman, 1989).

1.4.3 Sporulation mutants

Sporulation mutants have been identified which can be rescued by extracellular complementation by wild-type cells. Pairwise mixtures of certain developmental mutants also results in normal sporulation. The germinated spores give rise to the original mutant phenotypes, indicating that complementation is phenotypic and does not involve a permanent genetic change. Four complementation groups have been defined; asg, bsg, csg and dsg (Hagen et al., 1978). These mutant groups are defective in fruiting body morphogenesis as well as sporulation, although the four classes are blocked at different stages of development as judged by appearance and biochemical markers of development.

All of the asg mutations map to three genetic loci (Kuspa and Kaiser, 1989; Mayo and Kaiser, 1989). Developing asg^+ cells release a material called A factor that can rescue development of asg mutants (Kuspa et al., 1986). The asg mutants produce less than 5% of the wild type levels of A factor. A factor is produced at the onset of starvation, reaches a peak at two hours and declines to near negative levels by nine hours. Half the A factor activity obtained from conditioned media is heat-labile and nondialyzable, and the other half is heat-stable and passes through dialysis membrane (Plamann

et al., 1992). Heat-labile A factor has been purified and found to consist of a mixture of proteases (Plamann et al., 1992). Heat-stable A factor has also been purified. It is a mixture of amino acids and the small peptides that contain these amino acids (Kuspa et al., 1992a). Wild-type cells release a reproducible mix of amino acids when they are releasing A factor activity. Single amino acids rescue normal aggregation in all of the asg mutants and restore near wild-type levels of sporulation to the asgB and asgC mutants but not to asgA mutants (Plamann et al., 1992; Kuspa et al., 1992b). It appears that amino acids are the primary A signal molecules while the extracellular release of proteases and proteins by wild-type cells generates first peptides and then amino acids.

The bsg mutations map to several different loci. One locus, bsgA, has been identified and cloned (Gill et al., 1988). Mutants affecting bsgA aggregate very little on their own and substantially delay development of wild-type cells when present as a mixture (Gill and Cull, 1986). The BsgA gene product has been identified as an 90 kDalton protein which is not produced by bsgA cells (Gill and Bornemann, 1988). The protein is produced by vegetative cells and remains constant throughout the first 12 hours of development. Ninety percent of the BsgA protein was located in the cytoplasm. Since the bsgA product is not extracellular during development it may function in the synthesis or secretion of an extracellular molecule. It has recently been

demonstrated that the 90 kDalton protein product has ATP-dependent protease activity (Gill *et al.*, 1993). This suggests that the basis for *bsgA* mutants is a defect in intracellular proteolysis. The *bsgA* gene product has been shown to show 48% identity to the *E. coli lon* gene product, which encodes an ATP-dependent protease associated with cellular protein degradation, and 49% identity to the *M. xanthus lonV* gene product, which encodes an ATP-dependent protease essential for the vegetative growth of *M. xanthus* (Tojo *et al.*, 1993). There is no report as to the nature of the B signal itself.

All *csg* mutations affect a single gene, *csgA*, which encodes a protein with an apparent molecular weight of 17.7 kDalton (Hagen and Shimkets, 1990). Deletion of the *csgA* gene has no detectable effect on vegetative cells. However, when starved wild-type cells produce compact aggregation centres within 12 hours. Mutants affecting *csgA* form a reduced number of diffuse aggregates after 18 hours and also fail to ripple. A 17 kDalton protein has been purified which restores aggregation, sporulation and gene expression of *csg* cells to wild-type levels (Kim and Kaiser, 1990b,c,d) and has been shown to be the product of the *csgA* gene. Normally C factor is found tightly associated with the surface of the cell that produced it (Shimkets and Rafiee, 1990) and cell alignment appears to be necessary for efficient intercellular signalling by C factor expressed on cells (Kim and Kaiser, 1990a). C factor may function as a developmental timer that

entrains the natural sequence of morphological events of development (Kim and Kaiser, 1991; Li *et al.*, 1992). Murein components can also rescue sporulation of *csgA* mutants (Shimkets and Kaiser, 1982). N-Acetylglucosamine, N-acetylmuramic acid, diaminopimelic acid and D-alanine each increase the number of spores produced by *csgA* mutants. When all four components are included they have a synergistic effect raising the number of spores produced by *csgA* mutants to wild-type levels.

Mutants which do not produce D factor (*dsg*) map to two genetic loci. Two of the *dsg* mutations map to a gene known as *dsgA* (Cheng and Kaiser, 1989a; LaRossa *et al.*, 1983), which appears to be essential for vegetative function, as transposon insertions in the gene are lethal (Cheng and Kaiser, 1989b). Some point mutations, however, affect only development. The deduced amino acid sequence of the *dsgA* gene showed 50% identity with the translational initiation factor IF3 of *E. coli* (Kalman, Cheng and Kaiser, personal communication) Treatment of *dsgA* cells with the autocide AMI restores fruiting body formation and sporulation (Rosenbluh and Rosenberg, 1989a). AMI is a mixture of saturated and unsaturated fatty acids that accumulate in the culture medium during vegetative growth and kill the producing cells (Varon *et al.*, 1986). Whether AMI is the actual D signal is unclear. In lysing a small population of developing *dsgA* cells, AMI treatment may release the true D signal or other substances that can bypass this developmental step.

Recently a Tn*5* insertion, Ω258, has been identified which causes defects in sporulation and aggregation. Sporulation is decreased 10^4 - 10^5-fold and mutant cells have only a slight tendency to aggregate. This mutant has been designated *esg* (Downard, in Kaiser and Kroos, 1993). Additional insertions in the same region of the genome also generate the *esg* phenotype. Mutants of the *esg* phenotype sporulate when mixed with wild-type cells or mutants of the other complementation groups (*asg*, *bsg*, *csg* and *dsg*). Thus *esg* mutants represent a fifth extracellular complementation group, the E group.

1.4.4 Aggregation Mutants

Aggregation mutants fall into two phenotypic groups, *tag* mutants which show no aggregation (O'Connor and Zusman, 1990) and *frz* mutants which show defective aggregation (Zusman, 1982). Some aggregation mutants are unable to sporulate while others are capable of sporulation in the absence of aggregation into fruiting bodies (Morrison and Zusman, 1979). This would suggest that aggregation and sporulation are largely independent of each other.

The *frz* mutants are defective in aggregation as a result of mutations affecting the frequency of reversal of cell direction (Blackhart and Zusman, 1985b). The *frz* mutants are discussed in detail in Section 1.4.2.

All *tag* (temperature-dependent aggregation) mutants are able to aggregate at 28°C but not at 34°C (O'Connor and Zusman, 1990), in contrast to the wild-type which can

aggregate at both temperatures. This class of mutants was originally identified through screening of EMS-generated mutations (Morrison and Zusman, 1979). Subsequent work identified a linked insertion of Tn*5*, which was used to map EMS-generated mutations to four tightly linked loci (Torti and Zusman, 1981). Two of the four *tag* loci were cloned and Tn*5* mutagenesis of the cloned region was carried out. The resultant Tn*5* mutants had the same phenotype as the EMS-generated mutants. Mapping of 28 independent Tn*5* insertions identified nine *tag* complementation groups spanning 8.5 kilobases of DNA (O'Connor and Zusman, 1990). These observations suggest that the *tag* genes are required for normal development at 34^{o}C, but are not required for normal development at 28^{o}C

Extracellular complementation is evident in some aggregation mutants, e.g. *dsp* mutants, which lack cohesiveness and are unable to aggregate, but can undergo development when mixed with wild-type cells or *igl* aggregation mutants (Shimkets, 1986). However, both *dsp* and *igl* mutants have motility defects as well as defects in aggregation.

1.4.5 Glycerol-Resistant Mutants

Only a minority of mutants isolated for inability to sporulate under starvation conditions were also unable to sporulate when glycerol was added to liquid medium (Morrison and Zusman, 1979). Most mutants isolated for resistance to glycerol-induction undergo fruiting body formation and sporulation quite normally (Burchard and

Parish, 1975). Mutants which are not induced to sporulate by the addition of glycerol are described as glycerol-resistant. Glycerol-resistant mutants are able to grow on complex media containing 0.5 - 1.0M glycerol, while wild-type glycerol-sensitive cells are induced to sporulate and, hence, do not form colonies. Thus glycerol-resistant mutants can be selected for directly by plating a mutagenized culture on complex agar containing 0.5 - 1.0M glycerol.

A number of glycerol-resistant, non-fruiting mutants have been isolated by EMS and UV mutagenesis. The mutations in these mutants have been mapped by Mx8 transductions to two unlinked clusters (Figure 1.5). These clusters have been cloned into two series of plasmids; pKIA plasmids (Andreasson, Blea and Kaiser, personal communication).

1.4.6 Gene Fusions

The use of a promoterless *lacZ* either as Tn*5* *lac* or as a homology-based-integrative-plasmid provides a useful measurement of expression of a particular gene. This has been used successfully in the study of many genes, such as the genes for protein S, *ops* and *tps* (Downard *et al*., 1984; Downard and Zusman, 1985). The gene fusions were constructed as plasmids *in vitro* and transduced into *M. xanthus*, and ß-galactosidase activity was measured. The *tps* gene was transcribed early during development whereas *ops* transcription was correlated with the onset of sporulation. *ops* is transcribed in glycerol-induced

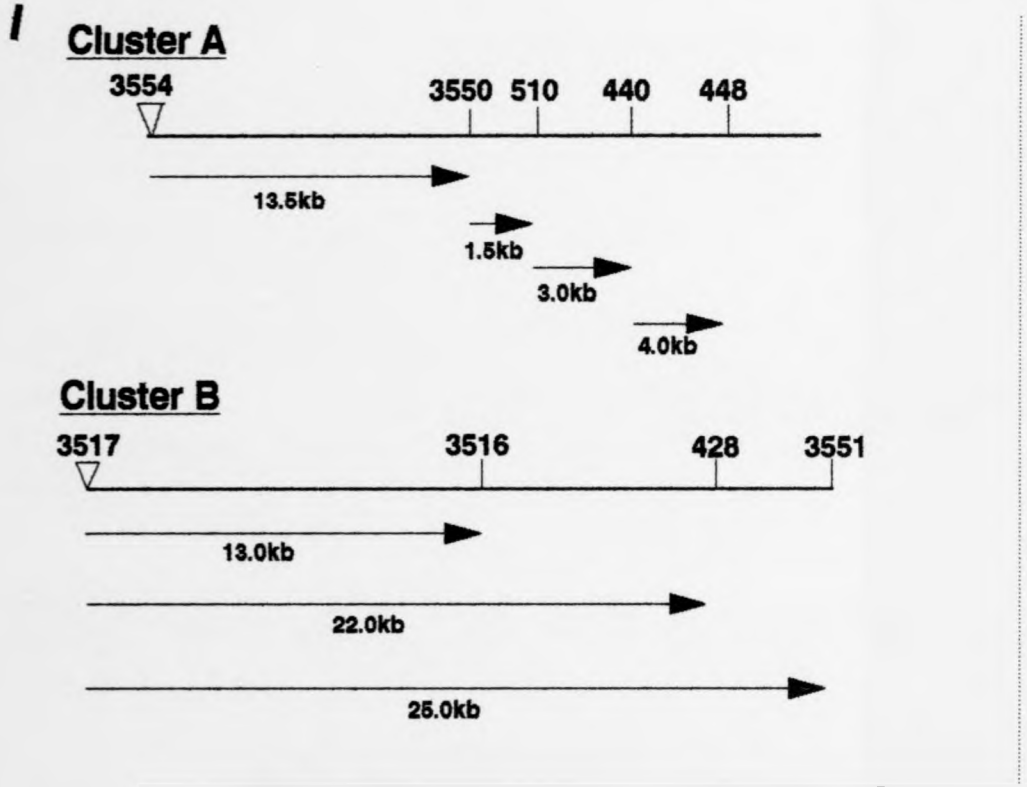


Fig. 1.5 Tentative Maps of Gly^{r} Fru^{-} Clusters.
(Andreasson, Blea and Kaiser, personal communication)

I **Tentative maps of the two clusters generated from Mx8 co-transduction data**

II **Shows the plasmids containing cloned DNA from each region**

Note. The plasmids bear no relation to the arrows drawn on the diagram

sporulation whilst tps is not.

Developmentally regulated gene fusions have also been identified by random Tn5 lac insertion (Kroos et al., 1986). Of 2374 kanamycin resistant transductants, 36 were identified which showed some increase in ß-galactosidase expression during development. These represent at least 29 different transcription units. Only seven of these 36 strains showed any developmental abnormalities. This suggests that many of the products of developmentally regulated genes are not essential for the completion of development.

1.4.6.1 Glycerol-Inducible Gene Fusions

Using a homology-based promoter probe a promoter has been identified which shows a spectacular increase in ß-galactosidase expression, up to 80-fold, during glycerol-induced sporulation (Hartree, 1989). During starvation-induced sporulation a two-fold increase was seen in spores. However, a 15-fold increase in expression occurred in spores during starvation-induced sporulation in a non-motile, mgl, strain which was unable to aggregate into fruiting bodies. Kim and Kaiser (1990a) reported that spores were still formed on starvation of DK306 cells, despite the absence of fruiting bodies. The gene was also expressed at intermediate levels in a strain where fruiting body sporulation was delayed due to a partial motility defect in the S motility system. That the gene was expressed in a non-motile strain, but not in a motile strain, suggests that the gene in question is

not essential for fruiting body sporulation but may have a role when spores are forced to form outside fruiting bodies. This led to the proposal that there are two pathways of starvation-induced development in M. xanthus: fruiting body sporulation and fruiting-independent sporulation (i.e. sporulation outside fruiting bodies) (Figure 1.6). It is thought that both these pathways can occur at once and that there is a fine balance between these pathways which is dependent on motility properties, cell density and other conditions.

Of the 2374 random Tn5 lac insertions generated by Kroos et al. (1986) one, ΩDK4530, was identified which was expressed at elevated levels during glycerol-induced sporulation (Kroos, 1986). The Tn5 insertion ΩDK4530 was also expressed during fruiting body sporulation (Kroos and Kaiser, 1987).

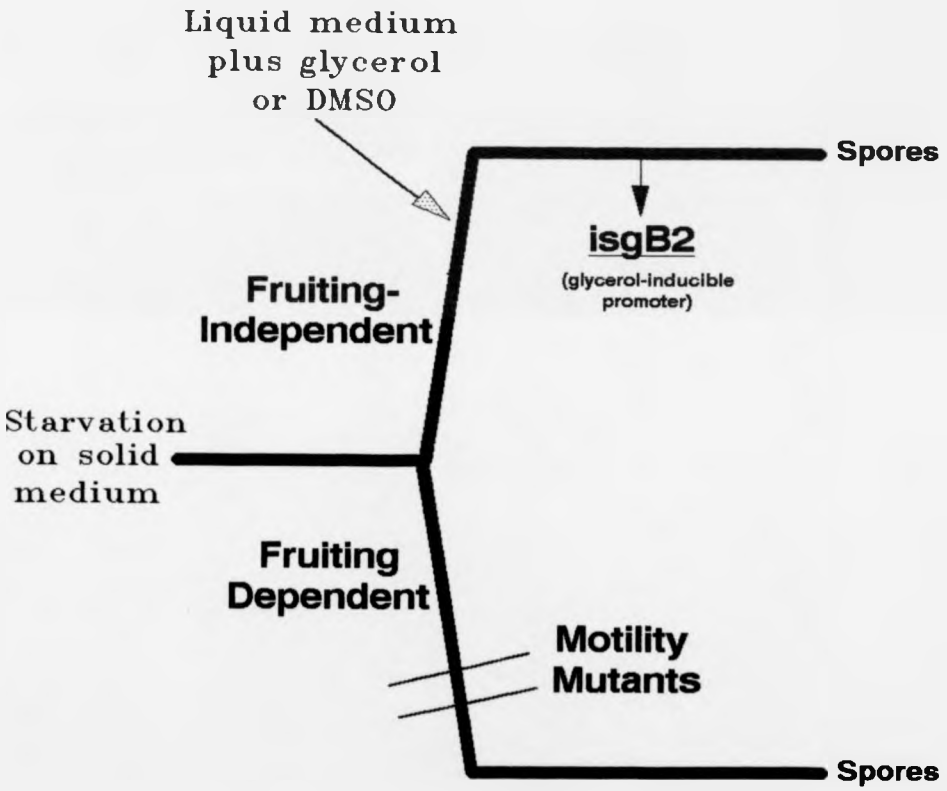


Fig. 1.6 Pathways of Development of M. xanthus.

Chapter 2

Materials and Methods

2.1 Strains

2.1.1 *Escherichia coli* K12

Strain	Genotype	Reference
MC1061	*araD139*, Δ(*araABC leu*)7697 Δ(*lacIPOZY*)X74, *galU*, *galK thi*, *rpsL*(*strA*), *hsdR* $hsdM^+$, *mcrB*	Casadaban and Cohen, 1980.
C600	*supE44*, *hsdR*, *thi-1*, *thr-1*, *leuB6*, *lacYI*, *tonA21*	
LE392	*hsdR514*, *supE44*, *supF58*, *lacY1* or Δ(*lacIZY*)*6*, *galK2*, *galT22*, *metB1*, *trpR55*, *mcrA*	
P2392	P2 lysogen of LE392	

2.1.2 *E. coli* Phages

Strain	Genotype	Reference
P1::Tn*9* ts	P1::Tn*9* *clr100*	Rosner, 1972.

2.1.3 *Myxococcus xanthus*

2.1.3.1 Existing Strains

These were obtained from the University of Warwick myxobacteria strain collection.

Strain	Genotype	Phenotype	Derivation	Reference
DK101	*sglA1*	partial motilty block	Spontaneous from FB	Hodgkin and Kaiser, 1977.
DK1622	"wild-type"	"wild-type"	Transduction of DK1217 to Agl^+	Kaiser, 1979.
DK306	*mgl sglA1*	non-motile	UV mutagenesis of DK101	Hodgkin and Kaiser, 1979a.
DK370	*cglF1 sglA1*	non-motile	UV mutagenesis of DK101	Hodgkin and Kaiser, 1979a.
DK440	*glrA1 sglA1 hsg-440*	Gly^r, Pru^-	UV mutagenesis of DK101	LaRossa *et al.*, 1983
DK510	*glrA2 sglA1 fru*	Gly^r, Pru^-	EMS mutagensis of DK101	Hagen *et al.*, 1978.

DK3516	glrB1 sglA1 fru	Gly^r, Fru^-	UV mutagenesis of DK101	Andreasson, personal communication
DK3517	sglA1 ΩDK3515	Kn^r	Random Tn5 insertion in glrB region	Andreasson, personal communication
DK3551	glrB2 sglA1 fru	Gly^r, Fru^-	UV mutagenesis of DK101	Andreasson, personal communication
DK3554	sglA1 ΩDK3554	Kn^r	Random Tn5 insertion in glrA region	Andreasson, personal communication
DK4530	sglA1 ΩDK4530 Tn5 lacZ	Glycerol-Inducible	Random Tn5 lacZ insertion	Kroos and Kaiser, 1987.
DZF1281	frz	Frizzy colony, Motility change, Deficient in chemotaxis		Zusman, personal communication

2.1.3.2 Strains Constucted During the Course of this Work

Strain	Genotype	Phenotype	Derivation
HMC1	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC2	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC3	glr fru	Gly^r, Fru^-	EMS mutagenesis of DK1622
HMC7	glr	Gly^r, Fru^+	NTG mutagenesis of DK1622
HMC10	glr fru	Gly^r, Fru^-	NTG mutagenesis of DK1622
HMC13	glr fru	Gly^r, Fru^-	UV mutagenesis of DK1622
HMC14	glr fru	Gly^r, Fru^-	UV mutagenesis of DK1622
HMC16	glr fru	Gly^r, Fru^-	UV mutagenesis of DK1622
HMC101	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC102	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC103	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC104	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC105	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC106	glr	Gly^r, Fru^+	UV mutagenesis of DK1622

HMC107	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC108	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC109	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC110	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC167	glr fru sglA1	Gly^r, Fru^-	NTG mutagenesis of DK101
HMC182	glr fru sglA1	Gly^r, Fru^-	NTG mutagenesis of DK101
HMC187	glr sglA1	Gly^r, Fru^+	NTG mutagenesis of DK101
HMC194	glrB3 sglA1	Gly^r, Fru^+	NTG mutagenesis of DK101
HMC201	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC202	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC203	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC204	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC205	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC206	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC207	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC208	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC209	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC210	glr	Gly^r, Fru^+	UV mutagenesis of DK1622
HMC215	glr fru	Gly^r, Fru^-	NTG mutagenesis of DK1622
HMC223	glr fru	Gly^r, Fru^-	NTG mutagenesis of DK1622
HMC225	glr fru	Gly^r, Fru^-	NTG mutagenesis of DK1622
HMC273	glr fru	Gly^r, Fru^-	NTG mutagenesis of DK1622
HMC281	glr	Gly^r, Fru^+	NTG mutagenesis of DK1622
HMC296	glr	Gly^r, Fru^+	NTG mutagenesis of DK1622
HMC300	glr mgl fis	Gly^r, non-motile, deficient in fruiting-independent sporulation	UV mutagenesis of DK306
HMC301	mgl fis	non-motile, deficient in fruiting-independent sporulation	UV mutagenesis of DK306
HMC2107	glr fru	Gly^r, Fru^-	NTG mutagenesis of DK1622

HMC2111	*glr fru*	Gly^r, Fru$^-$	NTG mutagenesis of DK1622
HMC2113	*glr*	Gly^r, Fru$^+$	NTG mutagenesis of DK1622
HMC2611	*glr*	Gly^r, Fru$^+$	NTG mutagenesis of DK1622
HMC2811	*glr*	Gly^r, Fru$^+$	NTG mutagenesis of DK1622
HMC400	*glrA1 sglA1 bsg-440* ΩDK4530 Tn*5* *lacZ*	Gly^r, Fru$^-$	Mx*4* transduction of ΩDK4530 into DK440 (*glrA1*, *bsg-440*)
HMC401	*glrA2 sglA1 fru* ΩDK4530 Tn*5* *lacZ*	Gly^r, Fru$^-$	Mx*4* transduction of ΩDK4530 into DK510 (*glrA2*, Fru$^-$)
HMC402	*glrB2 sglA1 fru* ΩDK4530 Tn*5* *lacZ*	Gly^r, Fru$^-$	Mx*4* transduction of ΩDK4530 into DK3551 (*glrB2*, Fru$^-$)
HMC403	*glrB3 sglA1* ΩDK4530 Tn*5* *lacZ*	Gly^r, Fru$^+$	Mx*4* transduction of ΩDK4530 into HMC194 (*glrB3*, Fru$^+$)

2.1.4 Myxophages

Mx*4*	Campos *et al.*, 1978; Geisselsoder *et al.*, 1978.
Mx*8cpl2*	Martin *et al.*, 1978.

2.2 Plasmids

2.2.1 Existing Plasmids

Plasmid	Contents	Derivation
pDAH101	pBR322 dimer, Tn*5*	Transposition of Tn*5* onto a pBR322 dimer
pDAH102	pBR322, Tn*5*	pDAH101 *Eco*RI 9.77kb fragment
pDAH114	*lacZYA*, 1/2Ap^r, p15A *ori*, Km^{r*}, *fd* term.	pMC871 *Bam*HI cut plus pLBU3 *Sau*3AI (*fd* term.)
pDAH122	*lacZYA*, Ap^r, p15A *ori*, Km^{r*}, P*linc*, *fd* term.	pDAH114 *Pst*I *Sal*I 9.98kb fragment (1/2Ap^r, *lacZYA*, p15A *ori*, Km^{r*}, *fd* term) plus pREG422 *Xho*I *Pst*I 6.13kb fragment (1/2Ap^r, P*linc*)
pDAH142	Km^r + 1/4IS*50*L, pBR322 *ori*, Ap^r, P*linc*.	Hodgson, 1993.
pDAH211	Ap^r, p15A *ori*, Km^{r*} + 4x(*fd* term.), P*linc*$^{\Delta}$	Hodgson, 1993.
pDAH212	*cer*, pUC *ori*, Ap^r.	McGowan *et al.*, 1993.

pDAH283 pDAH274	*lacZ*, P1*inc* , Ap^r, p15A *ori*, Km^{r*}, *fd* term.	Scanlan *et al*., 1990.
pDAH304	Ap^r, p15A *ori*, Km^{r*}, *fd* term., P1*inc*'	pDAH211 *Cla*I *Hind*III (filled) 6.32kb fragment (1/2Km^{r*}, p15A *ori*, Ap^r, P1*inc*') plus pDAH211 *Cla*I *Eco*RV 2.59kb fragment (1/2Km^{r*})
pDAH374	Ap^r, pUC9 *ori*, Km^r + IS*50*L	pDAH102 *Sal*I (Km^r + IS*50*L) plus pIC19H *Sal*I (pUC9 *ori*, Ap^r)
pBR322	Ap^r, Tc^r, pBR322 *ori*	Bolivar *et al*., 1977.
pBR329	Ap^r, Tc^r, Cm^r, pBR322 *ori*	Covarrubias and Bolivar, 1982.
pIC19H	Ap^r, *lacZ*α, polylinker. (pUC9 derivative with expanded polylinker)	Marsh *et al*., 1984.
pLBU3	Ap^r, *lacZ*α, *fd* term., Tc^r, pBR322 *ori*	Gentz *et al*., 1981.
pMC871	*lacZYA*, 1/2Ap^r, p15A *ori*, Km^{r*}	Casadaban *et al*., 1980.
pREG422	Tc^r, pBR322 *ori*, Ap^r, P1*inc*.	Gill *et al*., 1988.
pREG429	Km^r + 3/4IS*50*L, pBR322 *ori*, Ap^r, P1*inc*.	Gill *et al*., 1988.
ColE1:: Tn*5*-132	ColE1 *ori*, Colicin E', *mob*, IS*50*L' Is*50*R', Tc^r (from Tn*10*)	Rothstein *et al*., 1981.
pUWM5	*lacZYA*, Ap^r, p15A *ori*, Km^{r*}, P1*inc* *fd* term + 4.8kb Mx DNA (*isgB* region)	selection for glycerol-inducible promoters from a random DNA library in pDAH122 (Hartree, 1989)
pKIA1	Km^r + 3/4IS*50*L, pBR322 *ori*, Ap^r, P1*inc*, + 7.0kb Mx DNA from ΩDK3517 region.	*Bam*HI digest of DK3517[pREG429] DNA (Andreasson, personal communication)
pKIA2 pKIA7	Km^r + 3/4IS*50*L, pBR322 *ori*, Ap^r, P1*inc*, + 9.4kb Mx DNA from ΩDK3517 region.	*Bam*HI digest of DK3517[pREG429] DNA (Andreasson, personal communication)
pKIA3	Km^r + 3/4IS*50*L, pBR322 *ori*, Ap^r, P1*inc*, + 27.0kb Mx DNA from ΩDK3554 region.	*Eco*RI digest of DK3554[pREG429] DNA (Andreasson, personal communication)
pKIA4	Km^r + 3/4IS*50*L, pBR322 *ori*, Ap^r, P1*inc*, + 15.9kb Mx DNA from ΩDK3554 region.	*Eco*RI digest of DK3554[pREG429] DNA (Andreasson, personal communication)
pKIA6	Km^r + 3/4IS*50*L, pBR322 *ori*, Ap^r, P1*inc*, + 29.0kb Mx DNA from ΩDK3517 region.	*Eco*RI digest of DK3517[pREG429] DNA (Andreasson, personal communication)
pKIA9	Km^r + 3/4IS*50*L, pBR322 *ori*, Ap^r, P1*inc*, + 13.6kb Mx DNA from ΩDK3554 region.	*Bam*HI digest of DK3554[pREG429] DNA (Andreasson, personal communication)

2.2.2 Plasmids Constructed During the Course of this Work

Plasmid	Contents	Derivation
pHMC1	lacZYA, p15A ori, P1inc fd term., cer + 4.8kb Mx DNA (isgB)	pUWM5 ScaI cut plus pDAH212 EcoRV SmaI 0.32kb fragment (cer) (Fig. 7.1, 7.2)
pHMC2	pBR322 ori, Km^r + 3/4IS50L, Ap^r, P1inc + 4.3kb Mx DNA (glrB)	pREG422 SalI cut plus pKIA2 SalI 7.19kb fragment (Km^r + 3/4IS50L) (Fig. 5.12)
pHMC4	pBR322 ori, Ap^r, P1inc, Km^r + 1/2IS50L + 4.8kb Mx DNA (glrB)	pKIA2 SalI 15.52kb fragment (Mx DNA, P1inc, Ap^r, ori) plus pREG422 SalI 2.20kb fragment (Km^r + 1/2IS50L) (Fig. 5.12)
pHMC6	pBR322 ori, Ap^r, Km^r + 3/4IS50L P1inc + 6.8kb Mx DNA (glrA)	pKIA4 EcoRV 16.27kb fragment (Fig. 5.9)
pHMC7	pBR322 ori, Ap^r, P1inc + 9.1kb Mx DNA (glrA)	pKIA4 EcoRV 12.66kb fragment (Mx DNA + 1/2P1inc) plus pREG422 EcoRV 7.23kb fragment (Ap^r, ori + 1/2P1inc) (Fig. 5.9)
pHMC8	pBR322 ori, Ap^r, P1inc, Km^r + Mx DNA (glrA)	pHMC7 HindIII cut plus pDAH374 HindIII 1.54kb fragment (Km^r) (Fig. 5.9)
pHMC10	pBR322 ori, Ap^r, Tc^r + 11.0kb Mx DNA (isgB)	pBR329 EcoRI cut plus 11.0kb EcoRI Mx DNA (pUWM5 homolog) (Fig. 7.6)
pHMC12	lacZ, P1inc, Ap^r, p15A ori, Km^{r*}, fd term. + 4.7kb Mx DNA (isgB)	pDAH283 BamHI EcoRI cut plus pUWM5 EcoRI Sau3AI 4.7kb fragment (Mx DNA) (Fig. 7.10)
pHMC14	lacZ, P1inc, Ap^r, p15A ori, Km^{r*}, fd term. + 3.85kb Mx DNA (isgB)	pDAH283 BamHI SalI cut plus pHMC20 BamHI XhoI 3.85kb fragment (Mx DNA) (Fig. 7.10)
pHMC20	p15A ori, Km^{r*}, cer, P1inc' + 10.13kb Mx DNA (isgB)	BglII digest of DK101[pHMC1] DNA (Fig. 7.6, 7.7)
pHMC22	p15A ori, Km^{r*}, cer, Tc^r, + Mx DNA (isgB)	pHMC20 BamHI 22.27kb fragment plus Col E1::Tn5-132 BglII 2.78kb fragment (Tc^r) (Fig. 7.9)
pHMC24	p15A ori, Km^{r*}, cer, Tc^r, + Mx DNA (isgB)	pHMC20 SalI 21.65kb fragment plus Col E1::Tn5-132 XhoI 4.84kb fragment (Tc^r) (Fig. 7.9)
pHMC30	p15A ori, Ap^r, Km^{r*}, fd term., P1inc' + 3.8kb Mx DNA (glrB)	pDAH304 EcoRV cut plus pKIA2 MluI (filled) 3.8kb fragment (Mx DNA) (Fig. 5.8)
pHMC32	p15A ori, Km^{r*}, fd term., P1inc' + 1.35kb Mx DNA (glrB)	pDAH304 PstI cut plus pKIA2 PstI 1.35kb fragment (Mx DNA) (Fig. 5.8)
pHMC34	lacZ, P1inc, Ap^r, p15A ori, Km^{r*}, fd term. + 4.3kb Mx DNA (glrB)	pDAH283 SmaI cut plus pKIA2 SalI 7.19kb fragment (Mx DNA) (Fig. 5.8)

pHMC36	lacZ, Plinc, Ap^r, p15A ori, Km^{r*}, fd term. + 2.6kb Mx DNA (glrB)	pDAH283 SalI cut plus pKIA2 XhoI 2.6kb fragment (Mx DNA) (Fig. 5.8)
pHMC38	pBR322 ori, Km^r + 3/4IS50L, Ap^r, Tc^r, Plinc + Mx DNA (glrB)	pKIA2 partial SalI cut plus Col E1::Tn5-132 XhoI 4.84kb fragment (Tc^r) (Fig. 6.5)
pHMC40	lacZ, Plinc, Ap^r, p15A ori, Km^{r*}, fd term. + 2.8kb Mx DNA (glrA)	pDAH283 BamHI SalI cut plus pKIA4 BamHI XhoI 2.8kb fragment (Mx DNA) (Fig. 5.7)
pHMC42	Km^r + 3/4IS50L, pBR322 ori, Ap^r, Plinc + 2.0kb Mx DNA (glrA)	pREG429 SalI cut plus pKIA4 XhoI 2.0kb fragment (Mx DNA) (Fig. 5.7)
pHMC43	Ap^r, pBR322 ori, Plinc	pREG422 PstI XhoI 6.13kb fragment (Plinc, $1/2Ap^r$) plus pHMC8 PstI XhoI 5.12kb fragment (Mx DNA, ori, $1/2Ap^r$) (Fig. 5.11)
pHMC44	Ap^r, pBR322 ori, Plinc, Km^r + 1/4IS50L	pHMC43 XhoI cut plus pDAH142 SalI 1.49kb fragment (Km^r + 1/4IS50L) (Fig. 5.11)

2.3 Chemicals, Media and Antibiotics

2.3.1 Chemicals

Chemicals were obtained from either Fisons or Sigma. Restriction enzymes and DNA modification enzymes were obtained from Amersham International or Bethesda Research Laboratories (BRL). Radiochemicals were obtained from Amersham International.

2.3.2 Buffers

GTE (for alkaline lysis minipreps from *E. coli*)
50mM Glucose
10mM EDTA
25mM Tris-HCl pH8.0

Lysis mix (for large scale *E. coli* plasmid preps)
0.1% Triton X-100 (v/v)
50mM Tris-HCl pH8.0
5mM EDTA

PM (for resuspending *M. xanthus* cells)
10mM Potassium phosphate buffer pH7.4
8mM $MgSO_4$

SM (lambda phage buffer)
99mM NaCl
 8mM $MgSO_4$
50mM Tris-HCl pH7.5
0.01% Gelatin (w/v)

SSC (for Southern Blotting and Hybridization)
SSC is used at a variety of concentrations.
20x SSC is:

  3M NaCl
0.3M Sodium citrate

pH was adjusted to 7.0 with NaOH

STE (for large scale E. coli plasmid preps)
 25% Sucrose (w/v)
50mM Tris-HCl pH8.0
 5mM EDTA

STET (for boiling-water minipreps from E. coli)
  8% Sucrose (w/v)
0.5% Triton X-100
50mM EDTA
 5mM Tris-HCl pH8.0

TBE (used for agarose gel electrophoresis)
0.089M Tris base
0.089M Boric acid
 2.5mM EDTA

TE (used for dissolving DNA)
10mM Tris-HCl pH8.0
 1mM EDTA

TES (for large scale E. coli plasmid preps)
50mM Tris-HCl pH8.0
 5mM EDTA
50mM NaCl

TM (for resuspending M. xanthus cells)
10mM Tris-HCl pH8.0
8mM $MgSO_4$

Z-buffer (used in ß-galactosidase and protein assays)
 0.06M Na_2HPO_4
 0.04M NaH_2PO_4
 0.01M KCl
0.001M $MgSO_4$

2.3.3 Media

A1 medium (*M. xanthus* minimal media) (Bretscher and Kaiser, 1978)
10mM Tris-HCl pH7.6
0.8mM $MgSO_4$
1mM K_2HPO_4
10μM $CaCl_2$
0.1mM $FeCl_3$
3.79mM $(NH_4)_2SO_4$
50mg/l Leucine
100mg/l Isoleucine
100mg/l Phenylalanine
100mg/l Valine
10mg/l Methionine
1.5% Noble agar (w/v)
made up to 926ml with water

after autoclaving the following were added at the final concentrations indicated. All solutions had been filter sterilized:
0.5% Potassium aspartate (w/v)
0.4% Sodium pyruvate (w/v)
0.1mg/ml Asparagine
125μg/ml Spermidine
1μg/ml Vitamin B_{12}

CF (for starvation-induced sporulation)
0.015% Casitone (Difco) (w/v)
10mM Tris-HCl pH8.0
8mM $MgSO_4$
1mM K_2PO_4
1.5% Noble agar (w/v)
made up to 980ml with water

after autoclaving the following were added at the final concentrations indicated. All solutions had been filter sterilized:
0.02% $(NH)_2SO_4$ (w/v)
0.1% Sodium citrate (w/v)
0.2% Sodium pyruvate (w/v)

CTT (for *M. xanthus* cultures)
1% Casitone (Difco) (w/v)
10mM Tris-HCl pH8.0
8mM $MgSO_4$
1mM K_2PO_4 pH7.4

DCY (for *M. xanthus* cultures)
2% Casitone (Difco) (w/v)
0.2% Yeast extract (Difco) (w/v)
10mM Tris-HCl pH8.0
8mM $MgSO_4$

DCY agar is made as above but with 1.5% Bacto agar

(w/v) for normal agar and 0.7% Bacto agar (w/v) for soft agar.

DCY + 1.0M Glycerol (for selection/screen of glycerol-resistant mutants)
2% Casitone (Difco) (w/v)
0.2% Yeast extract (Difco) (w/v)
10mM Tris-HCl pH8.0
8mM $MgSO_4$
1.5% Bacto agar (w/v)
made up to 850ml with water

added after autoclaving:
50% Glycerol 150ml

LB (for E. coli cultures)
1% Bacto tryptone (Difco) (w/v)
0.5% Yeast extract (Difco) (w/v)
86mM NaCl

LB agar is made as above but with 1.5% Bacto agar (w/v) for normal agar and 0.7% Bacto agar (w/v) for soft agar.

LGC (for preparing P1 lysates)
1% Bacto tryptone (w/v)
0.5% Yeast extract (w/v)
86mM NaCl
5mM $CaCl_2$
0.3% Glucose (w/v)
1.1% Bacto agar (w/v)

LGC soft agar is made as above but with 0.6% (w/v) Bacto agar instead of 1.1% (w/v).

NZY (for growing host strains for lambda libraries)
86mM NaCl
8mM $MgSO_4.7H_2O$
0.5% Yeast extract (w/v)
1% NZ amine(casein hydrolysate) (w/v)

TB (for growing lambda libraries)
1% Bacto tryptone (w/v)
86mM NaCl
1.5% Bacto agar (w/v)

TMP (solid) (for starvation-induced sporulation)
10mM Tris-HCl pH8.0
8mM $MgSO_4$
1mM Potassium phosphate buffer pH7.5
1.5% Noble agar (w/v)

2.3.4 Antibiotics

The following antibiotics were used. Some could be stored as stock solutions, while others had to be freshly dissolved every time they were required. Antibiotics were dissolved in water unless otherwise stated.

Antibiotic:	Final Concentration:	Stock Solution:
Ampicillin (Ap)	50μg/ml	5mg/ml stored in aliquots at -20°C
Chloramphenicol (Cm)	12.5μg/ml	34mg/ml stored dissolved in absolute ethanol at -20°C
Kanamycin (Km)	50μg/ml	100mg/ml stored at 4°C
Oxytetracycline	12.5μg/ml	1.25mg/ml dissolved in 0.1N HCl freshly prepared when required. The HCl was neutralised with an equal volume of 0.1N NaOH added to the plate.
Tetracycline (Tc)	10μg/ml	10mg/ml dissolved in absolute ethanol and stored at -20°C

2.4 Growth and Maintenance of Cultures

E. coli cultures were grown at 37°C in LB broth (Section 2.3.3) or on LB agar. Liquid cultures were shaken at 280rpm in an orbital shaker. E. coli on plates could be maintained at 4°C for up to two months. For long term storage 10% DMSO was added to samples of a liquid culture and these were stored at -70°C.

M. xanthus was grown at 33°C in DCY broth (Section

2.3.3) or on DCY agar. Liquid cultures were shaken at 240rpm in an orbital shaker. M. xanthus could be maintained on plates at 17°C for 2 - 4 weeks. For longer term storage they were stored in 10% DMSO at -70°C.

2.5 NTG Mutagenesis of M. xanthus

A dense culture (A_{600} = 0.8) of M. xanthus DK101 was transferred to a universal and centrifuged at 4,500rpm in a Wifug Labor-50M bench-top centrifuge and the cell pellet was washed and resuspended in 0.5 volume PM buffer (Section 2.3.2). The cell suspension was transferred to a flask and assayed for colony forming units by plating out a series of serial dilutions. An equal volume of PM containing 200μg/ml NTG was added to give a final concentration of 100μg/ml. It was incubated with shaking at 33°C for 20 minutes and then the sample was assayed for colony forming units again. The cell suspension was transferred to a universal, DCY broth (Section 2.3.3) was added and it was centrifuged at 4,500rpm in a Wifug Labor-50M bench-top centrifuge. The pellet was washed and resuspended in 20ml DCY broth. After 1 - 2 days incubation the resulting culture was plated in 0.1ml aliquots onto appropriately modified DCY agar. This method was obtained from the myxobacteria laboratory manual.

2.6 EMS Mutagenesis of M. xanthus

A dense culture (A_{600} = 0.8) of M. xanthus was washed and resuspended in one volume PM buffer. The cell

suspension was assayed for colony forming units by plating out a series of serial dilutions. EMS was added to a final concentration of 1% and the culture incubated with shaking for 60 minutes at 33°C. The culture was transferred to a universal and an equivalent volume of DCY broth (Section 2.3.3) was added. The culture was centrifuged at 4,500rpm in an Wifug Labor-50M bench-top centrifuge. The cell pellet was washed and resuspended in 20ml DCY broth and assayed for colony forming units again. The culture was incubated at 33°C for 1 - 2 days and then plated in 0.1ml aliquots onto appropriately modified DCY agar. This method was obtained from the myxobacteria laboratory manual.

2.7 UV Mutagenesis of M. xanthus

The cells were prepared for mutagenesis in the same manner as for EMS mutagenesis except that the cells were resuspended in TM buffer (Section 2.3.2) rather than PM. Colony forming units were assayed before and after mutagenesis by plating out a series of serial dilutions. The UV mutagenesis was carried out by placing 1ml samples of the cell suspension in a 10ml petri dish and placing the dishes under a UV short wave lamp at 21ergs/mm^2/sec for 2.5 minutes. The cells were then centrifuged, washed and resuspended in 20ml DCY broth (Section 2.3.3) and incubated at 33°C for several days. The resulting culture was then plated onto appropriately modified DCY agar. This method was obtained from the myxobacteria laboratory manual.

2.8 Testing for Sporulation in M. xanthus

Samples were examined for spores under the phase-contrast microscope. The spherical spores were readily distinguishable from the rod shaped vegetative cells.

It was sometimes necessary to test whether spores once formed were viable. Where spores had been formed by starvation on a TMP plate (Section 2.3.3), the plate was incubated at 55^{o}C for two hours and overlaid with DCY soft agar (Section 2.3.3). The plate was incubated at 33^{o}C and viable spores germinated to form visible colonies.

2.9 Induction of Sporulation in M. xanthus by Starvation

Starvation of M. xanthus cells was induced by "spotting" exponential phase cells onto starvation media (Hartree, 1989). Dense cultures (A_{600} = 0.8) were centrifuged in universal bottles at 4,500rpm in a Wifug Labor-50M bench-top centrifuge. The cells were washed and resuspended in 1/10 volume of TM buffer (Section 2.3.2). The cell suspension was inoculated onto dried CF or TM agar (Section 2.3.3) in 20μl aliquots and the liquid was allowed to absorb. Fruiting bodies and spores were apparent after several days incubation.

2.10 Chemical-Induction of Sporulation in M. xanthus

Liquid cultures were grown in DCY broth (Section 2.3.3) at 33^{o}C to a density of A_{660} = 0.3-0.7. Cultures of densities in excess of this were found to sporulate

poorly or not at all (Hartree, 1989). Glycerol was added as a 50% solution in water to a final concentration of 0.5 - 1.0M. The culture was then incubated at 33°C with shaking. When the alternative inducer DMSO was used it was added as a 50% solution in water to a final concentration of 0.75M.

2.11 Collection and Sonication of M. xanthus Samples

Samples were collected and sonicated using methods developed by Hartree (1989).

Starvation samples on TMP plates (Section 2.3.3) were scraped off the agar using a coverslip into 1ml TM buffer (Section 2.3.2). Samples were pelleted in a 2ml Eppendorf tube at 13,000rpm in a microfuge and resuspended in 0.5ml of Z-buffer (Section 2.3.2).

Samples in liquid media were transferred to 2ml Eppendorf tubes and pelleted in a microfuge. As the liquid media usually used was DCY broth (Section 2.3.3) which would affect protein assays, the supernatant was removed as completely as possible. The cells were then washed with 1ml TM buffer before being resuspended in 0.5ml Z-buffer. Samples were stored in 2ml Eppendorf tubes at -20°C. Prior to sonication the samples were thawed at room temperature and kept on ice until needed.

Two methods of sonication were employed. Samples containing spores were sonicated using an MSE sonicator with a 3mm probe at 12 microns. To aid spore breakage 200µl of 0.10-0.11mm glass beads (B. Braun, Melsungen) were added. Samples were sonicated for 15 seconds at a

time with 15 second intervals to allow cooling. This was continued for six minutes to allow adequate spore breakage. The samples were cooled on dry ice throughout sonication.

The second method of sonication was to use a cup horn sonicator (W380 sonicator with a 431B cup horn, Heat Systems Ultrasonics Inc.) with cold water cooling. Two minutes sonication on full output easily disrupted vegetative cells without affecting spores. Up to six samples could be sonicated simultaneously. This method was useful for sonicating samples known to contain only vegetative cells.

To fractionate a sample into separate vegetative cell and spore lysates it was first sonicated in the cup horn sonicator in order to disrupt the vegetative cells. The remaining spores were then collected by centrifugation at 13,000rpm in a microfuge. The pellet was white, indicating that only mature spores were present. The supernatant was removed and kept as the vegetative fraction. The spores were resuspended in 0.5ml Z-buffer and sonicated with glass beads using the probe sonicator as already described. Sonicated samples were kept on ice until assayed the same day for both ß-galactosidase activity and protein content.

2.12 ß-Galactosidase Detection and Quantitative Assay

The enzyme ß-galactosidase was detected *in situ* using either the fluorogenic substrate 4-methyl umbelliferyl galactoside (4-MUG) (Youngman *et al.*, 1982)

or the chromogenic substrate 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal) (Maniatis et al., 1982; Sambrook et al., 1989). 4-MUG was dissolved in DMSO at a concentration of 2mg/ml and sprayed onto plates and the plates left at room temperature for 10-15 minutes. Long wave UV was used to visualise the blue fluorescence of 4-methyl umbelliferone which is released by hydrolysis of 4-MUG.

X-gal was added to solid media at a concentration of 40µg/ml. Plates were incubated at 33°C until the blue colour, produced by the release of indigo, developed.

Quantitative assays were carried out on sonicated samples using ortho-nitrophenyl-ß-D-galactoside (ONPG) using the method of Miller (1972; 1992). Two alterations were made to the method. Firstly, the Z-buffer did not contain ß-mercaptoethanol and secondly a separate blank was used for each sample, containing sonicate with Z-buffer with no ONPG. This was to eliminate the source of error caused by the fact M. xanthus sonicates are a similar yellow colour to that of the ortho-nitrophenol (ONP) released during the assay.

Units of enzyme were defined in terms of nanomoles of ONP produced per minute per milligram of protein (Kroos et al., 1986.) according to the equation:-

$$\frac{213 \times A_{420}}{[\text{ml}][\text{mg protein}][\text{min}]}$$

where 213 is derived from the extinction coefficient of ONP and converts absorbance into nanomoles ONP. [ml] and [mg protein] refer to the amount of sonicate used and

[min] is the time the assay was incubated before stopping the reaction.

2.13 Protein Determination

The amount of protein present in sonicates was assayed with Folin-Ciocalteu reagent using the method of Herbert et al. (1971).

2.14 P1 Mediated Transduction of M. xanthus

2.14.1 Preparation of P1 lysates

A 10^{-2} to 10^{-7} dilution series of phage was made in TM buffer (Section 2.3.2) from an existing phage lysate. E. coli C600 was grown in LB to a density of A_{660} = 0.3. To 0.5ml E. coli C600 culture was added 0.1ml of each phage dilution. The phage was allowed to adsorb for 20 minutes at room temperature and then 3ml molten LGC agar was added and the mixture poured onto a LGC agar plate(Section 2.3.3). After 16 hours incubation at 37°C plaques could be counted. A single plaque was picked into TM buffer (Section 2.3.2) using a pasteur pipette and mixed by vortexing. A dilution series of this phage stock was made and adsorbed to E. coli C600 and spread on LGC as before. Phage was eluted from plates showing almost confluent plaques by pouring 5ml TM buffer onto each plate and incubating at 4°C overnight. The liquid was then removed from the plates, briefly vortexed with a drop of chloroform and centrifuged at 5,000rpm in an MSE Chillspin. The supernatant containing the phage was removed and could be stored at 4°C over chloroform. The

lysate was titred and the process repeated until a titre of 10^9-10^{10} pfu/ml was obtained. This method was obtained from the myxobacteria laboratory manual.

2.14.2 Packaging of Plasmid into P1

Plasmid was packaged into P1 using the method described by Hodgson (1993). The phage used for packaging was P1::Tn*9* *clr100*. The plasmid was maintained in a Rec^+ strain of *E. coli* such as MC1061. The strain was cultured in LB containing the appropriate antibiotic selection. When the culture reached a density of A_{660} = 0.21 the phage were added to 0.5ml of the culture at a multiplicity of infection of ten and allowed to adsorb for 20 minutes at room temperature. The mixture was diluted into LB + 10mM $MgCl_2$ + antibiotic + 12.5μg/ml chloramphenicol. The chloramphenicol was added to select for phage-plasmid cointegrates. The culture was shaken overnight at 30°C. The next day, a one in 50 dilution was made and the culture incubated at 30°C until it reached a density of A_{660} = 0.21. Lysis was induced by transferring the culture to a shaking water bath at 42°C for 35 minutes followed by 37°C for two hours. Cell lysis was apparent after this treatment. To the lysate was added 0.1ml chloroform and shaking continued at 37°C for 15 minutes. After this, cell debris was removed by spinning in a universal bottle for ten minutes in an MSE Chillspin centrifuge at 5,000rpm at 4°C. The lysate could be stored over chloroform at 4°C for one month.

2.14.3 P1 Transduction of M. xanthus

The P1 phage containing packaged plasmid or other insert was used to transduce M. xanthus to kanamycin resistance, as described in Hodgson (1993). Dilutions of phage were made in TM buffer (Section 2.3.2) containing 100, 200 and 400µl of lysate to a total volume of 0.4ml. To each of these was added 0.1ml of 50mM $CaCl_2$ followed by 0.5ml of M. xanthus culture (A_{660} = 0.8-1.0). The phage was left to adsorb for 40 minutes at room temperature before 2.5ml of molten DCY soft agar (Section 2.3.3) was added to each sample which was overlaid on a DCY agar plate containing 20µg/ml kanamycin. The plate was incubated at 33°C overnight and the following day the kanamycin concentration was increased to 70µg/ml using a DCY soft agar overlay. Plates were incubated at 33°C for 3 - 5 days until transductant colonies appeared.

2.15 Myxophage Transduction

2.15.1 Preparation of Myxophage lysates

Serial dilutions from 10^0-10^7 were made from an existing myxophage stock in TM buffer (Section 2.3.2). 100µl of each dilution were then adsorbed to 0.5ml DK101 M. xanthus cells (4-5 x 10^8 cells/ml) for 20 minutes at room temperature. 3ml DCY soft agar (Section 2.3.3) was added to each adsorption reaction and they were poured onto DCY agar plates. After 1 - 2 days incubation at 33°C plaques could be seen. A single plaque was picked into TM buffer using a pasteur pipette. Serial dilutions

were made of this myxophage stock and adsorbed as described above to the M. xanthus strain of which a lysate was to be made. Phage was eluted from plates showing almost confluent plaques by pouring 5ml TM buffer onto each plate and incubating at 4°C overnight. The liquid was removed from the plates and centrifuged at 5,000rpm in an MSE Chillspin. The supernatant containing the phage was removed and stored over chloroform at 4°C. The lysate was titred and if necessary the process repeated until a titre of 10^8-10^9 was obtained. This method was obtained from the myxobacteria laboratory manual.

2.15.2 Myxophage Transduction

0.1 - 0.2ml of myxophage lysate in TM buffer (Section 2.3.2) was UV irradiated for five minutes at 16erg/mm^2/sec . For each transduction reaction 1-2 x 10^8 UV irradiated phage and 1-2 x 10^8 M. xanthus cells in 0.1 - 0.2ml DCY broth were mixed and the phage were allowed to adsorb for 20 minutes at room temperature. 40µl myxophage antisera was added to each reaction mixture, followed by 2.5ml DCY soft agar (Section 2.3.3) and the mixture was immediately poured onto DCY agar containing 50µg/ml kanamycin. The plates were incubated at 33°C for 4 - 6 days until transductant colonies appeared. UV irradiation of phage, and addition of myxophage antisera are necessary to prevent killing of potential transductants by the phage. This method was obtained from the myxobacteria laboratory manual.

2.16 Lambda Packaging

Lambda vectors EMBL3 BamH1 and EMBL4 EcoR1 were obtained from Stratagene. DNA was ligated into these vectors as recommended in the supplier's instructions. Lambda constructs were then packaged using the Gigapack II Gold Packaging Extract Kit from Stratagene in the manner recommended by the suppliers.

2.17 Immobilization of Lambda Plaques on Nitrocellulose Filters

The method used was that described by Maniatis et al. (1982). Nitrocellulose paper was obtained from Amersham.

2.18 Preparation of Lambda Phage DNA

This method was based on that described by Maniatis et al. (1982). Lambda phage DNA preps were always started from a single plaque. Once a discrete plaque had been obtained it was picked into 1ml SM buffer (Section 2.3.2) and a drop of chloroform was added. The sample was left at 4^{o}C overnight to allow the phage to elute into the buffer. 50μl of this phage stock were then adsorbed to 200μl of an overnight culture of E. coli LE392 cells for 20 minutes at 37^{o}C. 3ml TB (Section 2.3.3) containing 0.75% (w/v) agarose was added to the adsorption mixture and it was plated on TB agar. After 16 hours incubation at 37^{o}C almost confluent plaques were visible. The top agar was scraped off five plates into a

250ml centrifuge pot. 5ml SM buffer was used to wash each plate and poured into the centrifuge pot. 33µl chloroform was added, followed by 35µl RNase (1mg/ml) and 35µl DNase (1mg/ml) and the mixture was incubated at 37°C for 30 minutes. 2g NaCl was added and dissolved and the mixture was centrifuged at 8,000rpm for five minutes in an MSE H-21. The supernatant was taken off and transferred to an Oakridge tube where 3g polyethylene glycol 8000 was added and the sample placed on ice for two hours. The sample was centrifuged at 5,000rpm for ten minutes in an MSE H-21 and the supernatant was discarded. The pellet was resuspended in 3ml TM buffer and transferred to a universal. 3ml chloroform was added and the sample was vortexed and centrifuged at 5,000rpm for five minutes in an Wifug Labor-50M bench-top centrifuge. The top layer was removed and divided into 0.75ml aliquots in 1.5ml Eppendorfs. An equal volume of phenol was added to each tube and vortexed. The tubes were then centrifuged at 13,000rpm in a microfuge and the top layer of each sample was transferred to a new Eppendorf and the phenol step was repeated. 300µl 10M ammonium acetate and 800µl isopropanol was added to each sample and the samples were centrifuged at 13,000rpm in a microfuge for 15 minutes to precipitate the DNA. The DNA pellets were resuspended in a total of 200µl TE buffer.

2.19 Preparation of M. xanthus Chromosomal DNA

Chromosomal DNA was isolated by running a caesium chloride gradient of a total lysate of M. xanthus cells,

in the manner described by Hodgson (1993).

A dense 10ml culture of cells (A_{660} = 1) was centrifuged in a universal bottle at 5,000rpm in a Wifug Labor-50M bench-top centrifuge. The supernatant was poured off and the pellet resuspended in 1ml of STE (Section 2.3.2). To this was added and mixed by inversion: 5µl of 20mg/ml proteinase K, 200µl of 0.5M EDTA and 125µl of 10% N-laurylsarcosine. The tube was incubated at 50°C overnight. 28.5g CsCl was added and the volume of the lysate was increased to 36ml with TE (Section 2.3.2). 2ml of 5mg/ml ethidium bromide was added and the mixture was transferred to a 38ml Quickseal ultracentrifuge tube which was centrifuged for 18 hours at 45,000rpm in a VTi50 rotor (Beckman).

The band of chromosomal DNA was removed using a syringe and needle. The ethidium bromide was removed by repeated extraction with isopropanol which had been equilibrated with TE (Section 2.3.2) saturated with NaCl. The CsCl was removed by dialysing against two to three changes of TE for at least ten hours. The resulting DNA solution could then be concentrated, if necessary, by dehydrating the sample through the dialysis bags with polyethylene glycol 8000.

2.20 Isolation of Plasmid from E. coli

2.20.1 Rapid Small Scale Isolation of Plasmid DNA

A simplified version of the boiling method (Maniatis et al., 1982).

Cells were grown overnight as 2cm^2 patches on LB

agar plates. Each patch was scraped off using a toothpick dipped in STET (Section 2.3.2) into an Eppendorf tube containing 0.33ml STET. To this was added 10µl of 0.33mg/ml lysozyme and the mixture was shaken and incubated on ice for 30 minutes. The tube was transferred for three minutes to a boiling water bath that had just gone off the boil. The tube was centrifuged at 13,000rpm in a microfuge for 15 minutes and the pellet removed using a toothpick. The volume of the supernatant was increased to at least 0.3ml with STET and 0.33ml isopropanol was added and the sample mixed. The tube was kept at -20°C for two hours and centrifuged in a microfuge for 15 minutes. The supernatant was removed as completely as possible and the pellet was dried under vacuum. The pellet was dissolved in 50µl TE (Section 2.3.2) and could be stored at -20°C. For restriction analysis 4-6µl of the preparation was used per digest.

2.20.2 Rapid Small Scale Isolation of Plasmid DNA by the Alkaline Lysis Method.

This was used to obtain "cleaner" DNA samples than those achieved by the boiling-water method, and was also used to obtain plasmid DNA from tetracycline resistant strains. The method used was based on that described by Maniatis et al. (1982).

Cells were grown overnight in 3ml LB broth. The culture was transferred to a 1.5ml Eppendorf and centrifuged at 13,000rpm in a microfuge for five minutes.

The supernatant was removed and the cells were resuspended in 0.15ml ice-cold GTE (Section 2.3.2) containing 2mg/ml lysozyme, by vortexing. The cells were incubated at room temperature for five minutes then 0.2ml of a freshly prepared solution of 0.2N NaOH and 1% SDS was added and the tube rapidly inverted two to three times. The tubes were incubated on ice for five minutes or until lysis had occurred and 0.15ml ice-cold potassium acetate buffer pH4.8 (3M with respect to potassium and 5M with respect to acetate) was added and rapidly mixed. The sample was incubated on ice for five minutes, centrifuged in a microfuge at 13,000rpm for five minutes and the supernatant transferred to another Eppendorf tube. To this was added 0.4ml phenol chloroform and the tube was vortexed, then microfuged for two minutes. The supernatant was transferred to another Eppendorf tube and 0.4ml chloroform:isoamyl alcohol 24:1 was added. The tubes were vortexed then centrifuged in a microfuge for two minutes and the supernatant was collected. To this was added 1ml absolute ethanol and the sample was vortexed briefly and incubated on ice for five minutes. It was centrifuged in a microfuge for ten minutes and the supernatant was discarded. To the tube containing the pellet was added 1ml 70% ethanol without agitating the pellet and the tube was microfuged for five minutes. After centrifugation the supernatant was removed as completely as possible and the pellet dried under vacuum. The pellet was dissolved in 50μl TE and could be stored at 4^{o}C. For restriction analysis 4-6μl of the

preparation was used per digest.

2.20.3 Large Scale Preparation of Plasmid DNA

Larger quantities of plasmid were prepared by purification on a caesium chloride gradient (based on Maniatis et al., 1982).

Cells were grown in a litre LB broth (Section 2.3.3) and when the culture had reached an optical density of 0.9 at 660nm, 5ml of 34mg/ml chloramphenicol was added and incubation at 37°C continued overnight. The culture was centrifuged in 250ml pots at 5,000rpm in an MSE H-21 for 15 minutes and the supernatant was discarded. The pellets were resuspended in 20ml TES (Section 2.3.2) and transferred to an MSE Oakridge tube. The cell suspension was centrifuged at 5,000rpm in an MSE H-21 for ten minutes, the supernatant was removed and the cells were resuspended in 10ml STE (Section 2.3.2). The mixture was vortexed vigorously and stored at -20°C. After thawing, 1ml of 10mg/ml lysozyme was added and the mixture was incubated on ice for ten minutes before addition of 2.5ml 0.5M EDTA and incubation on ice for a further ten minutes. 16ml Lysis mix (Section 2.3.2) was added and the tube mixed by rocking and kept on ice for 20 minutes. The sample was centrifuged at 19,000rpm for 35 minutes in an MSE H-21 and the supernatant was decanted through muslin into a measuring cylinder containing 28.5g CsCl. This was mixed by rocking and made up to 38ml with TES, then transferred to Oakridge tubes. To this was added 2ml of 5mg/ml ethidium bromide and the suspension was

kept on ice for an hour. It was centrifuged at 15,000rpm at 4°C in an MSE H-21 for 15 minutes and the supernatant was decanted into a 38ml Quickseal tube by pouring through a layer of glass wool in a syringe, to filter out any solids. The tube was centrifuged for at least 16 hours at 45,000rpm in a VTi50 rotor (Beckman). The supercoiled DNA was clearly visible, either as the lower of two bands or as the only band. The band, containing the supercoiled circular DNA, was collected with a syringe and needle in a volume of approximately 5ml. This was transferred to a 5ml Quickseal tube which was centrifuged for at least five hours at 55,000rpm in a VTi65 rotor (Beckman). The lower band was again collected and the ethidium bromide was removed by repeated extraction with isopropanol which had been equilibrated with TE (Section 2.3.2) saturated with NaCl. To the sample was added two volume units of sterile distilled water and six volume units ethanol and it was stored at -20°c overnight. The sample was centrifuged at 15,000rpm for 60 minutes in an MSE H-21. The supernatant was removed as completely as possible and the pellet was dried under vacuum. The pellet was dissolved in 0.2ml TE.

2.21 Transformation of E. coli

Cells were transformed by plasmid DNA using a method based on the calcium chloride transformation method of Maniatis et al. (1982). A 0.1ml sample of an overnight culture of cells was diluted in 30ml LB broth (Section

2.3.3) in a 100ml flask and incubated at 37°C with vigorous shaking. When the cells had reached a density of A_{660} = 0.2 (for *recA*$^+$ strains) the culture was chilled on ice for ten minutes. The culture was divided between prechilled plastic universals and centrifuged in an MSE Chillspin at 4,500rpm for ten minutes. The cells were resuspended in half the original volume of an ice-cold, sterile solution of 50mM $CaCl_2$ and 10mM MOPS (morpholinopropanesulfonic acid) pH6.8, and chilled on ice for 15 minutes. The suspension was centrifuged as before and resuspended in 1/15 the volume of 50mM $CaCl_2$ and 10mM MOPS pH6.8. This mixture was dispensed in 0.2ml aliquots into prechilled 1.5ml Eppendorf tubes and stored at 4°C overnight. The DNA (not more than 40ng/aliquot) was added and the tubes were kept on ice for 30 minutes. Following heat shock at 42°C for two minutes, 1ml LB broth was added to each tube. The contents of each tube was transferred to a bijou bottle and incubated at 37°C without shaking for 60 minutes. To each bijou bottle was added 3ml of soft LB agar and the mixtures were poured onto well dried LB agar plates containing the appropriate antibiotic and allowed to set. Transformant colonies were obtained after overnight incubation at 37°C.

2.22 Restriction Endonuclease Digestion

Restriction digests were carried out using the conditions recommended by the suppliers. In addition to the recommended buffers 1mM dithiothreitol (DTT), 4.0mM spermidine and 0.1mg/ml Bovine Serum Albumin (BSA) were

also added to the mixtures.

2.23 Ligations

T4 DNA ligase (BRL) was used with the buffer recommended by the suppliers. Reactions were always carried out at 15°C overnight. The concentrations of insert and vector were those recommended by Legerski and Robberson (1985), and the reaction volume was always 20µl.

2.24 Phenol Extractions

Protein was removed from DNA using Darbyshire's Reagent, in a manner based on the methods described by Maniatis et al. (1982). It consisted of a solution of 100g phenol, 100ml chloroform, 4ml isoamyl alcohol and 0.1g 8-hydroxyquinoline. It was equilibrated with 2 x 40ml changes of TE (Section 2.3.2), and stored under TE at 4°C. It was allowed to reach room temperature before use.

Phenol extraction was typically used to remove restriction or other DNA modifying enzymes before proceeding to the next stage of a preparation. The volume of the solution was increased to at least 0.1ml and it was vortexed with an equal volume of Darbyshire's Reagent. The upper aqueous layer was removed and extracted twice with diethylether saturated with water. The DNA was precipitated with ethanol.

2.25 Ethanol Precipitation

Ethanol precipitation of DNA was carried out according to Maniatis et al. (1982). Sodium acetate was added to a final concentration 0.3M. Three volumes of ethanol was added and the sample was stored at -20°C for two hours.

2.26 Agarose Gel Electrophoresis

Flat bed agarose gels were run as described by Maniatis et al. (1982). The buffer used was TBE (Section 2.3.2). All gels were run with ethidium bromide in the buffer (0.5µg/ml).

2.27 Elution of DNA from Agarose Gels

Bands containing DNA were cut out of gels and eluted into TE (Section 2.3.2) using a Gene clean kit II from Bio 101 used according to the supplier's instructions.

Where the fragment to be eluted was less than 500 base pairs or larger than 15kb, the DNA was eluted into 7.5M ammonium acetate using an IBI (International Biotechnologies Inc.) electroeleuter used according to manufacturers' instructions.

2.28 Labelling of DNA by Nick Translation

The method was based on that of Maniatis et al. (1982). DNaseA was added to the reaction (1ng/µl). The reaction was incubated at 14°C for three hours. The reaction was stopped by adding 2µl of stop buffer (0.5% (w/v) bromo-phenol blue, 0.5% (w/v) xylene cyanole, 2.0%

(w/v) SDS, 60.0% (w/v) glycerol). Denatured salmon sperm was added as a carrier and the unincorporated radionucleotides were removed on a spin column containing Sephadex G25 in TE (Figure 2.1). The labelled DNA could be stored frozen until ready for use.

2.29 Southern Transfer

DNA in agarose gels was transferred to nitrocellulose membrane by Southern transfer. The method was that described by Maniatis et al. (1982). Nitrocellulose paper was obtained from Amersham.

2.30 Hybridization Conditions

Nitrocellulose filters were hybridized with radioactive probe labelled by nick translation. The method was based on that of Maniatis et al. (1982) for hybridization in the absence of formamide. All hybridizations were carried out in a Hybaid Oven. The solution used for both hybridizing and prehybridizing was 5x SSC (Section 2.3.2), 0.02M Tris-HCl pH7.4, 0.5% SDS and 0.1mg/ml denatured salmon sperm DNA. The nitrocellulose was prehybridized for an hour in 10ml of this solution at 65^{o}C. The probe, labelled by nick translation, was denatured by heating at 100^{o}C for five minutes and added to 10ml of hybridization solution. The nitrocellulose was hybridized overnight at 65^{o}C. The following day, the nitrocellulose was washed in two changes of 2 x SSC for 15 minutes, followed by two changes of 2 x SSC + 0.1% SDS for 15 minutes and 0.1 x

Fig. 2.1

Spin Column Technique Used to Purify DNA Labelled by Nick Translation.

a) shows the method used to prepare a G25 spin column. The assembly was then spun in an MSE bench-top centrifuge with a swinging bucket rotor at 2,500rpm.

b) shows the arrangement for removing unincorporated nucleotides from the labelled probe. The 0.5ml Eppendorf contained compacted Sephadex G25. This tube was placed in an intact 1.5ml Eppendorf tube and the nick translation reaction was pipetted onto the column. The cap was closed and the assembly was spun at 2,500rpm for five minutes.

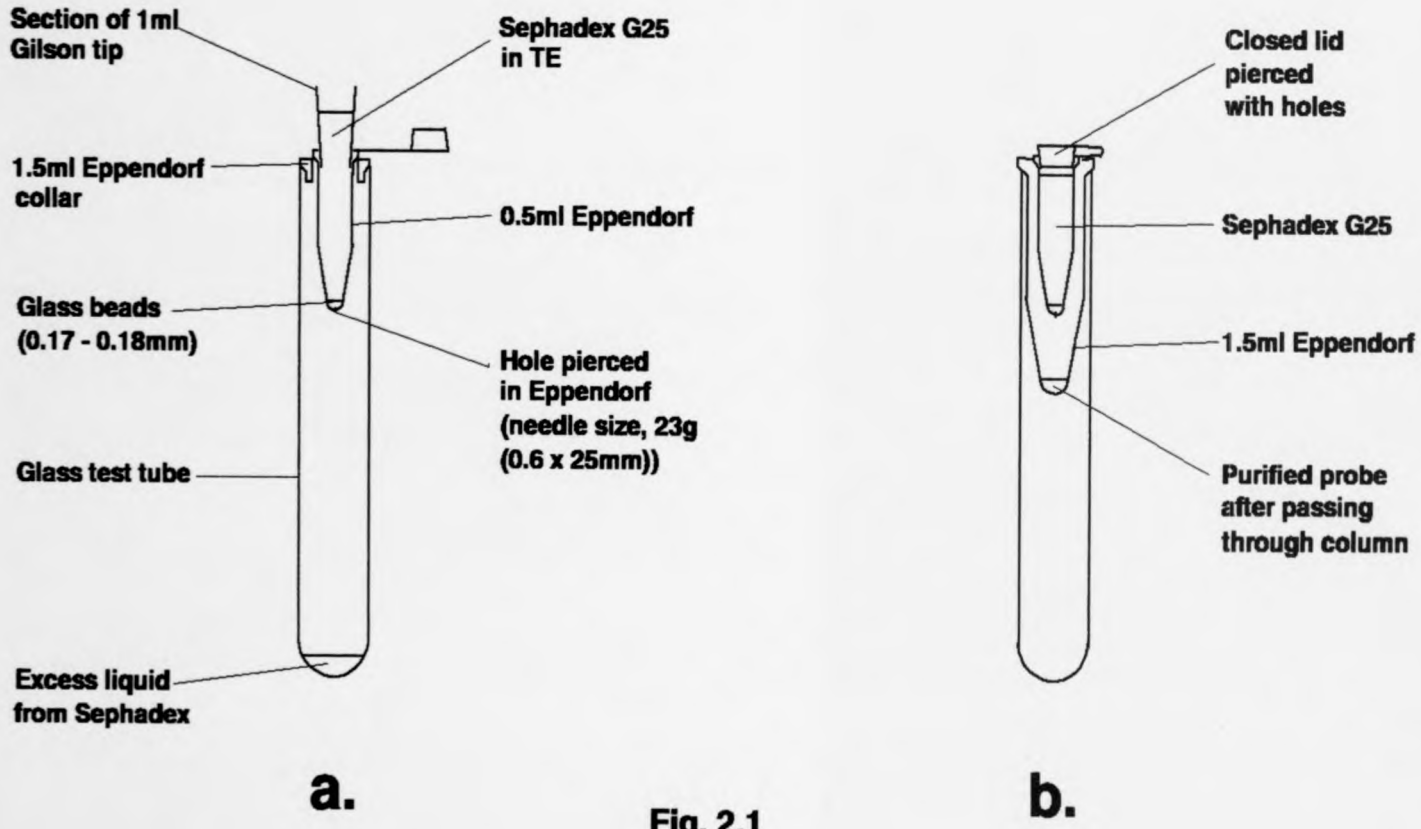

Section of 1ml Gilson tip
Sephadex G25 in TE
1.5ml Eppendorf collar
0.5ml Eppendorf
Glass beads (0.17 - 0.18mm)
Hole pierced in Eppendorf (needle size, 23g (0.6 x 25mm))
Glass test tube
Excess liquid from Sephadex
a.
Closed lid pierced with holes
Sephadex G25
1.5ml Eppendorf
Purified probe after passing through column
b.

Fig. 2.1

SSC for ten minutes. All washes were carried out at 65°C and all wash solutions were prewarmed.

The nitrocellulose filter was air dried, sealed in a polythene bag and exposed to X-ray film at -70°C.

2.31 Alkaline Phosphatase Reactions

Alkaline phosphatase reactions were performed in order to prevent vector fragments with compatible ends from self-ligating (Maniatis et al., 1982). Calf intestinal alkaline phosphatase was used. 2-5μg DNA in TE was made up to 48μl volume with 10 x reaction buffer and water. 1μl calf intestinal alkaline phosphatase was added, followed by a further 1μl after 15 minutes incubation. Fragments with blunt ends or 3' overhangs were incubated at 37°C for 15 minutes followed by 55°C for 45 minutes. Fragments with 5' overhangs were incubated at 37°C for 30 minutes.

2.32 End-filling Reactions

End-filling reactions were carried out using the method described by Maniatis et al. (1982). 2-3μg DNA was mixed with 0.05mM deoxynucleotides and 10 x polymerase buffer and made up to 30μl volume. One unit of the Klenow fragment was added to the reaction mixture and it was incubated at room temperature for 30 minutes.

Chapter 3

Isolation of Glycerol-Resistant Mutants from the Wild-Type DK1622 and the Non-motile Strain DK306

3.1 Introduction

Development in *M. xanthus* usually occurs under starvation conditions, when cells aggregate into fruiting bodies and spores form within. This will only occur if the cells are at a high enough density and are located on a solid surface. However, spore formation can also be induced by adding high concentrations of low molecular weight compounds such as glycerol (Dworkin and Gibson, 1964) or dimethylsulphoxide (DMSO) (Zusman in Bacon and Rosenberg, 1967) to exponential phase cultures of *M. xanthus* growing in complex liquid medium. The best inducers of sporulation are fully saturated aliphatic compounds containing two to four carbons with at least one primary or secondary alcohol group (Sadler and Dworkin, 1966). In a series of primary aliphatic alcohols, there is an optimal chain length for effectiveness, and the effective concentration required for induction decreases with increasing chain length. High concentrations of non-penetrating solutes such as glucose do not induce spore formation, suggesting that induction is not the result of a simple osmotic effect (Sadler and Dworkin, 1966).

Glycerol is the most commonly used inducer of sporulation and was used throughout this study. Glycerol-induced sporulation is very rapid and synchronous, taking about two hours for the formation of spherical spores in complex media (Dworkin and Gibson, 1964). Aeration and either magnesium or calcium ions are essential for glycerol-induced sporulation. Induction is

independent of the cell cycle and does not require a solid surface. However, it has been observed that approximately 1% of cells in a glycerol-induced population fail to form myxospores (Dworkin and Sadler, 1966). The mechanism of glycerol-induction is uncertain. Glycerol was shown to be incorporated by the cells at a very low rate, but other inducers such as ethylene glycol were not incorporated by the cells at all. These observations suggested that morphogenesis may be brought about by a continuous interaction between the inducer and a peripheral structure of the cell envelope (Sadler and Dworkin, 1966). The presence of glycerol is required continuously until the appearance of non-refractile spores. Removal of glycerol at any stage prior to this results in the exact reversal of the sequence of morphological changes leading to spore formation, so that the cells revert to vegetative rods. Hence, it appears that glycerol is not acting as a simple trigger but is required to maintain a continuous altered state of the cell structure or organisation (Dworkin and Sadler, 1966).

Mutants unable to form myxospores in response to 0.5M glycerol have been isolated (Burchard and Parish, 1975) by spreading mutagenized cultures of M. xanthus cells onto complex media containing 0.75M glycerol. Mutants unable to form spores continue to grow and form colonies and are described as glycerol-resistant (Gly^r). Glycerol-resistant mutants of M. xanthus FB_t arose spontaneously at a frequency of 1-3 x 10^{-5} and

ultraviolet mutagenesis increased this frequency to a maximum of 7% of the survivors.

Four glycerol-resistant mutants were examined and found not to form myxospores in response to the alternative inducers ethylene glycol and DMSO (Burchard and Parish, 1975). However, all four mutants produced myxospores with low efficiency in response to phenylethyl alcohol. This suggests that ethylene glycol, DMSO and glycerol all induce sporulation by the same mechanism while phenylethyl alcohol appears to induce sporulation by some other means. Alternatively, phenylethyl alcohol may have a different receptor on the cell surface to that of glycerol, ethylene glycol and DMSO.

Of 117 glycerol-resistant mutants tested, 109 formed fruiting bodies containing mature spores (Burchard and Parish, 1975). Hence, mutations to the glycerol-resistance phenotype do not normally block myxospore formation within the fruiting body sporulation pathway. This may suggest that induction of fruiting body sporulation and glycerol-induced sporulation use different pathways involving separate genes. This is consistent with the observation that only a minority of mutants isolated for the inability to sporulate under starvation conditions were also unable to sporulate when glycerol was added to liquid medium (Hagen et al., 1978; Morrison and Zusman, 1979).

Isolation of a glycerol-inducible promoter from M. xanthus and analysis of its expression in different genetic backgrounds (Hartree, 1989) led to the hypothesis

that there may be two pathways of starvation-induced sporulation in M. xanthus: (1) Fruiting body sporulation and (2) Fruiting-independent sporulation (sporulation outside of fruiting bodies), as shown in Figure 1.6. It is thought that both these pathways of sporulation can occur simultaneously in a population of M. xanthus cells under starvation conditions and that the proportion of spores formed by each of the two pathways is at least partially dependent on the motility properties of the cells, and the cell density. It was also proposed that chemical-induced sporulation "short circuited" the fruiting-independent sporulation pathway.

There were two aims to this area of work. Firstly to isolate glycerol-resistant mutants from the wild-type strain and look at the ability of these mutants to undergo fruiting body sporulation. This was to confirm the observations of Burchard and Parish (1975) that only a minority of glycerol-resistant mutants were also unable to undergo fruiting body sporulation and, therefore, that glycerol-induced sporulation and fruiting body sporulation do not share the same pathway of induction. The second aim was to isolate glycerol-resistant mutants from the non-motile, mgl, strain DK306. DK306 cells are unable to aggregate into fruiting bodies. However, Kim and Kaiser (1990a) reported that spores were still formed on starvation of non-motile cells. These spores are proposed to have been formed by the fruiting-independent pathway. Glycerol-resistant mutants derived from DK306 were then analysed for their ability to form spores in

the absence of fruiting bodies. This work was carried out in order to ascertain whether mutants with a block in the glycerol-induced sporulation pathway were also blocked in starvation-induced, fruiting-independent sporulation.

3.2 Isolation of Glycerol-Resistant Mutants from the Wild Type Strains DK101 and DK1622

Before attempting mutagenesis it was necessary to have an adequate system for selection of glycerol-resistant mutants. Therefore, the wild-type strains, DK101 and DK1622, and several glycerol-resistant mutants isolated previously (Andreasson, Blea and Kaiser, personal communication) were streaked onto DCY agar (Section 2.3.3) containing 0.5M, 0.75M and 1.0M glycerol. Media containing 0.5M and 0.75M glycerol still permitted some growth of the wild-type strains DK101 and DK1622. Concentrations of 1.0M glycerol allowed growth of the glycerol-resistant mutants but almost completely inhibited growth of the glycerol-sensitive wild-type strains. Hence, it was decided to use DCY agar containing 1.0M glycerol as the media to select for glycerol-resistant mutants.

Both DK101 and DK1622 could be used for isolation of glycerol-resistant mutants. However, preliminary experiments into methods for screening for fruiting ability showed that DK1622 underwent fruiting body sporulation more quickly and was easier to analyse for fruiting defects than DK101. In DK101 fruiting body

sporulation is delayed due to a partial motility defect in the social motility system. Hence, DK1622 was used as the parental strain for mutagenesis experiments.

Mutagenesis of M. xanthus DK1622 was carried out on exponential phase DCY broth (Section 2.3.3) cultures using the mutagens N-methyl-N'-nitro-N-nitrosoguanidine (NTG), ethyl methyl sulphonate (EMS) and ultraviolet (UV) as described in Sections 2.5, 2.6 and 2.7 respectively. Glycerol-resistant mutants were selected for on DCY agar containing 1.0M glycerol. After 4 - 5 days growth single colonies were picked from the selection plates onto fresh DCY media containing 1.0M glycerol to ensure that the colonies were truly glycerol-resistant. 1179 glycerol-resistant mutants were isolated using NTG, 1234 using EMS and 1118 using UV. For the mutagenesis conditions used the frequency of isolation of glycerol-resistance was as follows; 3.5×10^{-5} for NTG; 2×10^{-6} for EMS and 1.5×10^{-6} for UV. Spontaneous mutants were found to arise at a frequency of 2×10^{-7} when mutants were selected for on DCY agar containing 1.0M glycerol and 211 glycerol-resistant mutants were isolated. This is a lower frequency of spontaneous mutation than recorded by Burchard and Parish (1975). However, this is probably primarily because they selected for glycerol-resistant mutants on media containing 0.75M glycerol as oppose to the final concentration of 1.0M glycerol used in this study.

In order to assess the fruiting ability of the glycerol-resistant mutants, they were picked onto CF

(Clone-fruiting) agar (Section 2.3.3). CF agar contains 0.015% casitone, which is just enough to allow a single M. xanthus cell to replicate to a high enough cell density to allow fruiting body formation once starvation of the cells occurs. After several days incubation at 33^{o}C the colonies were examined for fruiting bodies using a plate microscope. Colonies may not have undergone fruiting body formation for one of two reasons; (1) because the cells contain a mutation which blocks the fruiting body sporulation pathway or (2) because the cells had not grown to a high enough density for fruiting body formation once starvation began. Colonies where fruiting body formation had not occurred were inoculated into 5ml DCY broth in a 50ml conical flask and incubated with shaking at 33^{o}C overnight. The cells were sedimented at 4,500rpm in a Wifug Labor-50M bench-top centrifuge, washed in 5ml TM buffer (Section 2.3.2) and resuspended in 0.5ml TM buffer. 20μl aliquots were spotted on to TM agar and allowed to dry into the agar. The plates were incubated at 33^{o}C for several days to confirm the fruiting phenotypes of the glycerol-resistant mutants. The results are shown in Figure 3.1. Only a small minority (0.08 - 1.69%) of the glycerol-resistant mutants were unable to form fruiting bodies containing mature myxospores.

In order to further assess the ratio of common genes between glycerol-induced sporulation and fruiting body sporulation, mutations in fruiting body sporulation were screened for without prior selection for glycerol-

Frequency of Fruiting Mutations in Glycerol-Resistant Mutants Derived from DK1622

MUTAGEN	No. of glycerol-resistant mutants	No. non-fruiting	Percent non-fruiting
EMS	1,234	1	0.08%
NTG	1,179	20	1.69%
UV	1,118	2	0.18%
Spontaneous	211	0	0.0%

Frequency of Fruiting Mutations in Colonies Not Selected for Glycerol-Resistance

MUTAGEN	No. of colonies screened	No. non-fruiting	Percent non-fruiting
EMS	1,192	0	0.0%
NTG	1,203	4	0.33%
UV	2,236	2	0.09%

Fig. 3.1 The Frequency of Mutations in the Fruiting Phenotypes of Glycerol-Resistant Mutants and Non-resistant Colonies.

resistance. Mutagenized cultures of M. xanthus DK1622 were diluted serially from 10^{-3} - 10^{-7} and plated in 100μl aliquots onto DCY agar with no selection. Single colonies were picked from these plates after 3 - 4 days incubation at 33^{o}C and screened for fruiting ability on CF agar as before. The results are also shown in Figure 3.1. The frequency of fruiting mutations which arose in cells which had not been previously selected for glycerol-resistance was only slightly lower than that observed in glycerol-resistant mutants. The results suggest that most of the lesions in the glycerol-resistant mutants were in genes involved solely in glycerol-induced sporulation and these findings are consistent with the observations of Burchard and Parish (1975). This data suggests that the pathways of induction of glycerol-induced sporulation and fruiting body sporulation share few, if any, common genes.

For NTG mutagenesis, the frequency of fruiting defects in glycerol-resistant mutants is higher than the frequency of fruiting defects in cells which had not been selected for glycerol-resistance prior to screening for fruiting mutations. However, NTG is known to generate a higher frequency of double site mutations than EMS or UV, and this probably accounts for the higher frequency of glycerol-resistant, non-fruiting mutants attained using NTG mutagenesis. Evidence that glycerol-resistant, non-fruiting mutants are the result of double site mutations is given in Chapter 6.

3.3 Isolation of Glycerol-Resistant Mutants from the Non-motile (mgl) Strain DK306

Glycerol-resistant mutants of DK306 were selected as this strain is non-motile and is, therefore, unable to aggregate into fruiting bodies. Kim and Kaiser (1990a) reported that despite the absence of fruiting bodies, spores were still formed when non-motile cells were starved. These spores formed in the absence of fruiting bodies are proposed to have been formed by the starvation-induced, fruiting-independent pathway. If there are common genes between the induction pathways of glycerol-induced sporulation and starvation-induced, fruiting-independent sporulation, one might expect that a proportion of glycerol-resistant mutants derived from DK306 would be unable to form spores by the starvation-induced, fruiting-independent pathway. As DK306 cells are unable to form fruiting bodies it is easy to assess whether fruiting-independent sporulation occurred when the cells were starved.

Glycerol-resistant mutants of DK306 were isolated using UV mutagenesis as described in Section 2.7 and were selected for on DCY agar containing 1.0M glycerol. Single colonies were picked from the selection plates onto fresh DCY agar containing 1.0M glycerol in order to confirm the glycerol-resistance of the colonies. 2011 glycerol-resistant colonies derived from DK306 were obtained.

In order to accurately assess whether the glycerol-resistant mutants derived from DK306 were still able to

sporulate using the starvation-induced, fruiting-independent pathway it was necessary to develop a method for screening for the presence of spores, as it was obviously impractical to analyse over 2000 mutants under the microscope. Sporulation in non-motile cells requires the alignment of cells in order to allow the transmission of C factor between the cells (Kim and Kaiser, 1990a). Therefore, the media used for screening for fruiting-independent sporulation must allow the cells to grow up sufficiently that some cell alignment occurs. Therefore, CF agar and TMP containing 0.015% casitone (TMPC) were used initially to assess which allowed better starvation-induced, fruiting-independent sporulation. Ten colonies of DK306 cells, from a fresh streak plate, were inoculated onto CF and TMPC agar and incubated at 33^{o}C for seven days. The plates were incubated at 55^{o}C for 2½ hours to kill vegetative cells and overlaid with DCY soft agar. Overlaying the plates with complex media soft agar allowed the heat-resistant spores to germinate. After 4 - 5 days incubation at 33^{o}C the plates were examined for growth which would indicate that sporulation and germination had occurred. All ten sample spots contained cells that had sporulated and germinated on TMPC agar, while only five out of ten sample spots on CF agar showed signs of survival of 55^{o}C temperatures. Therefore, it was decided to use TMPC as the starvation media for fruiting-independent sporulation. To avoid displacement of some of the spores during the overlay step, each individual mutant to be tested was inoculated into a

separate well of a 10cm square 25 well plate containing TMPC agar. The final assay method is shown in Figure 3.2.

All 2011 glycerol-resistant mutants derived from DK306 were inoculated onto TMPC agar in 10cm square 25 well plates and the plates were incubated at 33°C for seven days. Following incubation at 55°C for 2½ hours each well of the plates was overlaid with 1ml DCY soft agar. The plates were incubated at 33°C for 4 - 5 days and were then examined for signs of germination of spores. Mutants that gave rise to no signs of growth could be either sporulation or germination mutants. Such mutants were inoculated onto TMPC agar again. After seven days incubation at 33°C samples of cells were examined under the microscope for the presence of spores. The results are shown in Figure 3.3. Only one out of 2011 (approximately 0.05%) of the glycerol-resistant mutants derived from DK306 was also unable to undergo starvation-induced, fruiting-independent sporulation. This mutant, designated HMC300, is of interest as it was completely deficient in sporulation, i.e. it was unable to undergo glycerol-induced sporulation, fruiting body sporulation or starvation-induced, fruiting-independent sporulation. However, this strain may only be deficient in fruiting body sporulation as a result of the mgl motility defect, which renders the cells unable to aggregate.

As before, a number of colonies were screened for mutations in fruiting-independent sporulation without

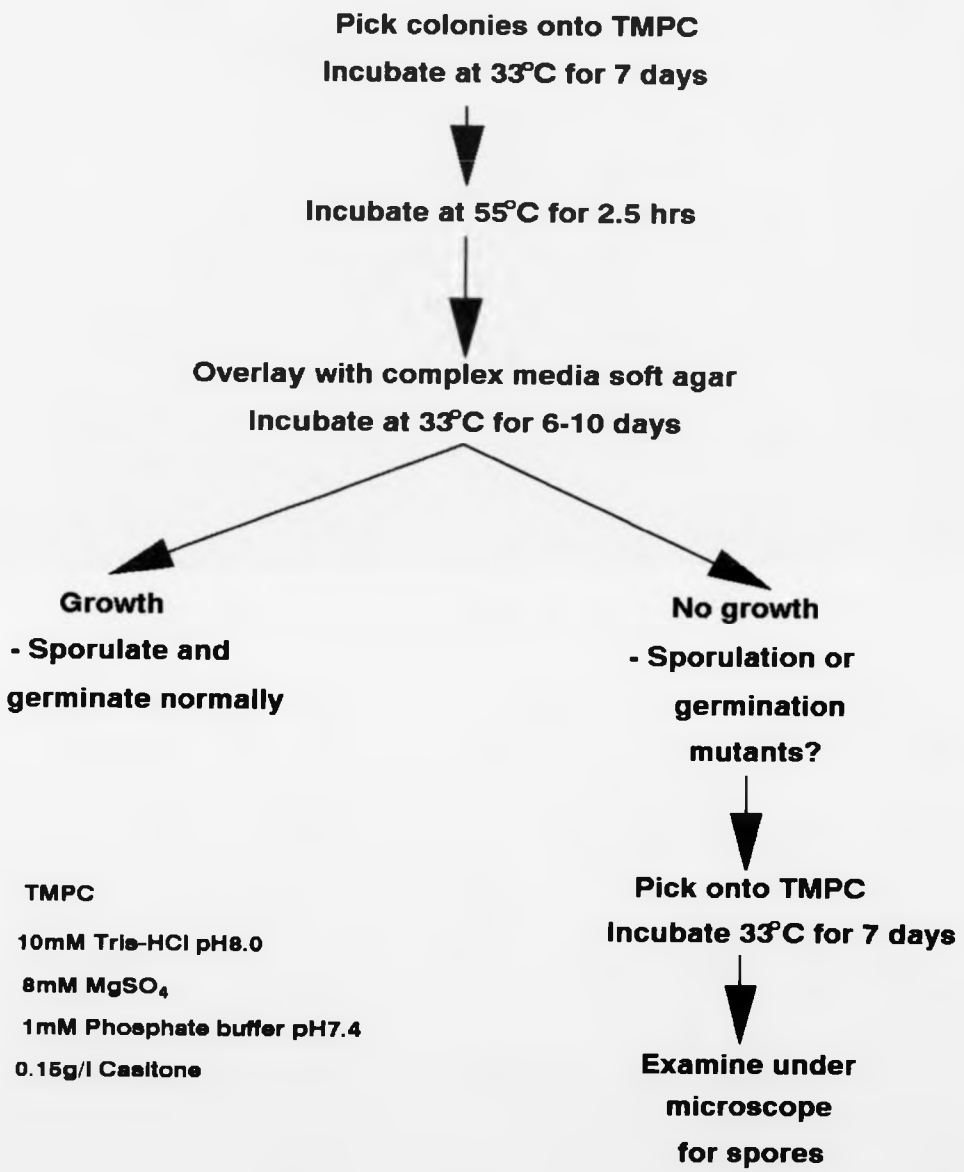


Fig. 3.2 Method for Screening for Fruiting-Independent Sporulation.

Frequency of Fruiting-Independent Mutations in Glycerol-Resistant Mutants Derived from the Non-motile (mgl) Strain DK306

MUTAGEN	No. of glycerol-resistant mutants	No. Non-sporulating	Percentage non-sporulating
UV	2,011	1	0.05%

Frequency of Fruiting-Independent Mutations in Colonies Not Selected for Glycerol-Resistance

MUTAGEN	No. Colonies screened	No. Non-sporulating	Percentage non-sporulating
UV	2,044	1	0.049%

Fig. 3.3 The Frequency of Mutations in Fruiting-Independent Sporulation in Glycerol-Resistant Mutants Derived from DK306.

prior selection for glycerol-resistance. Of 2044 colonies screened only one (0.049%), designated HMC301, was unable to undergo fruiting-independent sporulation, as shown in Figure 3.3. This frequency is almost identical to the frequency of glycerol-resistant mutants unable to undergo fruiting-independent sporulation. These data suggest that there are very few common genes in the pathways of induction of glycerol-induced sporulation and starvation-induced, fruiting-independent sporulation.

3.4 Discussion

It has been suggested that glycerol acts directly on the mechanism of myxospore formation that normally operates within fruiting bodies (Witkin and Rosenberg, 1970). However, previous data were limited to two strains which did not form glycerol myxospores in shake culture, one of which did not form fruiting bodies and the other formed rudimentary fruiting bodies without myxospores (Dworkin and Sadler, 1966). Burchard and Parish (1975) isolated 117 glycerol-resistant mutants of which only eight (6.84%) were unable to form fruiting bodies. In this study 3742 glycerol-resistant mutants were isolated of which only 23 (0.61%) were unable to form fruiting bodies containing mature myxospores. It would appear, therefore, that the pathways of induction of glycerol-induced sporulation and fruiting body sporulation share few, if any, common genes.

The isolation of a glycerol-inducible promoter

(Hartree, 1989) and analysis of its expression in different genetic backgrounds led to the hypothesis that there are two pathways of starvation-induced sporulation; (1) fruiting body sporulation and (2) fruiting-independent sporulation. The glycerol-inducible promoter was also expressed when spores are forced to form outside of fruiting bodies. This led to the proposal that glycerol may act on the induction pathway involved in fruiting-independent sporulation. However, of 2011 glycerol-resistant mutants derived from DK306 only one (0.05%) was unable to undergo starvation-induced, fruiting-independent sporulation. This would suggest that there are very few common genes in the pathways of induction of glycerol-induced sporulation and starvation-induced, fruiting-independent sporulation. The pathway of chemically-induced sporulation must, therefore, be independent of starvation-induced, fruiting-independent sporulation. As at least one gene is induced both by chemical-induced sporulation and starvation-induced, fruiting-independent sporulation there must be at least two independent "receptors" for initiation of the pathway.

The mutant HMC300 was of interest as it was completely deficient in sporulation i.e. it was unable to undergo glycerol-induced sporulation, fruiting body sporulation or starvation-induced, fruiting-independent sporulation. However, HMC300 may only be deficient in fruiting body sporulation as a result of the *mgl* motility defect which renders the cells unable to aggregate. It

would be of interest to transduce a Tn*5*-linked *mgl*$^+$ gene into HMC300 which should restore motility. The resultant transductants could then be assessed for fruiting body sporulation. This would allow one to determine whether HMC300 is deficient in fruiting body sporulation because of the motility defect, or is a true sporulation-deficient mutant. The mutant HMC301 was glycerol-sensitive but was unable to undergo starvation-induced, fruiting-independent sporulation or fruiting body sporulation. This mutant should be examined for glycerol-induced spore formation in liquid culture to determine if spores are indeed formed. If the mutation which results in the block in starvation-induced, fruiting-independent sporulation were in a gene encoding for a structural or physical property of spores the mutant might be unable to sporulate by any means. Such a mutant would not necessarily be glycerol-resistant, since the mutation may be in a gene which is not activated until after the glycerol signal has been recognised and the sporulation pathway initiated. HMC301 should also be transduced back to *mgl*$^+$, using a Tn*5*-linked *mgl*$^+$ gene, and the transductants assessed for fruiting body sporulation, in order to ascertain whether fruiting body formation and sporulation is blocked by the mutation. Isolating mutants from the non-motile strain, DK306, which are unable to form spores in the absence of fruiting bodies might provide a method for isolating mutants which are truly deficient in sporulation.

Chapter 4

Southern Blot Analysis of the glrA and glrB Regions

4.1 Introduction

Several mutants have been isolated which are unable to aggregate and form mature fruiting bodies under starvation conditions (Hagen *et al*., 1978). A small minority of these mutants have been found to be also unable to undergo glycerol-induced sporulation, i.e. are glycerol-resistant (Gly^r). In this work the following mutants were studied; DK440 (*glrA1*, *bsg-440*) (LaRossa *et al*., 1983), DK510 (*glrA2*, Fru^-) (Hagen *et al*., 1978), DK3516 (*glrB1*, Fru^-) and DK3551 (*glrB2*, Fru^-). The mutations leading to glycerol-resistance in these mutants have been mapped to two unlinked loci, *glrA* and *glrB*, using Mx*8* transduction (Andreasson, Blea and Kaiser, personal communication). The *glr* genes were linked to two unlinked Tn*5* markers; ΩDK3517 (*glrB*) and ΩDK3554 (*glrA*). Preliminary maps of the two loci are shown in Figure 1.5. Preliminary Mx*8* transduction data also revealed that the mutations causing the glycerol-resistant (Gly^r) and non-fruiting (Fru^-) phenotypes were not 100% linked (Andreasson, Blea and Kaiser, personal communication).

In order to clone genomic DNA from around the Tn*5* markers ΩDK3517 and ΩDK3554, the wild-type, kanamycin resistant transposons were replaced with a modified Tn*5*, Tn*5*-132. Tn*5*-132 is a variant of Tn*5* in which the kanamycin resistance determinant has been replaced by tetracycline resistance, but most of the IS*50* elements that comprise the long terminal inverted repeats of Tn*5* have been retained (Rothstein *et al*., 1981). The *in situ*

replacement of Tn*5* was accomplished by infecting the Tn*5*-containing strain with P1::Tn*5*-132 and selection for resistance to tetracycline. The Tn*5*-132 can "hop" into the chromosome resulting in a kanamycin resistant, tetracycline resistant strain, or undergo homologous recombination with the endogenous Tn*5*. Recombination will be restricted to the IS*50* regions, since these are the only sequences of shared homology. In those cells where recombination occurs the kanamycin resistance of the endogenous Tn*5* will be replaced by resistance to tetracycline, and thus the cells will be tetracycline resistant but sensitive to kanamycin. This replacement results in the strain having a single transposon insertion at precisely the same chromosomal position as Tn*5* yet being distinguishable on the basis of its antibiotic resistance (Avery and Kaiser, 1983).

Having replaced the Tn*5* with Tn*5*-132, the *M. xanthus* DNA adjacent to the Tn*5* insertion can be cloned using the integrative plasmid pREG429 (Gill *et al*, 1988). pREG429 is capable of replication in *E. coli* but not in *M. xanthus*. In addition to the plasmid replication functions and ampicillin resistance derived from pBR322, this plasmid contains a fragment of bacteriophage P1 which confers P1 incompatibility (Sternberg and Hoess, 1983), and a portion of Tn*5* which encodes kanamycin resistance, a strong selectable marker in both *E. coli* and *M. xanthus*, and also contains most of the left IS*50* element. Hence, pREG429 can be easily transferred from *E. coli* to *M. xanthus* by P1 specialized transduction and

once introduced into *M. xanthus* the left IS*50* element provides the homology to integrate the plasmid at chromosomal Tn*5*-132 insertion sites in the *M. xanthus* chromosome.

The strategy used to clone *M. xanthus* DNA adjacent to a modified Tn*5* insertion using pREG429 is shown in Figure 4.1. pREG429 does not replicate autonomously once transferred to *M. xanthus* via coliphage P1. Hence, it can only be stably maintained and give rise to kanamycin resistance by integration into the host chromosome by homologous recombination between the IS*50* sequences contained on the plasmid and the IS*50* elements of the endogenous Tn*5*. Once the cloning vector is integrated into the Tn*5* element it is now physically joined to the DNA to be cloned. Digestion of DNA from these cells produces a fragment of DNA which contains all the vector sequences required for selection and maintenance in *E. coli* plus a sequence of chromosomal DNA extending outward from the Tn*5*. After ligation at low DNA concentration, to promote circularization of the restriction fragments, the recombinant plasmid can be recovered by transformation into *E. coli*. When pREG429 is introduced into a *M. xanthus* strain containing a Tn*5*-132 insertion, homologous recombination between the plasmid and the IS*50*L (left) or IS*50*R (right) flanking sequences of the transposon occurs at approximately equal frequency. Hence, it is possible to clone chromosomal DNA from either side of the Tn*5* insertion, depending on which IS*50* element the vector pREG429 has inserted into.

Fig. 4.1 General Cloning Scheme Using the Integrating Vector pREG429 (Gill et al., 1988).

Transduction of pREG429 into an M. xanthus strain which contains Tn5-132 results in homologous recombination between the IS50 sequences of the plasmid and the IS50 sequences of the chromosomal Tn5-132. IS50 sequences are designated by open boxes with arrows denoting their relative orientation. The location of the unique EcoRI (E), ClaI (C) and BamHI (B) sites on the plasmid are indicated. Arbitrary chromosomal markers I - VI are shown. Recombination between pREG429 and the left or right IS50 element of Tn5-132 occurs at approximately equal frequency; only one of the alternatives is shown here. The result of this recombination is integration of the plasmid into the chromosome as shown in b. Cleavage of DNA prepared from such a transductant with EcoRI, ClaI or BamHI generates the fragments shown. Each fragment contains all of the essential portions of the integrated vector plus flanking chromosomal DNA, up to the first recognition site for the respective enzyme. The plasmid shown in c depicts the possible outcome from ClaI digested DNA, if a ClaI site were located between chromosomal markers I and II.

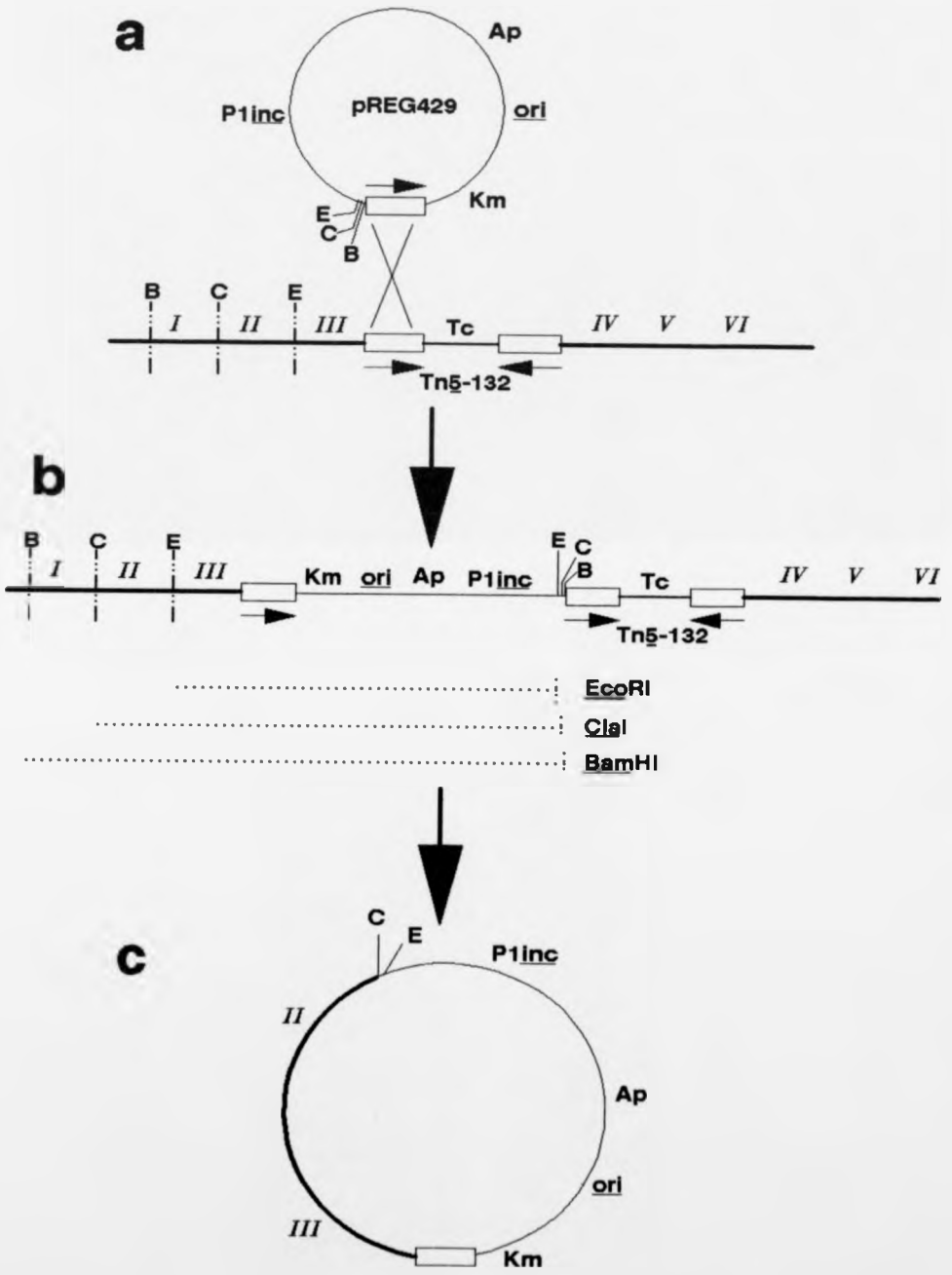

a
Ap
P1inc
pREG429
ori
Km
E
C
B
B
C
E
I
II
III
Tc
IV
V
VI
Tn5-132
b
B
C
E
I
II
III
Km
ori
Ap
P1inc
E
C
B
Tc
IV
V
VI
Tn5-132
EcoRI
ClaI
BamHI
c
C
E
P1inc
II
Ap
ori
III
Km

Fig. 4.1

Two series of plasmids were cloned in this manner; (1) pKIA4 and pKIA9 contain DNA from adjacent to ΩDK3554 designated locus glrA, and (2) pKIA1, pKIA2, pKIA3, pKIA6 and pKIA7 contain DNA from adjacent to ΩDK3517 designated locus glrB (Andreasson and Kaiser, personal communication).

Our aim was to further analyse these plasmids by cross hybridization studies and by probing M. xanthus chromosomal DNA from the wild-type strain DK1622 and the strains containing the Tn5 insertions ΩDK3517 (DK3517) and ΩDK3554 (DK3554). This would confirm from which locus, and from which side of the transposon in each locus, the DNA in each plasmid originated.

Two other loci involved in glycerol-induced sporulation were also included in this study. The first of these is represented by the plasmid pUWM5 which contains a glycerol-inducible promoter (Hartree, 1989). This glycerol-inducible gene has been designated isgB. This plasmid was included in the cross hybridization studies with the pKIA plasmids. The second locus was represented by a Tn5 lac fusion [ΩDK4530] in the wild-type background (Kroos and Kaiser, 1987). This Tn5 lac fusion shows increased β-galactosidase expression during both glycerol-induced sporulation (Kroos, 1986) and fruiting body sporulation (Kroos et al., 1986). Chromosomal DNA from the strain DK4530, which contains the ΩDK4530 Tn5 lac, was probed with the inserts of plasmids representing each of the families of pKIA plasmids and pUWM5.

4.2 Preparation of Probe Fragments

The pKIA series of plasmids were all cloned in pREG429. Restriction maps of pREG429 and all the pKIA plasmids are given in Chapter 5. The probe fragments were determined by comparing EcoRI HpaI digests of pREG429 and each of the pKIA plasmids. One or more extra fragments appears in the digests of each of the pKIA plasmids when compared with the digest of pREG429 and it was these extra fragments that were used as probes. The restriction fragments that result from complete EcoRI HpaI digestion of pREG429 and each of the pKIA plasmids are shown in Figure 4.2. The fragments used as probes are indicated for each of the pKIA plasmids. Hence, EcoRI HpaI digested plasmid DNA was subject to electrophoresis on a 0.5% agarose gel (Section 2.26). The fragments to be used as probes were cut out from the gel and electroeluted into 7.5M ammonium acetate as described in Section 2.27. The DNA was precipitated using ethanol (Section 2.25) and the DNA was resuspended in 20μl TE buffer (Section 2.3.2). 0.5μg of DNA was radiolabelled by nick translation and unincorporated nucleotides were removed using a G25 Sephadex column (Section 2.28).

The probe fragment of the glycerol-inducible promoter cloned in pUWM5 was isolated by complete digestion with Sau3AI. Sau3AI cuts frequently within the vector DNA but cuts M. xanthus DNA very infrequently. Complete digestion of pUWM5 with Sau3AI leaves the 4.8kb cloned M. xanthus genomic fragment containing the

pREG429	pKIA3	pKIA4	pKIA9
8.09kb	**27.00kb**	**16.85kb**	**14.16kb**
4.24kb	8.09kb	8.09kb	8.09kb
0.89kb*	4.24kb	4.24kb	4.24kb
0.45kb	0.89kb*	0.89kb*	0.89kb*
0.44kb	0.45kb	0.45kb	0.45kb
	0.44kb	0.44kb	0.44kb

pKIA1	pKIA2	pKIA6	pKIA7
8.09kb	**9.96kb**	**17.42kb**	**9.96kb**
5.75kb	8.09kb	8.09kb	8.09kb
4.24kb	4.24kb	4.24kb	4.24kb
1.82kb	0.89kb*	0.89kb*	0.89kb*
0.89kb*	0.45kb	0.45kb	0.45kb
0.45kb	0.44kb	0.44kb	0.44kb
0.44kb			

* = Result of partial digestion of 0.45kb and 0.44kb fragments

 = Fragments used as probes

Fig. 4.2 Restriction Fragments Obtained by Complete Digestion with EcoRI and HpaI.

glycerol-inducible promoter. This 4.8kb fragment was isolated and used as a probe representing the *isgB* region, as for the pKIA *Eco*RI *Hpa*I fragments.

4.3 Cross Hybridization Studies

This work was carried out to find out which of the pKIA plasmids shared sequence homology and to see if any of the pKIA plasmids shared homology with the glycerol-inducible promoter. The plasmids pKIA1, pKIA2, pKIA3, pKIA4, pKIA6, pKIA7, pKIA9 and pUWM5 were completely digested with *Sac*I and subject to electrophoresis on a 0.7% agarose gel (Section 2.26). The digested DNA was transferred to nitrocellulose membrane by Southern transfer (Section 2.29). Membranes prepared in this manner were probed with each of the aforementioned plasmid inserts in turn using the hybridization conditions described in Section 2.30. The restriction fragments obtained by complete *Sac*I digestion of each of the plasmids is shown in Figure 4.3. Significant cross hybridization was evident between the inserts in pKIA3, pKIA4 and pKIA9, as shown in Figures 4.4 and 4.5, suggesting that these three plasmids all contain DNA cloned from the same side of one of the two Tn*5* markers and, hence, represent a family. This was also suggested by the large number of *Sac*I restriction fragments common to all three plasmids. The plasmid pKIA3 had been thought to contain DNA cloned from the *glrB* region. The inserts in pKIA4 and pKIA9 showed no cross hybridization with any of the other pKIA plasmids or pUWM5. However,

pKIA1	pKIA2	pKIA6	pKIA7
18.10kb	uncut	22.20kb	uncut
2.20kb		17.50kb	
		4.10kb	
		3.10kb	
		2.40kb	
		2.20kb	
		1.80kb	
		1.40kb	
		1.10kb	
		0.79kb	

pKIA3	pKIA4	pKIA9	pUWM5
17.50kb	21.80kb	17.50kb	16.15kb
7.40kb	4.00kb	4.00kb	4.60kb
5.10kb	3.60kb	3.60kb	
4.80kb		2.80kb	
4.00kb			
3.60kb			
2.00kb			
1.65kb			
1.30kb			
0.74kb			
0.35kb			

Fig. 4.3 Restriction Fragments Obtained by Complete Digestion of the pKIA Plasmids and pUWM5 with SacI.

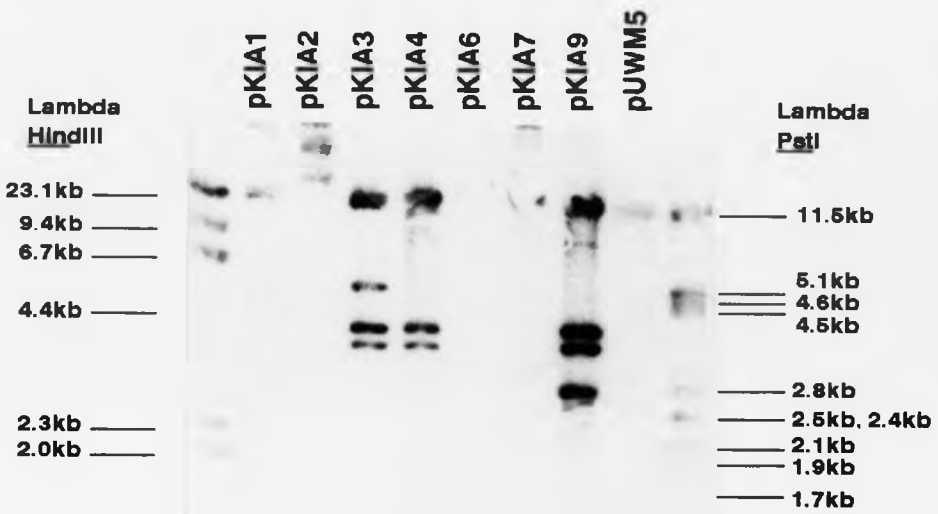
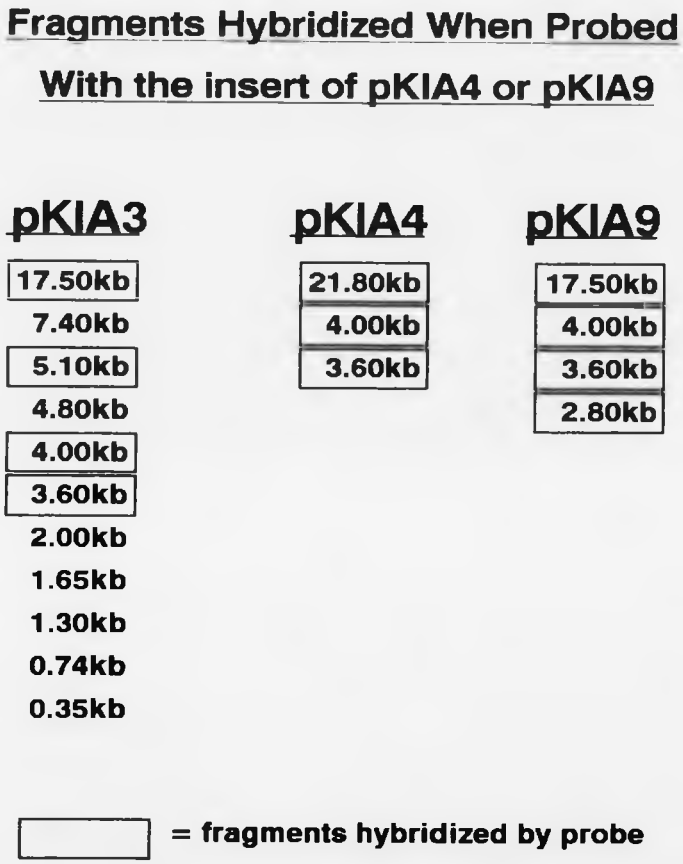


Fig. 4.4 Autoradiograph of SacI Digested pKIA Plasmid and pUWM5 DNA Probed With the Insert of pKIA9.



When probed with the insert of pKIA3 all restriction fragments of pKIA3, pKIA4 and pKIA9 were hybridized.

Fig. 4.5 Cross Hybridization Between pKIA3, pKIA4 and pKIA9.

some hybridization was evident between the 1.3kb SacI fragment of pKIA3 and the 2.2kb SacI fragment of pKIA1. Cross hybridization between the other plasmids was unclear. The plasmids pKIA2 and pKIA7 contain no sites for SacI and, hence, were uncut. However, the sizes and preliminary restriction maps of these two plasmids suggested that they were identical. This was consistent with the observation that when filters of the SacI digested pKIA plasmids were probed with the insert of either pKIA2 or pKIA7, the pKIA2 and pKIA7 tracks both sequestered equal amounts of the radiolabelled probe. To analyse this possibility further pKIA2 and pKIA7 DNA was digested with Sau3AI, subject to electrophoresis on a 0.7% agarose gel and transferred to nitrocellulose membrane as before. Duplicate filters were then probed independently with pKIA2 and pKIA7. Sau3AI digestion of both plasmids resulted in a ladder of DNA. Two fragments of 4.1kb and 1.9kb were identifiable in both the pKIA2 and pKIA7 tracks and the ladder of restriction fragments attained for both plasmids was identical. The inserts of pKIA2 and pKIA7 both gave identical hybridization signals in both tracks. It was concluded that pKIA2 and pKIA7 were indeed identical.

In order to further analyse pKIA1, pKIA2 (pKIA7), pKIA6 and pUWM5 these plasmids were digested with Sau3AI, subject to electrophoresis on a 0.7% agarose gel and transferred to nitrocellulose membrane. Digests of pREG429 and pDAH122, the parents of the pKIA plasmids and pUWM5 respectively, were used as controls. Filters

prepared in this manner were probed with the inserts from pKIA1, pKIA2, pKIA6 and pUWM5 in turn. Sau3AI digestion of a plasmid containing M. xanthus DNA resulted in vector DNA being digested to very small fragments while several larger fragments of M. xanthus genomic DNA remained, because Sau3AI cuts much less frequently in M. xanthus DNA. The distinguishable restriction fragments obtained by Sau3AI digestion of the aforementioned plasmids are shown in Figure 4.6. No cross hybridization occurred between pKIA1 and any of the other plasmids or between pUWM5 and any of the other plasmids. However, some cross hybridization did occur between pKIA2 and pKIA6. The fragments hybridized when the plasmids were probed with pKIA2 are shown in Figure 4.6. When the plasmids were probed with pKIA6 all restriction fragments of both pKIA2 and pKIA6 were hybridized. This suggests that these two plasmids share a common region of homology and, hence, may belong to the same family.

To confirm that the insert in pUWM5 did not share homology with any of the pKIA plasmids it was used to probe Sau3AI digested DNA of all the pKIA plasmids. However, no cross hybridization was observed. Hence, the isgB gene shows no homology to either of the loci represented by the pKIA plasmids.

From this cross hybridization analysis it has been possible to elucidate four groups of plasmids which show no cross-hybridization between the four groups; (1) pKIA3, pKIA4, and pKIA9, (2) pKIA1, (3) pKIA2, pKIA6 and pKIA7 and (4) pUWM5. The isgB region present on pUWM5

Cross Hybridization When Probed With the pKIA2 Insert

pKIA1	pKIA2	pKIA6	pUWM5
2.50kb	**4.10kb**	9.60kb	4.80kb
	1.90kb	8.60kb	
		4.60kb	
		4.10kb	
		2.30kb	
		1.90kb	
		1.50kb	

[ ] = fragments hybridized when probed with the pKIA2 insert

When probed with the pKIA6 insert all restriction fragments of pKIA2 and pKIA6 were hybridized. No cross hybridization was evident between pKIA1 and any of the other plasmids or between pUWM5 and any of the other plasmids.

Fig. 4.6 Restriction Fragments Obtained by Complete Digestion of pKIA1, pKIA2, pKIA6 and pUWM5 with Sau3AI.

showed no homology to either the glrA or glrB loci and, therefore, represents an independent locus.

4.4 Probing the M. xanthus Chromosome

The strains DK3517 and DK3554 contain the Tn5 insertions ΩDK3517 and ΩDK3554 respectively. These were the Tn5 markers used in mapping the glycerol-resistant mutants and cloning the pKIA plasmids which contain DNA from adjacent to the Tn5 markers. Chromosomal DNA was isolated from these two strains and the wild-type strain DK1622 in the manner described in Section 2.19. The DNA was digested completely with SacI and subject to electrophoresis on a 0.4% agarose gel. The digested DNA was transferred to nitrocellulose membrane by Southern transfer (Section 2.29). Membranes prepared as described were probed with each of the inserts from the pKIA plasmids in turn and also with that from pUWM5. Tn5 is approximately 5.7kb in size (Rothstein et al., 1981) and does not contain any restriction sites for SacI. Therefore, when the chromosomal DNA from the wild-type and the two Tn5 insertion strains were probed with insert DNA from a pKIA plasmid one would expect that the wild-type strain and one of the two Tn5 insertion strains would have an identical pattern of hybridized fragments. In the other Tn5 insertion strain one of the hybridized fragments should increase by the size of the Tn5 insertion. This will indicate that the probe contains DNA cloned from adjacent to the Tn5 insertion. For example when chromosomal DNA from DK1622, DK3517 and

DK3554 were probed with the insert of pKIA2 a single fragment of approximately 13.0kb was observed in the strains DK1622 and DK3554. However, a single fragment of about 18.5kb was observed in the DK3517 track. This increase in fragment size approximately corresponded to the size of Tn*5* and indicated that pKIA2 contained DNA cloned from adjacent to the ΩDK3517 Tn*5* insertion. The fragments hybridized and the bandshifts observed for all the pKIA plasmids are shown in Table 4.1. The data show that pKIA3, pKIA4 and pKIA9 contain DNA cloned from adjacent to the ΩDK3554 Tn*5* insertion, while pKIA1, pKIA2, pKIA6 and pKIA7 all contain DNA cloned from adjacent to the ΩDK3517 Tn*5* insertion.

When pUWM5 was used to probe DK1622, DK3517 and DK3554 chromosomal DNA, identical fragments were hybridized from all three strains, as shown in Table 4.1. This indicates that the *isgB* locus is not linked to either of the Tn*5* insertions, ΩDK3517 or ΩDK3554 and, therefore, represents an independent locus from the *glrA* and *glrB* loci.

DK4530 contains a Tn*5* *lac* insertion [ΩDK4530] (Kroos and Kaiser, 1987) that shows increased ß-galactosidase expression during glycerol-induced sporulation (Kroos, 1986). Chromosomal DNA was prepared from this strain, as described in Section 2.19. *Sac*I digested DK4530 DNA and wild-type DK1622 DNA was transferred to nitrocellulose membrane by Southern transfer (Section 2.29) following electrophoresis on a 0.5% agarose gel. Probes were prepared from (1) pKIA3 (*glrA*), (2) pKIA2 (*glrB*) and (3)

Probe	Fragments Hybridized in Strain			DNA Cloned From Adjacent to:-	Locus
	DK1622	DK3517	DK3554		
pKIA1	12.50kb	17.00kb	12.50kb	Ω3517	B
	2.20kb	2.20kb	2.20kb		
pKIA2	13.00kb	18.50kb	13.00kb	Ω3517	B
pKIA3	10.50kb	10.50kb	10.50kb	Ω3554	A
	7.80kb	7.80kb	13.00kb		
	6.40kb	6.40kb	6.40kb		
	5.10kb	5.10kb	5.10kb		
	4.80kb	4.80kb	4.80kb		
	4.00kb	4.00kb	4.00kb		
	3.60kb	3.60kb	3.60kb		
	2.00kb	2.00kb	2.00kb		
	1.70kb	1.70kb	1.70kb		
	1.30kb	1.30kb	1.30kb		
	0.74kb	0.74kb	0.74kb		
	0.35kb	0.35kb	0.35kb		
pKIA4	10.50kb	10.50kb	10.50kb	Ω3554	A
	7.80kb	7.80kb	13.00kb		
	4.10kb	4.10kb	4.10kb		
	3.60kb	3.60kb	3.60kb		
pKIA6	22.20kb	22.20kb	22.20kb	Ω3517	B
	17.50kb	23.00kb	17.50kb		
	4.10kb	4.10kb	4.10kb		
	3.10kb	3.10kb	3.10kb		
	2.40kb	2.40kb	2.40kb		
	2.20kb	2.20kb	2.20kb		
	1.80kb	1.80kb	1.80kb		
	1.40kb	1.40kb	1.40kb		
	1.10kb	1.10kb	1.10kb		
	0.79kb	0.79kb	0.79kb		
pKIA7	13.00kb	18.50kb	13.00kb	Ω3517	B
pKIA9	10.50kb	10.50kb	10.50kb	Ω3554	A
	7.80kb	7.80kb	13.00kb		
	4.00kb	4.00kb	4.00kb		
	3.60kb	3.60kb	3.60kb		
pUWM5	7.00kb	7.00kb	7.00kb		
	5.80kb	5.80kb	5.80kb		

Table 4.1 Probing Chromosomal DNA from Strains DK1622, DK3517 and DK3554 With the Inserts of the pKIA Plasmids and pUWM5.

pUWM5 (*isgB*), which were chosen as representatives of the three loci involved in glycerol-induced sporulation which had so far been identified. If one of the probes contained DNA from adjacent to the Tn*5* *lac* an extra band would be observed in the DK4530 track, than in the wild-type track, as Tn*5* *lac* has a restriction site for *Sac*I in the *lacZ* region. Also the total size of the DK4530 hybridizing fragments would be approximately 12.0kb longer, corresponding to the Tn*5* *lac* insertion. If the probe did not contain DNA from adjacent to the Tn*5* *lac* insertion, then the hybridizing fragments in the DK4530 and wild-type tracks would be identical. For all three probes used the fragments hybridized were identical in both the wild-type and DK4530 tracks, indicating that the ΩDK4530 Tn*5* *lac* insertion represents an independent locus from *glrA*, *glrB* and *isgB*.

4.5 Discussion

This series of experiments revealed four independent loci involved in glycerol-induced sporulation. These loci are *glrA*, *glrB*, *isgB* and ΩDK4530. Probing the chromosomal DNA of the *M. xanthus* strains DK3517 and DK3554 showed that pKIA3, pKIA4 and pKIA9 contain DNA from adjacent to ΩDK3554 (*glrA*) while pKIA1, pKIA2, pKIA6 and pKIA7 contain DNA from adjacent to ΩDK3517 (*glrB*). Probing DK4530 chromosomal DNA revealed that the ΩDK4530 Tn*5* *lac* insertion represents an independent locus from both the ΩDK3517 and ΩDK3554 Tn*5* insertions. Using the cloned *isgB* region from pUWM5 to probe DK3517, DK3554 and

DK4530 has shown that the *isgB* locus is independent from ΩDK3517, ΩDK3554 and ΩDK4530.

Cross hybridization studies between the pKIA plasmids showed that pKIA3, pKIA4 and pKIA9 show considerable cross hybridization and, therefore, contain DNA cloned from the same side of the ΩDK3554 insertion. It would seem that DNA has only been successfully cloned from one side of the ΩDK3554 Tn*5* insertion, as shown in Figure 4.7. Cross hybridization was also evident between pKIA2, pKIA6 and pKIA7 suggesting that these three plasmids contain DNA from one side of the ΩDK3517 Tn*5* insertion, also shown in Figure 4.7. The plasmids pKIA2 and pKIA7 were subsequently found to be identical. Plasmid pKIA1 also contains DNA from adjacent to the ΩDK3517 Tn*5* insertion but shares no homology to pKIA2 or pKIA6 and is, therefore, thought to contain DNA from the opposite side of the ΩDK3517 Tn*5* insertion to pKIA2 and pKIA6 (Figure 4.7).

Hence, we have identified four independent loci that appear to be involved in glycerol-induced sporulation; (1) *glrA*, (2) *glrB*, (3) *isgB* and (4) ΩDK4530. The pKIA plasmids contain DNA cloned from the *glrA* and *glrB* loci and can be separated into three families; (1) pKIA3, pKIA4 and pKIA9, (2) pKIA1 and (3) pKIA2, pKIA6 and pKIA7. The first of these families contains plasmids where the cloned DNA comes from adjacent to ΩDK3554 (*glrA*), while the other two families are plasmids where the cloned DNA comes from adjacent to ΩDK3517 (*glrB*).

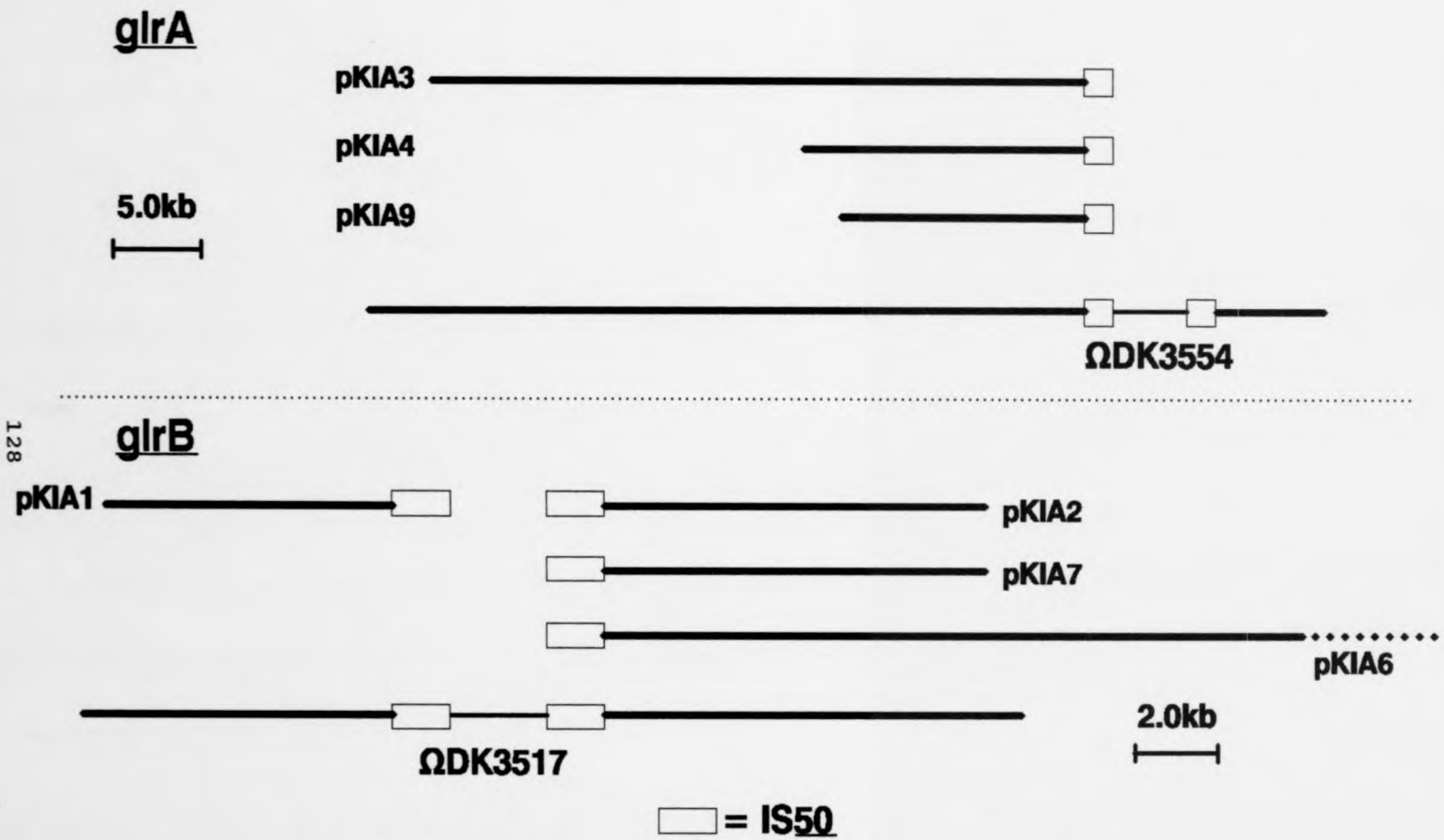


Fig. 4.7 Preliminary Maps of the glrA and glrB Loci.

Chapter 5

Restriction Analysis of the pKIA Plasmids and Subcloning of the *glrA* and *glrB* Loci

5.1 Introduction

Cross hybridization studies with the pKIA plasmids indicated that the plasmids could be divided into three families; (1) pKIA3, pKIA4 and pKIA9, (2) pKIA1 and (3) pKIA2, pKIA6 and pKIA7 (Section 4.3). Probing the chromosomal DNA from the wild-type *M. xanthus* strain, DK1622, and two strains, DK3554 and DK3517, carrying the Tn*5* insertions, ΩDK3554 and ΩDK3517 respectively, revealed that pKIA3, pKIA4, and pKIA9 contained DNA from adjacent to ΩDK3554 and pKIA1, pKIA2, pKIA6 and pKIA7 all contained DNA from adjacent to the Tn*5* insertion ΩDK3517. The locus defined by ΩDK3554 has been designated *glrA*, while the ΩDK3517 locus has been named *glrB*.

The primary aim of this work was to map the restriction sites of all the pKIA plasmids. A range of enzymes with six base pair recognition sequences were used. This should allow one to confirm that the pKIA plasmids represent three families and ascertain how the chromosomal inserts in the plasmids overlap.

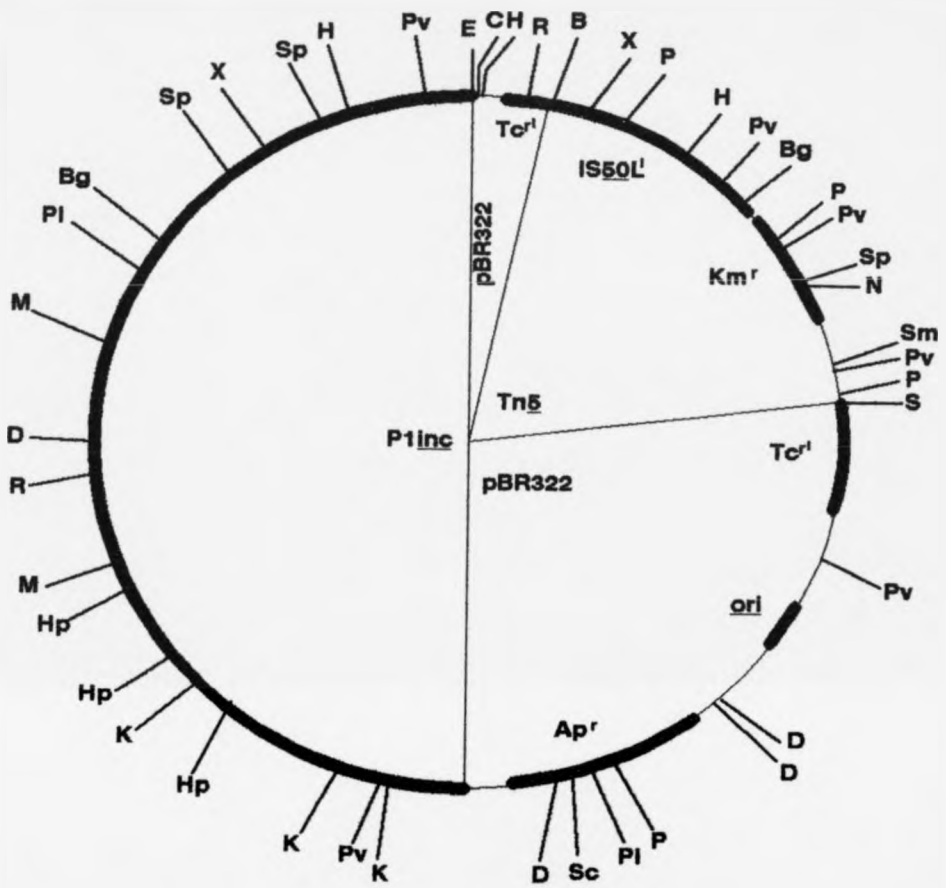
Complementation analysis with the pKIA plasmids is discussed in detail in Chapter 6 (Section 6.2). This analysis revealed that pKIA3, pKIA4 and pKIA9 complemented the mutants DK440 (*glrA1*, *bsg-440*) and DK510 (*glrA2*, Fru$^-$) from glycerol-resistance to glycerol-sensitivity. However, the fruiting phenotypes of these mutants were not complemented. Hence, it seems that the plasmids pKIA3, pKIA4, and pKIA9 all contain the *glrA* gene or genes. The plasmids pKIA2 and pKIA7 were found to complement the mutant DK3551 (*glrB2*, Fru$^-$) from

glycerol-resistance to glycerol-sensitivity. Again neither plasmid complemented the fruiting phenotype of this mutant. Thus, it would appear that pKIA2 and pKIA7 contain the glrB gene or genes.

A secondary aim was, therefore, to subclone smaller regions of the glrA and glrB loci. These subclones could then be used in further complementation studies to locate the glrA and glrB genes within the original clones. pKIA4 was used as an example of the glrA region for subcloning and pKIA2 was used as an example of the glrB region. The subclones derived from pKIA4 (glrA$^+$) and pKIA2 (glrB$^+$) and the subcloning methods are described in this chapter, while the use of these subclones for complementation analysis is discussed in Chapter 6 (Sections 6.3 and 6.4).

5.2 Restriction Mapping of the pKIA Plasmids

The restriction sites for 19 restriction endonucleases was determined for each of the pKIA plasmids. All the pKIA plasmids are cloned in the vector pREG429 (Gill et al., 1988). Hence, this plasmid was digested alongside the pKIA plasmids for comparison. A restriction map of pREG429 is shown in Figure 5.1. The restriction enzymes used in this analysis were mainly enzymes which have a six base pair recognition sequence. The restriction sites were determined by digestion of each of the plasmids with each restriction enzyme and combinations of two enzymes. Where digestion with two separate enzymes could not be carried out in the same



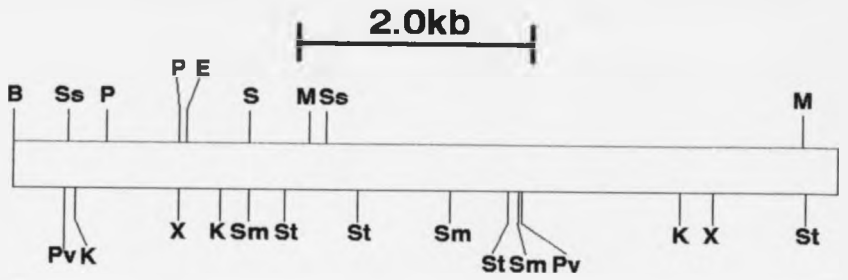
pREG429 13.22kb

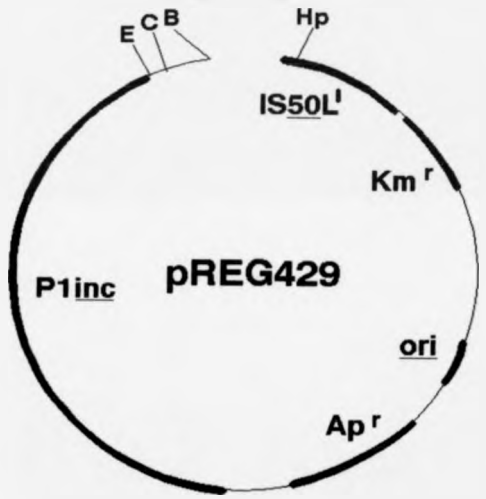
B = BamHI
Bg = BglII
C = ClaI
D = DraI
E = EcoRI
R = EcoRV
H = HindIII
Hp = HpaI
K = KpnI
M = MluI
N = NcoI
P = PstI
Pl = PvuI
Pv = PvuII
S = SalI
Sc = ScaI
Sm = SmaI
Sp = SphI
X = XhoI

Fig. 5.1 Restriction Map of pREG429.

restriction buffer, the DNA was first digested with the enzyme with the lowest salt concentration requirements. The restriction enzyme was then removed by phenol extraction (Section 2.24) and the DNA was precipitated with ethanol (Section 2.25) and resuspended in TE buffer (Section 2.3.2). The DNA was then digested with the second restriction enzyme. Samples of digested DNA were then electrophoresed, as described in Section 2.26, in duplicate on 0.4% and 1.2% agarose gels and photographed. Lambda DNA samples digested with HindIII and PstI were used as size markers. The 0.4% agarose gel allowed more accurate size determination of large fragments of DNA, while the 1.2% agarose gel allowed accurate size determination of the small DNA fragments.

Complete restriction maps of pKIA1, pKIA2 (pKIA7), pKIA4 and pKIA9 are shown in Figures 5.2, 5.3, 5.4 and 5.5 respectively. The plasmid pKIA7 was found to be identical to pKIA2, while pKIA3 and pKIA6 proved to be too large for accurate restriction mapping. However, restriction analysis of pKIA3 did confirm that this plasmid contained shared regions of homology with pKIA4 and pKIA9, as shown in Figure 5.6. However, the data for pKIA3 was inconsistent. The plasmid was isolated using EcoRI, as was pKIA4, but pKIA3 contained only one site for EcoRI. From comparison with pKIA4 one would expect two EcoRI sites in pKIA3 if both pKIA3 and pKIA4 are EcoRI clones. Preliminary restriction analysis of pKIA6 suggested that the plasmid contained all the DNA cloned in pKIA2 and another 8.0 - 10.0kb of additional



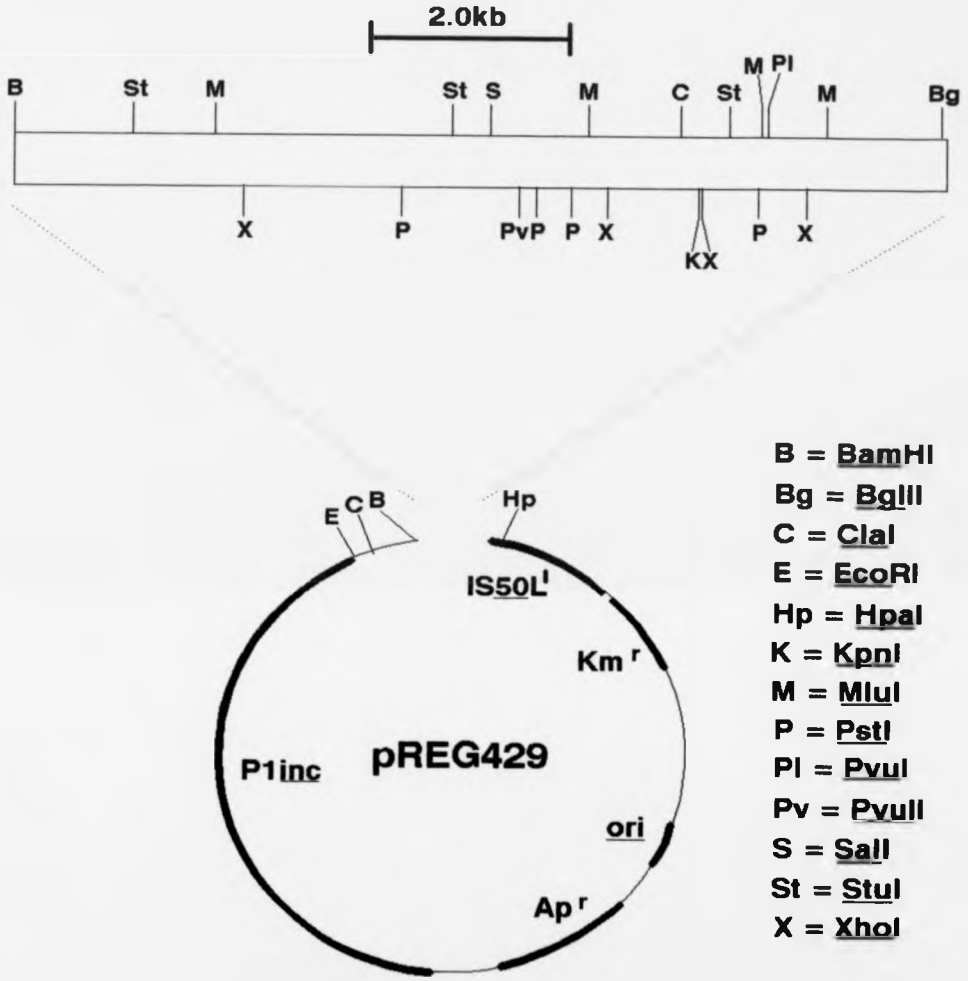


B = BamHI
C = ClaI
E = EcoRI
Hp = HpaI
K = KpnI
M = MluI
P = PstI
Pv = PvuII
S = SalI
Sm = SmaI
Ss = SacI
St = StuI
X = XhoI

The cloned region of M. xanthus DNA contained no sites for the following restriction enzymes:-

BglII
ClaI
DraI
EcoRV
HindIII
HpaI
PvuI
ScaI

Fig. 5.2 Restriction Map of pKIA1.



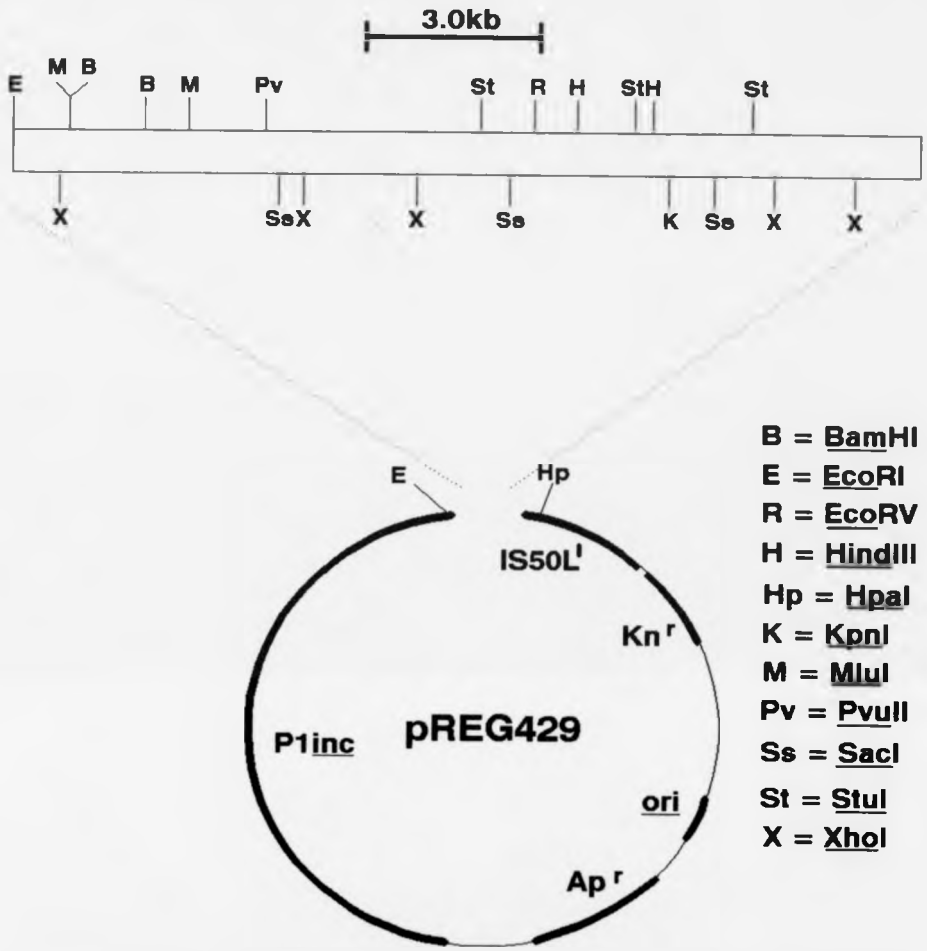
B = BamHI
Bg = BglII
C = ClaI
E = EcoRI
Hp = HpaI
K = KpnI
M = MluI
P = PstI
Pl = PvuI
Pv = PvuII
S = SalI
St = StuI
X = XhoI

The cloned region of M. xanthus DNA contained no sites for the following restriction enzymes:-

DraI
EcoRI
EcoRV
HindIII
HpaI
SacI
ScaI

There were too many SmaI sites within the cloned region to map.

Fig. 5.3 Restriction Map of pKIA2 (pKIA7).

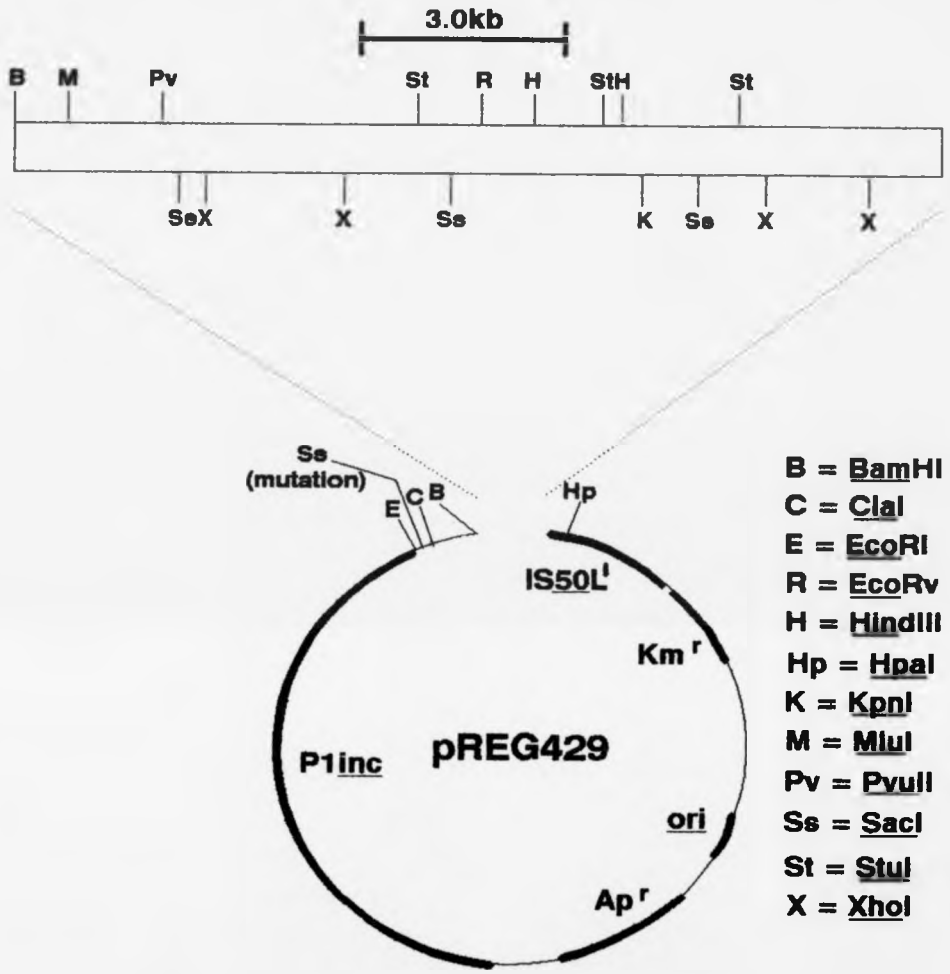


The cloned region of M. xanthus DNA contained no sites for the following restriction enzymes:-

BglII
ClaI
DraI
HpaI
PvuI
ScaI

There were too many PstI, PvuII, SalI, and SmaI sites within the cloned region to map.

Fig. 5.4 Restriction Map of pKIA4.

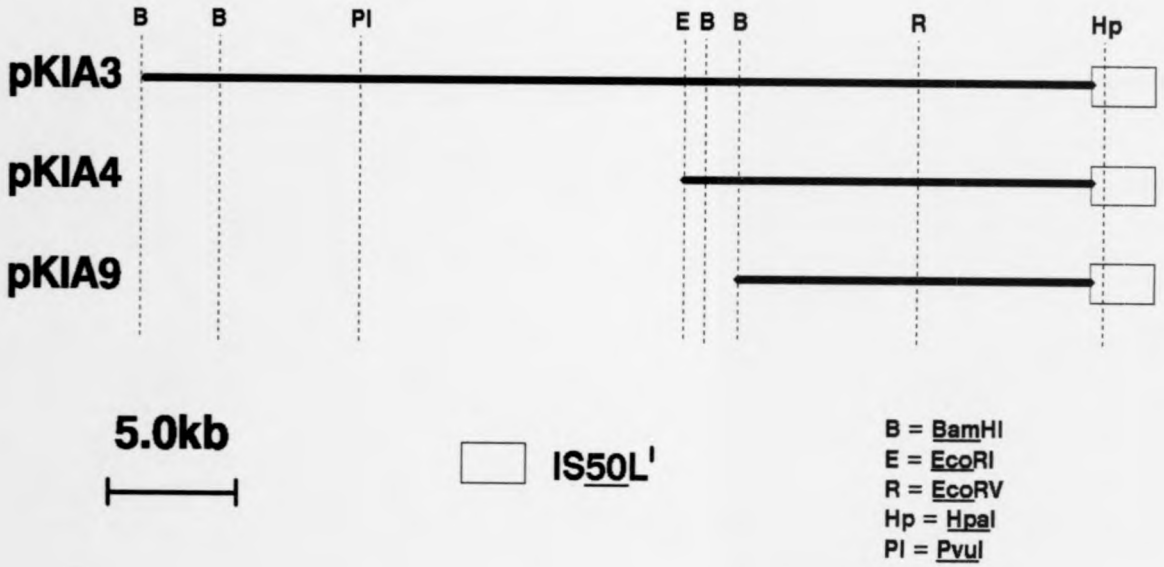


The cloned region of M. xanthus DNA contained no sites for the following restriction enzymes:-

BglII
ClaI
DraI
EcoRI
HpaI
PvuI
ScaI

There were too many PstI, SalI and SmaI sites within the cloned region to map.

Fig. 5.5 Restriction Map of pKIA9.



The cloned region of M. xanthus DNA in pKIA3 contains no sites for the following enzymes:-

BglII
DraI

Fig. 5.6 Preliminary Restriction Map of pKIA3.

M. xanthus DNA. The exact amount of additional DNA cloned is unclear as the plasmid appeared to contain a deletion within the pREG429 vector DNA.

5.3 Subcloning the glrA and glrB Regions

This was carried out so that the subclones derived from the glrA and glrB regions could be used in complementation studies in order to try and pin-point the glrA and glrB genes within the regions of cloned DNA. pKIA4 was used in subcloning as the representative of the glrA region, while pKIA2 was used as the representative of the glrB region. The fragments subcloned from the glrA region are shown in Figure 5.7 together with the names of the resultant plasmids. The subclones derived from the glrB region are shown in Figure 5.8.

The plasmids pHMC6, pHMC8, pHMC40, pHMC42 and pHMC44 are all subclones of the glrA region while pHMC2, pHMC4, pHMC30, pHMC32, pHMC34 and pHMC36 all contain DNA subcloned from the glrB region. DNA fragments for subcloning were isolated by electrophoresis of DNA digested with the appropriate enzymes on agarose gels. Fragments of interest were then cut out from the gel and eluted from the agarose. Fragments between 500 base pairs and 15.0kb were eluted into TE buffer (Section 2.3.2) using the Gene clean kit II (from Bio 101). Fragments larger than 15.0kb were eluted into 7.5M ammonium acetate using an IBI electroeluter and were then precipitated using ethanol (Section 2.25) and resuspended

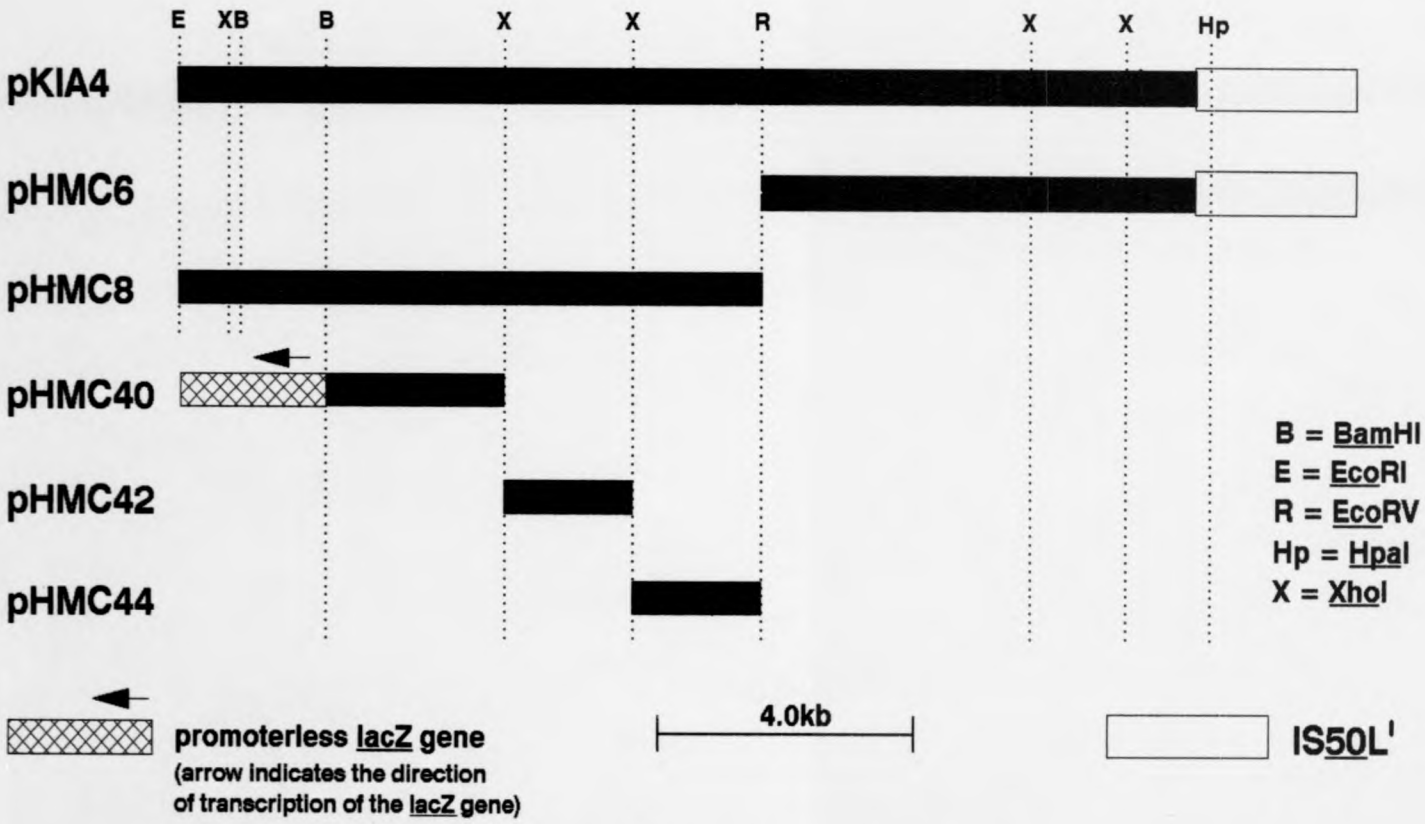


Fig. 5.7 Subclones Derived from the glrA Region.

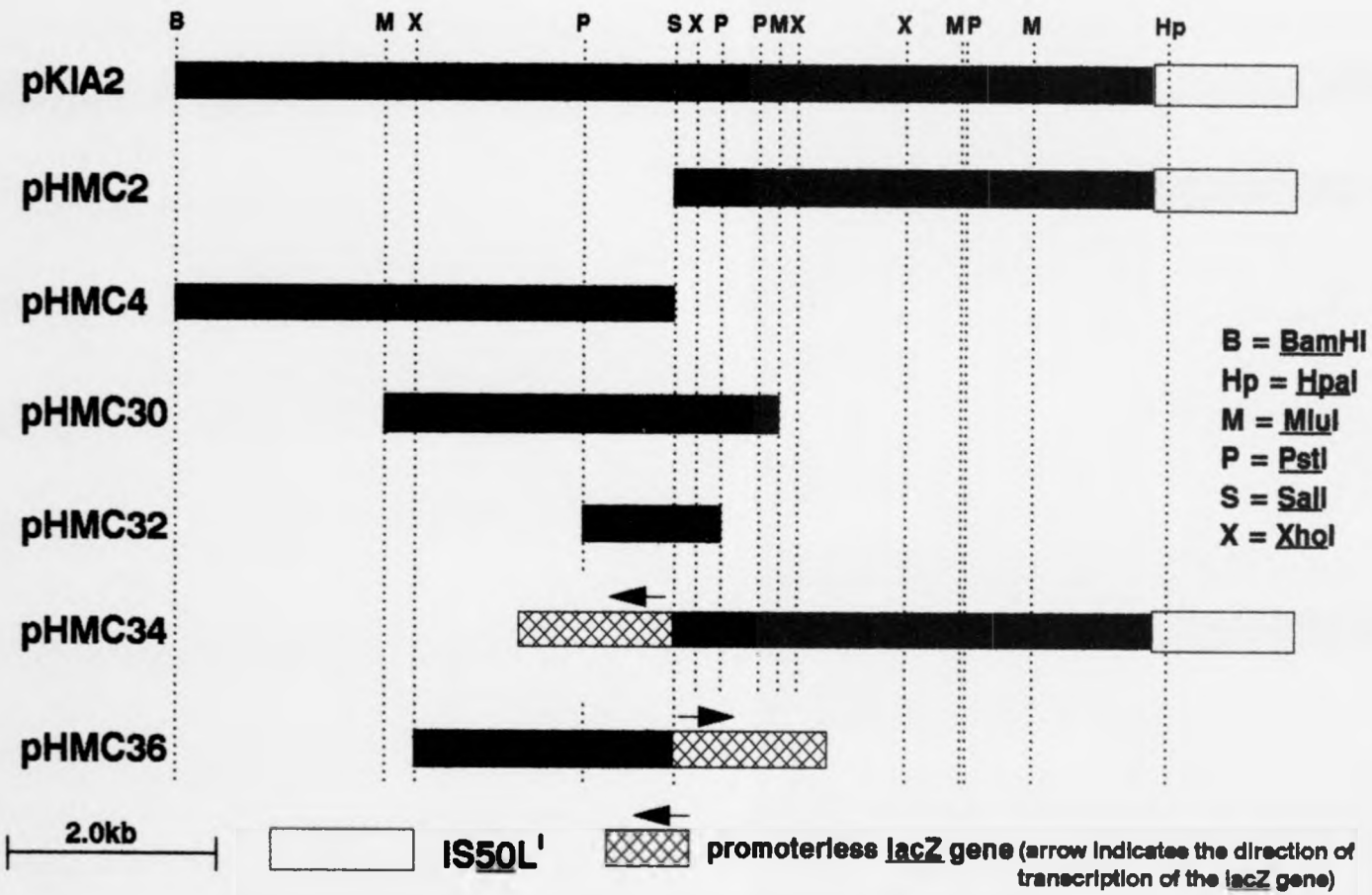


Fig. 5.8 Subclones Derived from the girB Region.

in TE buffer. Ligation of DNA fragments was carried out as described in Section 2.23, and all recombinant plasmids were recovered by calcium chloride transformation of E. coli MC1061 with ligated DNA (Section 2.21). The orientation of inserted fragments was determined for each plasmid by restriction analysis. Where end-filling reactions were necessary before ligation of the fragments, this was carried out as described in Section 2.32. Alkaline phosphatase reactions to stop vector self-ligation was used in the construction of some of these subclones. Calf intestinal alkaline phosphatase was used as described in Section 2.31.

5.3.1 Subclones Derived from the glrA Region

5.3.1.1 Construction of pHMC6

The plasmid pKIA4 was digested with EcoRV. EcoRV digestion of pKIA4 results in two fragments of 12.66kb and 16.27kb. The 16.27kb EcoRV fragment containing 6.8kb of cloned M. xanthus DNA (see Figure 5.7), kanamycin and ampicillin resistance determinants and a replication origin was isolated. This fragment was self-ligated and used to transformed E. coli. The resultant plasmid was termed pHMC6 and construction of this plasmid is shown in Figure 5.9.

5.3.1.2 Construction of pHMC8

The plasmid pKIA4 was digested with EcoRV and the 12.66kb fragment was isolated. This fragment contains

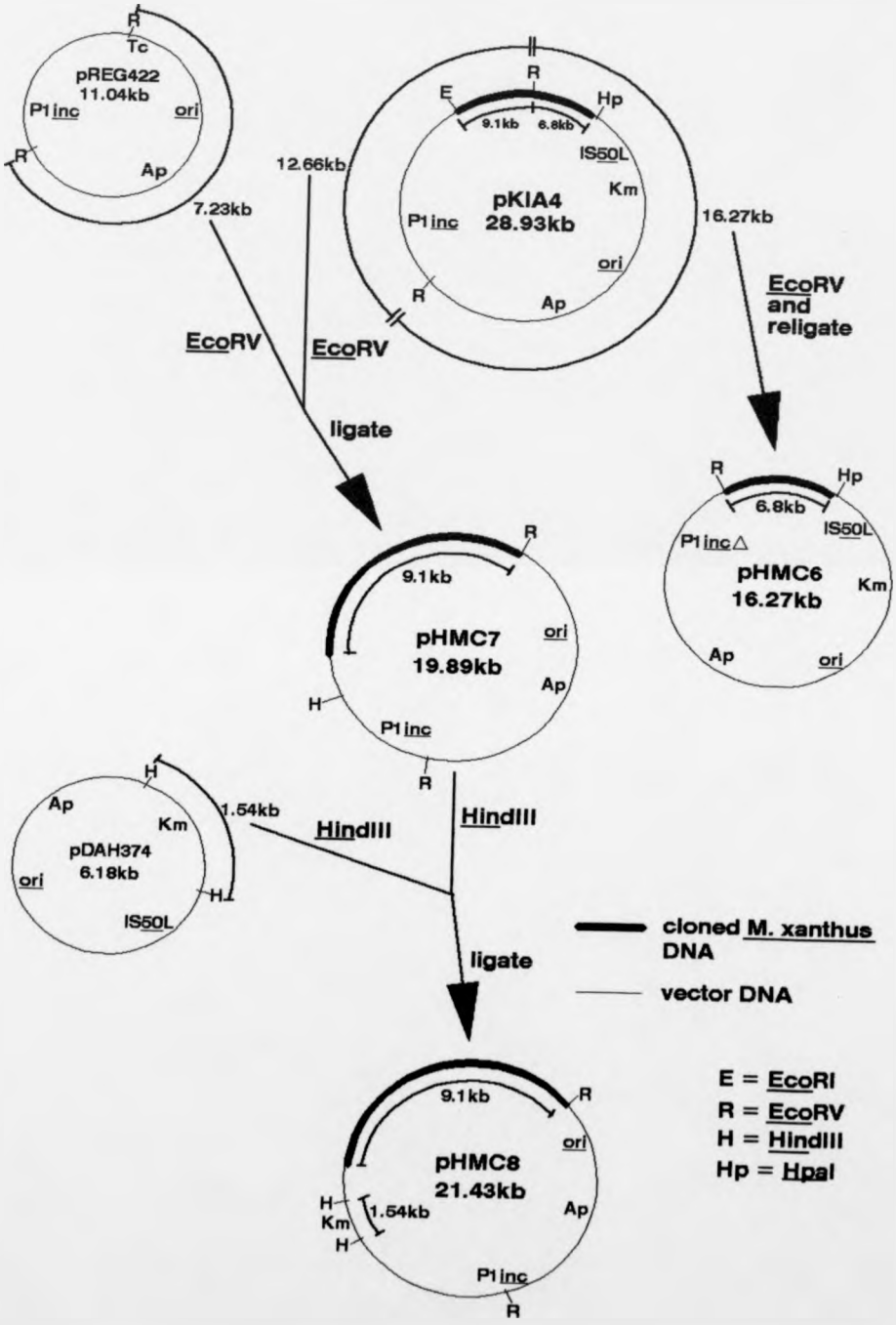


Fig. 5.9 Construction of pHMC6 and pHMC8.

9.1kb of cloned M. xanthus DNA. It was ligated with a 7.23kb EcoRV fragment obtained from pREG422 which contains an ampicillin resistance determinant and a replication origin, as shown in Figure 5.9. The fragment derived from pREG422 was treated with calf intestinal alkaline phosphatase to stop self-ligation. The ligation mixture was used to transform E. coli. Restriction analysis was used to identify transformants containing plasmids where the two EcoRV fragments were ligated together in such a way as to reconstruct the P1inc region of pREG422 (pREG429). Such a plasmid was designated pHMC7. Plasmid pHMC7 was linearized with HindIII and a 1.54kb fragment derived from pDAH374, containing a kanamycin resistance determinant, was ligated into pHMC7. The resultant plasmid was termed pHMC8 and construction of this plasmid is shown in Figure 5.9.

5.3.1.3 Construction of pHMC40

A 2.8kb BamHI XhoI fragment from pKIA4, shown in Figure 5.7, was isolated and ligated into pDAH283 which had been linearized by BamHI SalI digestion. A restriction map of pDAH283 is shown in Figure 5.10. pDAH283 contains a promoterless lacZ gene and the 2.8kb BamHI XhoI fragment was cloned in such a way that the BamHI end of the fragment was adjacent to the promoterless lacZ gene.

5.3.1.4 Construction of pHMC42

A 2.0kb XhoI fragment derived from pKIA4 (see Figure

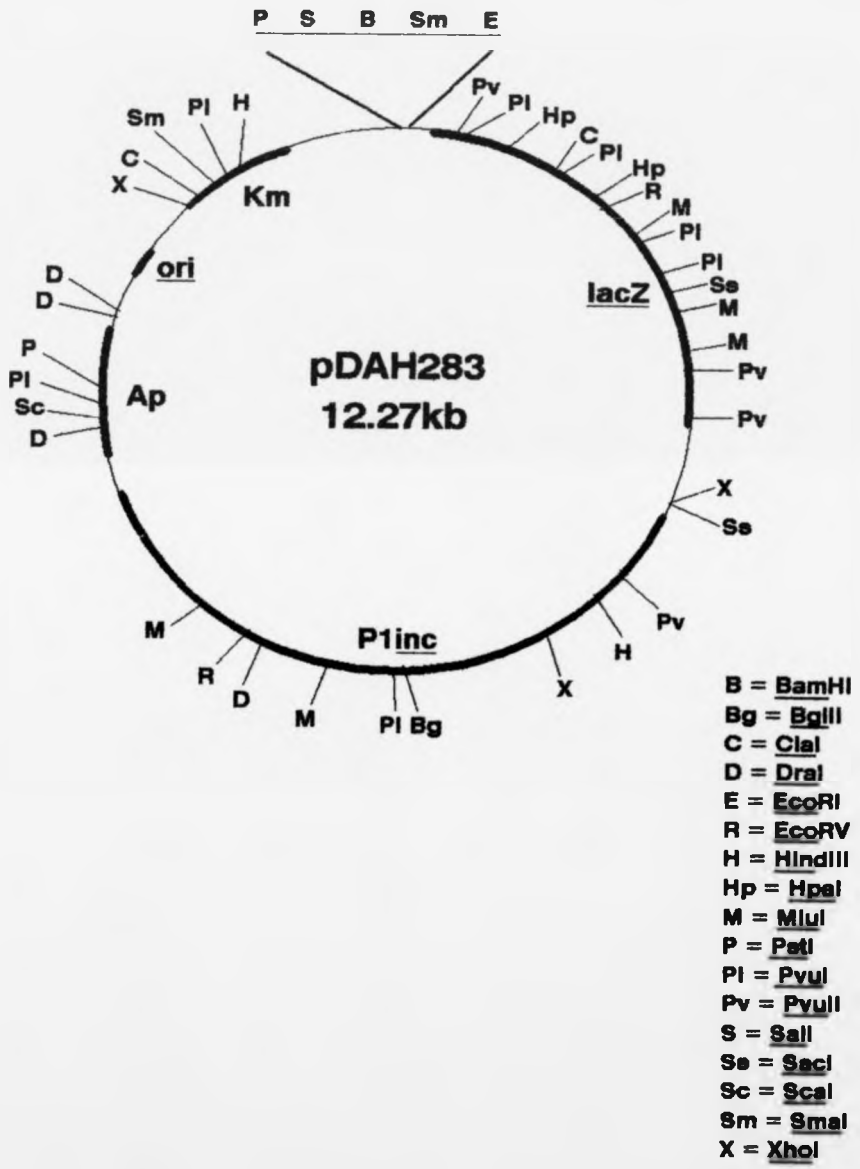


Fig. 5.10 Restriction Map of pDAH283.

5.7) was isolated and ligated into pREG429 (see Figure 5.1) which had been linearized with SalI. The linearized pREG429 plasmid was treated with alkaline phosphatase to stop self-ligation. The orientation of the 2.0kb XhoI fragment within the pREG429 vector DNA was indistinguishable without further restriction mapping of the fragment.

5.3.1.5 Construction of pHMC44

Plasmid pHMC8, a subclone of pKIA4, was digested with PstI and XhoI and a 5.12kb fragment was isolated. This fragment contained 1.7kb of cloned M. xanthus DNA as shown in Figure 5.7 and a replication origin. This fragment was ligated to 6.13kb PstI XhoI fragment derived from pREG422 which contained the P1inc region. Ligation of the two fragments resulted in the reconstruction of the ampicillin gene. The resultant plasmid was obtained by transformation of E. coli and was designated pHMC43. Plasmid pHMC43 was then linearized with XhoI and a 1.49kb SalI fragment derived from pDAH142, containing a kanamycin resistance determinant, was ligated in. A recombinant plasmid was obtained in E. coli MC1061 and was designated pHMC44. The construction of pHMC43 and pHMC44 is shown in Figure 5.11.

5.3.2 Subclones Derived from the glrB Region

5.3.2.1 Construction of pHMC2

Plasmid pKIA2 was digested with SalI. This generated two fragments of 15.52kb and 7.19kb. The

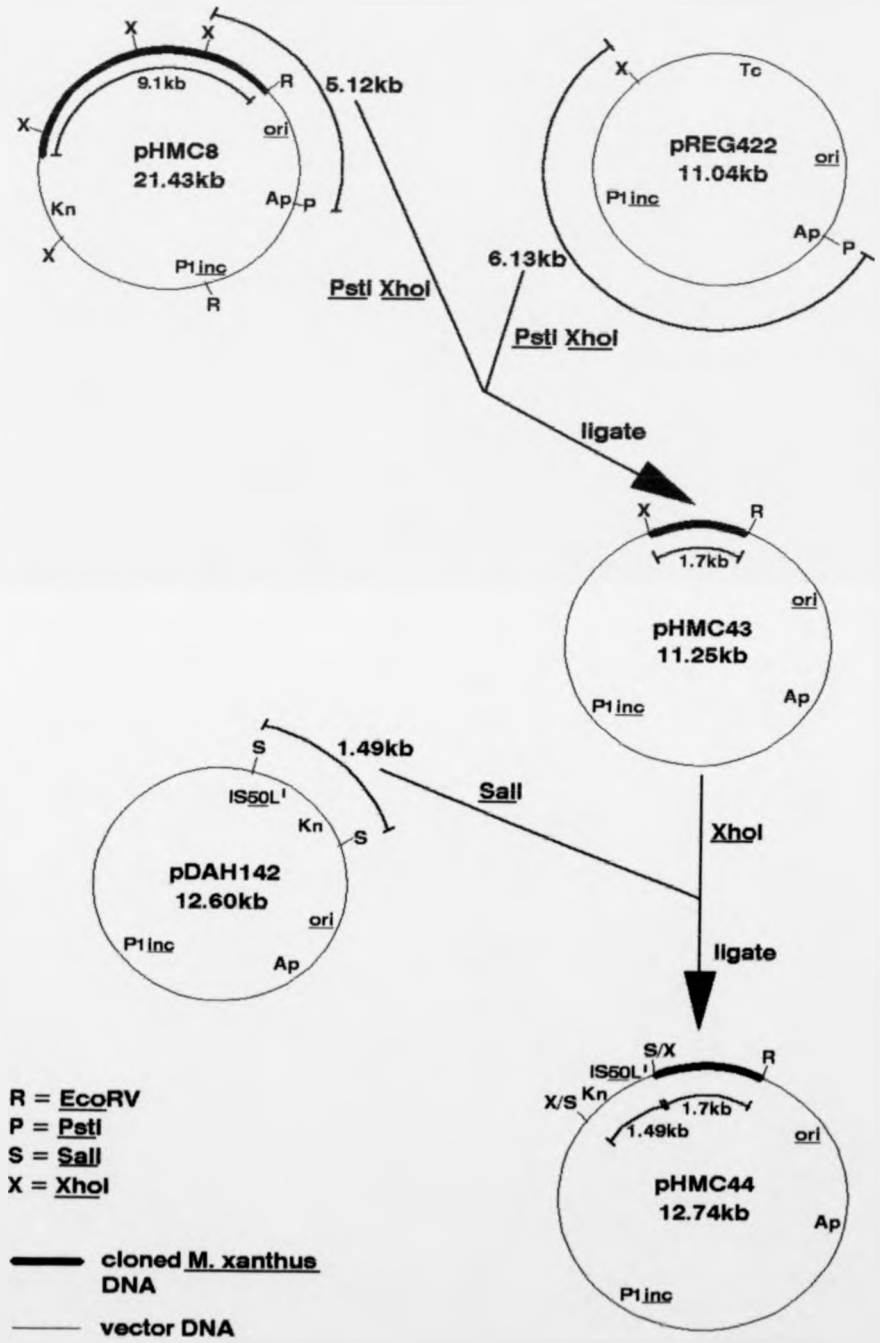


Fig. 5.11 Construction of pHMC44.

7.19kb fragment, which contained 4.3kb of cloned M. xanthus DNA, as shown in Figure 5.8, and a kanamycin resistance determinant, was isolated. This fragment was ligated into pREG422 which had been linearized with SalI and treated with calf intestinal alkaline phosphatase to stop self-ligation. Construction of pHMC2 is shown in Figure 5.12.

5.3.2.2 Construction of pHMC4

Plasmid pKIA2 was digested with SalI and the 15.52kb fragment was isolated. This fragment contains 4.8kb of cloned M. xanthus DNA, the P1inc region, an ampicillin resistance determinant and a replication origin. The fragment was ligated to a 2.20kb SalI XhoI fragment derived from pREG429 which contains a kanamycin resistance determinant. Construction of pHMC4 is shown in Figure 5.12.

5.3.2.3 Construction of pHMC30

Plasmid pKIA2 was digested with MluI and a 3.8kb fragment (Figure 5.8) was isolated. This fragment was end-filled and ligated into pDAH304 which had been linearized with EcoRV and treated with alkaline phosphatase. A restriction map of pDAH304 is shown in Figure 5.13.

5.3.2.4 Construction of pHMC32

Plasmid pKIA2 was digested with PstI and a 1.35kb fragment (Figure 5.8) was isolated. This 1.35kb fragment

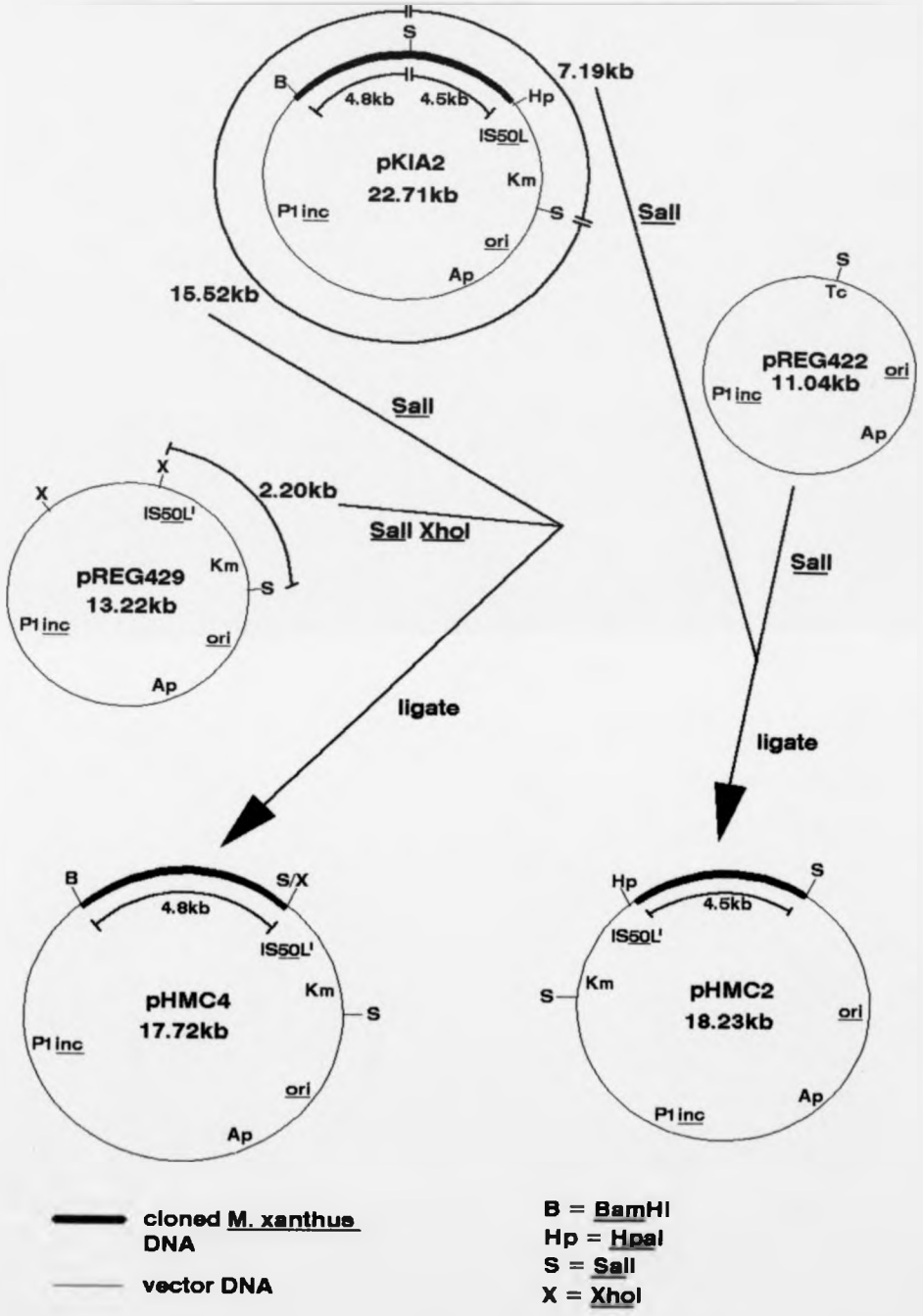


Fig. 5.12 Construction of pHMC2 and pHMC4.

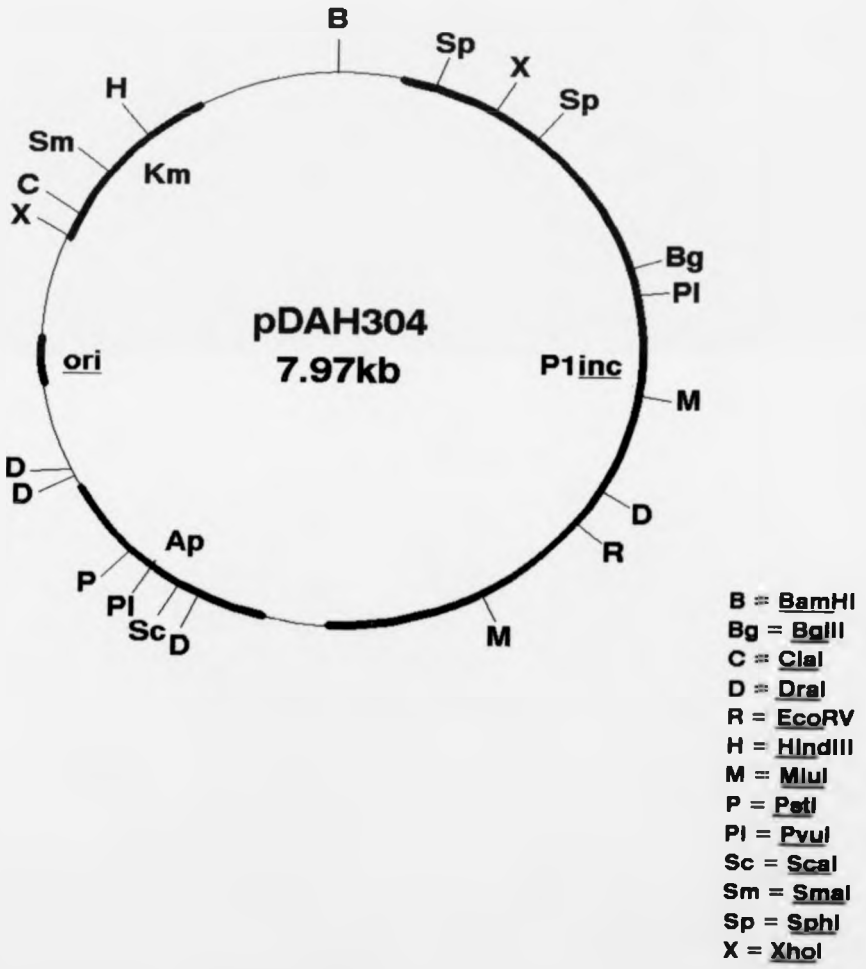


Fig. 5.13 Restriction Map of pDAH304.

was ligated into pDAH304 (Figure 5.13) which had been linearized with PstI and treated with alkaline phosphatase.

5.3.2.5 Construction of pHMC34

Plasmid pKIA2 was digested with SalI and the 7.19kb fragment (cloned previously in pHMC2) was isolated and end-filled. The fragment was then ligated into pDAH283 (Figure 5.10) which had been linearized with SmaI. Recombinant plasmids were obtained in E. coli MC1061 and restriction analysis was used to identify those plasmids where the cloned M. xanthus DNA within the 7.19kb fragment derived from pKIA2, was adjacent to the promoterless lacZ of pDAH283.

5.3.2.6 Construction of pHMC36

Plasmid pKIA2 was digested with SalI and XhoI and a 2.6kb fragment (Figure 5.8) was isolated. This fragment was ligated into pDAH283 (Figure 5.10) which had been linearized with SalI and treated with alkaline phosphatase to stop self-ligation. Recombinant plasmids were obtained in E. coli and restriction analysis was used to identify those plasmids where the SalI end of the 2.6kb SalI XhoI fragment derived from pKIA2, was adjacent to the promoterless lacZ gene of pDAH283.

5.4 Discussion

Restriction analysis of the pKIA plasmids confirmed that the chromosomal inserts in pKIA3, pKIA4 and pKIA9

overlapped and contained DNA from the same region, as shown in Figure 5.6, and that these plasmids were completely different from pKIA1, pKIA2, pKIA6 and pKIA7. This analysis also confirmed that pKIA2 and pKIA7 are identical and that the chromosomal inserts in both these plasmids share some regions of homology to the insert of pKIA6. The plasmids pKIA3 and pKIA6 could not be fully mapped, however, due to their large size, and the deletion in the latter plasmid.

Chapter 6

Analysis of the glrA and glrB Regions

6.1 Introduction

The aim of this study was to assess whether the mutations in the glycerol-resistant mutants DK440 (*glrA1*, *bsg-440*) (LaRossa *et al*., 1983), DK510 (*glrA2*, Fru$^-$) (Hagen *et al*., 1978), DK3516 (*glrB1*, Fru$^-$) and DK3551 (*glrB2*, Fru$^-$) could be complemented/gene converted by any of the pKIA plasmids. This was achieved by using coliphage P1 to transduce each plasmid in turn into each mutant background. The plasmids cannot replicate in *M. xanthus*, hence, there are two possible outcomes of introduction of a plasmid containing cloned *M. xanthus* DNA into an *M. xanthus* strain: (1) A double crossover event occurs which results in a substitution of cloned DNA for homologous chromosomal DNA. These transductants are haploid. (2) A single crossover event occurs resulting in the recombinant plasmid integrating into the chromosome at a region of homology. This results in the formation of a duplication of the cloned DNA sequence and the integration of vector DNA into the chromosome. This results in heterozygous merodiploids in which the chromosomal copy and the plasmid copy of the genes of interest are tandemly arranged, as shown in Figure 6.1. Homozygous merodiploids, which contain two identical copies of the genes of interest, with the alteration of either the plasmid or chromosomal copy, arise at 10 - 30% (O'Connor and Zusman, 1983; Shimkets *et al*., 1983). These homozygous merodiploids are the result of gene conversion. The frequency of homozygous merodiploids is the same whether the mutant allele comes from the donor

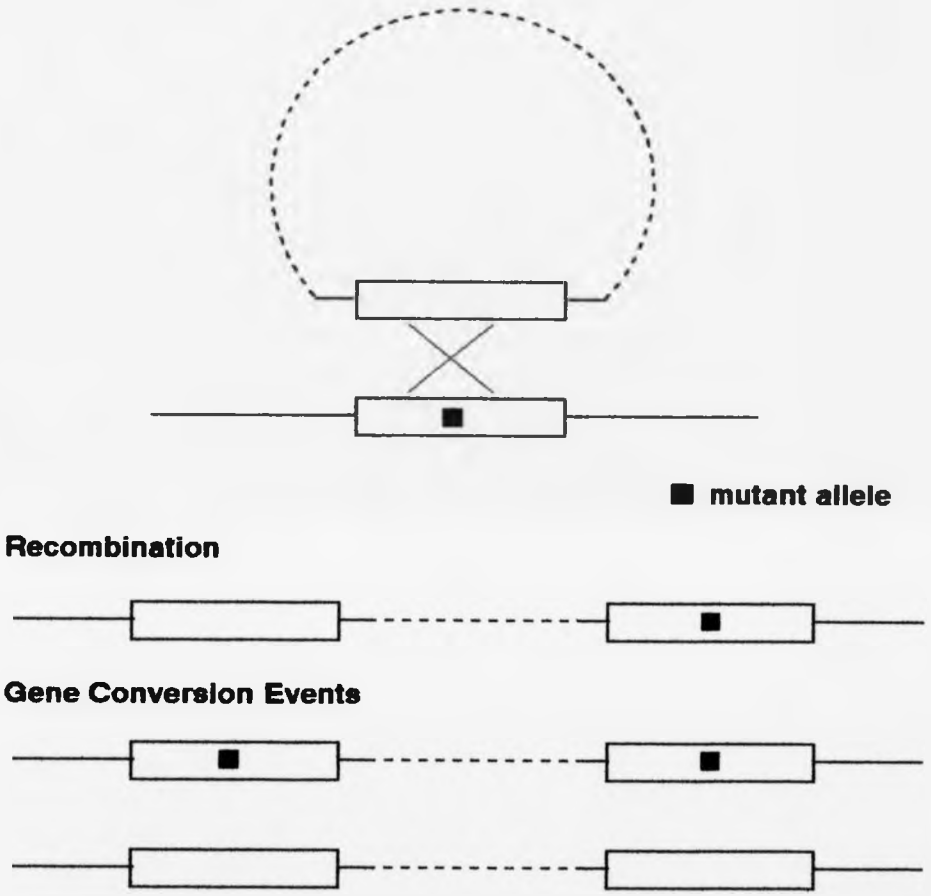


Fig. 6.1 Recombinational Repair and Gene Conversion.

Following recombination between a plasmid bearing M. xanthus DNA and the homologous region on the chromosome, a heterozygous merodiploid is formed.

Gene conversion events can lead to homozygotes containing either two mutant alleles or two wild-type alleles.

plasmid or the recipient strain (Shimkets et al., 1983). Hence, both the recipient and donor DNA must make similar contributions. It is hypothesized that the generation of homozygotes occurs via formation of a heteroduplex and mismatch repair which generates homozygotes containing two mutant alleles or homozygotes containing two wild-type alleles from heterozygotes containing one wild-type allele and one mutant allele (Shimkets et al., 1983).

Merodiploids can be isolated by direct selection of the antibiotic resistance genes carried on the vector. If a plasmid carrying a kanamycin resistance determinant in the vector DNA is transduced into a kanamycin sensitive recipient then transductants which are kanamycin resistant must contain the plasmid integrated into the chromosome and, hence, are merodiploids. Haploid cells in which a double crossover event had occurred would be sensitive to kanamycin. Once isolated, merodiploids are relatively stable and can be analysed for complementation/gene conversion of a mutant phenotype (O'Connor and Zusman, 1983; Shimkets et al., 1983). The proportion of transductants with wild-type and mutant phenotypes allows one to determine whether the mutant allele is recessive or dominant in comparison to the wild-type allele. A dominant mutant allele results in approximately 70% of the transductants showing the mutant phenotype, while the remaining 30% would have a wild-type phenotype. In contrast a recessive mutant allele results in approximately 70% of transductants having the wild-type phenotype, while the remaining 30% show the mutant

phenotype.

Once complementation of the glrA and glrB mutants by the pKIA plasmids had been assessed, subclones of pKIA4 (glrA$^+$) and pKIA2 (glrB$^+$) were constructed as described in Section 5.3. These subclones were assessed for their ability to complement DK440 (glrA1, bsg-440), DK510 (glrA2, Fru$^-$), DK3516 (glrB1, Fru$^-$) and DK3551 (glrB2, Fru$^-$). This was carried out in order to locate the glrA gene(s) and glrB gene(s) within the cloned regions of DNA.

6.2 Complementation Analysis with the pKIA Plasmids

In order to assess the ability of each of the pKIA plasmids to complement the glrA and glrB mutants each plasmid in turn was transduced, using coliphage P1 (Section 2.14), into the following strains ; DK101 (wild-type; Gly^S, Fru$^-$), DK440 (glrA1, bsg-440), DK510 (glrA2, Fru$^-$), DK3516 (glrB1, Fru$^-$) and DK3551 (glrB2, Fru$^-$). Merodiploids were obtained by selecting for transductants resistant to kanamycin. Transductant colonies were picked onto fresh DCY media containing 50μg/ml kanamycin to confirm resistance. One hundred colonies of each transductant strain were then screened on DCY agar containing 1.0M glycerol (Section 2.3.3) and on CF agar (Section 2.3.3) in order to assess the glycerol-resistance and fruiting phenotypes of the resultant transductants. The percentages of glycerol-resistant, non-fruiting (Gly^r, Fru$^-$), glycerol-sensitive, non-fruiting (Gly^S, Fru$^-$), glycerol-resistant, fruiting

(Gly^r, Fru^+) and glycerol-sensitive, fruiting (Gly^s, Fru^+) transductants from each host/plasmid combination are shown in Table 6.1. When pKIA3, pKIA4 and pKIA9 were transduced into the glycerol-resistant mutants DK440 (glrA1, bsg-440) and DK510 (glrA2, Fru^-) large numbers of glycerol-sensitive, non-fruiting (Gly^s, Fru^-) transductants were obtained. In these transductants the plasmid DNA appeared to complement the glrA mutations of the parental strains, resulting in the glycerol-sensitive phenotype. This suggested that pKIA3, pKIA4 and pKIA9 all contain the glrA gene(s). This was consistent with the Southern blot analysis (Chapter 4) and restriction analysis (Chapter 5) of these plasmids which showed that the chromosomal inserts in the three plasmids formed a nested series of DNA cloned from the ΩDK3554 region, which had been shown to co-transduce with the glrA mutations. That the fruiting mutations in these strains were not also complemented suggested that the fruiting mutation was the result of a mutation at a second locus which has been shown to be co-transduced with ΩDK3554 (Andreasson, Blea and Kaiser, personal communication) but is distinct from the glrA locus. A small minority (18% to 28%) of transductants were glycerol-resistant, non-fruiting (Gly^r, Fru^-) and, hence, were probably homozygotes carrying two copies of the mutant version, arising from gene conversion. The frequency of homozygotes carrying two copies of the wild-type allele arising in this study is unknown, as they were indistinguishable from heterozygotes on the basis of the

Donor	Recipient	Transductant Phenotype			
		Gly^{r}Fru^{-}	Gly^{s}Fru^{-}	Gly^{r}Fru^{+}	Gly^{s}Fru^{+}
pKIA3	DK101 (wild-type)	0%	0%	0%	100%
	DK440 (glrA1, Fru-)	27%	73%	0%	0%
	DK510 (glrA2, Fru-)	18%	82%	0%	0%
	DK3516 (glrB1, Fru-)	100%	0%	0%	0%
	DK3551 (glrB2, Fru-)	100%	0%	0%	0%
pKIA4	DK101	0%	0%	0%	100%
	DK440	22%	78%	0%	0%
	DK510	23%	77%	0%	0%
	DK3516	100%	0%	0%	0%
	DK3551	100%	0%	0%	0%
pKIA9	DK101	0%	0%	0%	100%
	DK440	17%	83%	0%	0%
	DK510	28%	72%	0%	0%
	DK3516	100%	0%	0%	0%
	DK3551	100%	0%	0%	0%
pKIA2	DK101	0%	0%	0%	100%
	DK440	100%	0%	0%	0%
	DK510	100%	0%	0%	0%
	DK3516	100%	0%	0%	0%
	DK3551	42%	58%	0%	0%
pKIA6	DK101	0%	0%	0%	100%
	DK440	100%	0%	0%	0%
	DK510	100%	0%	0%	0%
	DK3516	100%	0%	0%	0%
	DK3551	99%	1%	0%	0%
pKIA7	DK101	0%	0%	0%	100%
	DK440	100%	0%	0%	0%
	DK510	100%	0%	0%	0%
	DK3516	100%	0%	0%	0%
	DK3551	39%	61%	0%	0%
pKIA1	DK101	0%	0%	0%	100%
	DK440	100%	0%	0%	0%
	DK510	100%	0%	0%	0%
	DK3516	37%	0%	63%	0%
	DK3551	100%	0%	0%	0%

Table 6.1 Complementation Analysis Using the pKIA Plasmid Series.

phenotypes tested. These observations clearly indicate that the *glrA*$^{+}$ allele was dominant. No complementation of the *glrB* mutants, DK3516 and DK3551, by plasmids pKIA3, pKIA4 and pKIA9 was evident and the introduction of any of these plasmids into the wild-type strain, DK101, had no effect on the phenotype of the wild-type.

Mx*8* transduction mapping of the *glrA* mutations indicated that the *glrA2* (DK510) mutation is situated 15.0kb away from the ΩDK3554 Tn*5* insertion and that the *glrA1* (DK440) mutation is situated 18.0kb away from the Tn*5* insertion ΩDK3551, as shown in Figure 1.5 (Andreasson, Blea and Kaiser, personal communication). The plasmids pKIA3, pKIA4 and pKIA9 contain 27.0kb, 15.9kb and 13.6kb of cloned DNA respectively, with each clone starting from immediately adjacent to the Tn*5* insertion. Hence, one would have predicted that pKIA3 would complement both mutations; pKIA4 would have complemented the *glrA2* (DK510) mutation but not the *glrA1* (DK440) mutation and pKIA9 would not complement either mutation. The observation that all three plasmids complemented both mutations suggested that both the *glrA1* and *glrA2* mutations are located within 13.6kb from the ΩDK3554 Tn*5* insertion.

When pKIA2 and pKIA7 were transduced into DK3551 (*glrB2*, Fru^{-}) a large number of the resultant transductants were glycerol-sensitive, non-fruiting (Gly^{S}, Fru^{-}) (Table 6.1) suggesting that pKIA2 and pKIA7 contain DNA that complements the *glrB2* mutation and, thus, could contain a *glrB* gene. The fruiting mutation

was not complemented suggesting that the abnormality in fruiting may be the result of a second mutation at a locus which has been shown to be co-transduced with ΩDK3517 (Andreasson, Blea and Kaiser, personal communication), but is distinct from the glrB locus. Again a small minority (39% to 42%) of glycerol-resistant, non-fruiting (Gly^r, Fru^-) transductants were obtained, which were believed to be homozygotes containing two mutant alleles, arising from gene conversion. These observations suggested that the $glrB^+$ allele was dominant. The plasmid pKIA6, which had been shown by Southern blot analysis to contain a region of shared homology with pKIA2 and pKIA7, did not appear to complement this mutant. Restriction mapping of pKIA6 indicated that this plasmid contained a deletion within the vector pREG429 DNA. However, this deletion was not in the region of shared homology with pKIA2 (pKIA7). Preliminary complementation analysis by Blea (personal communication) had suggested that pKIA2, pKIA6 and pKIA7 all complemented DK3516 (glrB1, Fru^-) to glycerol-sensitivity. The plasmid pKIA7 also appeared to complement DK3551 (glrB2, Fru^-) to the glycerol-sensitive phenotype, while pKIA2 and pKIA6 were not tested in DK3551.

The plasmid pKIA1 did not complement the glycerol phenotype of any of the mutants. However, on transduction of pKIA1 into DK3516 (glrB1, Fru^-) several glycerol-resistant, fruiting (Gly^r, Fru^+) transductants were observed. Thus pKIA1 appeared to complement the

fruiting mutation in this strain. That the fruiting mutation can be complemented without complementation of the mutation in glycerol-induced sporulation suggests that the two abnormalities was the result of two independent mutations at separate, but co-transducible loci. The plasmid pKIA1 contains DNA from the opposite side of the chromosomal Tn*5* insertion ΩDK3517 to pKIA2, pKIA6 and pKIA7, as shown by Southern blot analysis (Chapter 4). Hence, it would appear that the DNA cloned in pKIA2 and pKIA6 contains the *glrB* region. Neither the *glrA1* (DK440) or *glrA2* (DK510) mutations were complemented by pKIA1, pKIA2, pKIA6 or pKIA7 and none of these plasmids had any effect on the phenotype of the wild-type strain when introduced into DK101.

Plasmids pKIA3 and pKIA2 were chosen as representatives of the *glrA* and *glrB* regions respectively and were transduced into a further 43 glycerol-resistant mutants in order to see if any of the mutations in these mutants could be complemented by DNA from the *glrA* or *glrB* regions. The mutants had been isolated using EMS, NTG and UV mutagenesis, as described in Chapter 3. Thirteen of these glycerol-resistant mutants were also unable to undergo fruiting body sporulation while the remaining thirty fruited quite normally. The results of this complementation analysis are shown in Tables 6.2 and 6.3. The mutant HMC194 (Gly^r, Fru^+) appeared to be complemented by pKIA2 as 58% of the resultant transductants screened were glycerol-sensitive (Table 6.3) and, hence, had been complemented to the wild-type

Recipient	Donor	Transductant	Phenotype	Donor	Transductant	Phenotype
		Gly^rFru^-	Gly^sFru^-		Gly^rFru^-	Gly^sFru^-
HMC3	pKIA3 (girA)	100%	0%	pKIA2 (girB)	100%	0%
HMC10		100%	0%		100%	0%
HMC13		100%	0%		100%	0%
HMC14		100%	0%		100%	0%
HMC16		100%	0%		100%	0%
HMC167		100%	0%		100%	0%
HMC182		100%	0%		100%	0%
HMC216		100%	0%		100%	0%
HMC223		100%	0%		100%	0%
HMC225		100%	0%		100%	0%
HMC273		100%	0%		100%	0%
HMC2107		100%	0%		100%	0%
HMC2111		100%	0%		100%	0%

Table 6.2 Complementation Analysis of Gly^rFru^- Mutants Using pKIA3 (girA) and pKIA2 (girB).

Recipient	Donor	Transductant	Phenotype	Donor	Transductant	Phenotype
		$Gly^r Fru^+$	$Gly^s Fru^+$		$Gly^r Fru^+$	$Gly^s Fru^+$
HMC1	pKIA3 (glrA)	100%	0%	pKIA2 (glrB)	100%	0%
HMC2		100%	0%		100%	0%
HMC7		100%	0%		100%	0%
HMC101		100%	0%		100%	0%
HMC102		100%	0%		100%	0%
HMC103		100%	0%		100%	0%
HMC104		100%	0%		100%	0%
HMC105		28%	72%		34%	66%
HMC106		100%	0%		100%	0%
HMC107		92%	8%		100%	0%
HMC108		100%	0%		94.4%	5.6%
HMC109		96%	4%		100%	0%
HMC110		100%	0%		100%	0%
HMC187		100%	0%		100%	0%
HMC194		100%	0%		17%	83%
HMC201		100%	0%		100%	0%
HMC202		100%	0%		100%	0%
HMC203		100%	0%		100%	0%
HMC204		100%	0%		100%	0%
HMC205		100%	0%		100%	0%
HMC206		100%	0%		100%	0%
HMC207		100%	0%		100%	0%
HMC208		100%	0%		100%	0%
HMC209		100%	0%		100%	0%
HMC210		100%	0%		100%	0%
HMC281		100%	0%		100%	0%
HMC296		100%	0%		100%	0%
HMC2113		100%	0%		100%	0%
HMC2611		100%	0%		100%	0%
HMC2811		100%	0%		100%	0%

Table 6.3 Complementation Analysis of $Gly^r Fru^+$ Mutants Using pKIA3 (glrA) and pKIA2 (glrB).

phenotype. This observation was confirmed by repeating the analysis using pKIA7 which has an identical restriction map to the insert of pKIA2. Hence, the mutation in HMC194 was designated *glrB3*.

An interesting result was also observed with the mutant HMC105 (Gly^r, Fru^+). Upon introduction of the plasmid pKIA3 (*glrA*$^+$) into this mutant 66% of the resultant transductants were glycerol-sensitive, while the other 34% remained glycerol-resistant (Table 6.3). This suggested that the mutation in the mutant HMC105 was complemented by DNA from the *glrA* region. However, when pKIA2 (*glrB*$^+$) was introduced into the same mutant 72% of the resultant transductants were glycerol-sensitive while the other 18% remained glycerol-resistant, suggesting that the mutation in HMC105 could be complemented by DNA from the *glrB* region. It is highly unlikely that both the *glrA* and *glrB* regions of DNA can complement the same mutation as Southern blot analysis (Chapter 4) and restriction analysis (Chapter 5) showed that the two regions do not share any homology. A more likely explanation for this observation is that transduction of vector DNA into the mutant results in a high frequency of reversal from the mutant phenotype to the wild-type phenotype. This hypothesis could be tested by introducing a plasmid containing the same vector sequences but an unrelated region of *M. xanthus* chromosomal DNA, e.g. genes involved in the regulation of carotenogenesis.

A minority (4% to 8%) of glycerol-sensitive

transductants were obtained upon introduction of pKIA3 into HMC107 (Gly^r, Fru^+) and HMC109 (Gly^r, Fru^+) and also upon transduction of pKIA2 into HMC108 (Gly^r, Fru^+). As the frequency of glycerol-sensitive transductants was below 10% it is thought that these transductants were probably revertants. None of the other mutants tested were affected by the presence of either plasmid.

6.3 Analysis of the glrA Region

6.3.1 Complementation Analysis Using Subclones Derived from pKIA4

The complementation analysis with the pKIA plasmids suggested that pKIA3, pKIA4 and pKIA9 all contain DNA which complements both DK440 (*glrA1*, *bsg-440*) and DK510 (*glrA2*, Fru^-) from glycerol-resistant to the wild-type glycerol-sensitive phenotype. The plasmid pKIA4 was chosen as a representative of this region. The plasmids pHMC6, pHMC8, pHMC40, pHMC42 and pHMC44 all contain DNA subcloned from the insert in pKIA4, as shown in Figure 5.7. The construction of these plasmids is discussed in Chapter 5 (Section 5.3). All the plasmids were designed so that they contained a kanamycin resistance determinant in the vector DNA. Each of the subclones was transduced into the wild-type, DK101 and DK440 (*glrA1*, *bsg-440*) and DK510 (*glrA2*, Fru^-) using the coliphage P1 (Section 2.14). Kanamycin resistant merodiploids were selected for. Transductant colonies were picked onto DCY agar containing 50μg/ml kanamycin to confirm the antibiotic resistance. Two hundred transductant colonies were

screened on DCY agar containing 1.0M glycerol and CF agar to assess the glycerol-sensitivity and fruiting phenotypes of the transductant strains.

The plasmids pHMC6 and pHMC8 were constructed and assessed for complementation ability first. The results are shown in Table 6.4. Neither plasmid had any effect when introduced into the wild-type, DK101. Plasmid pHMC6 did not complement either DK440 (*glrA1*, *bsg-440*) or DK510 (*glrA2*, Fru^-). All 200 transductant colonies tested for each strain remained glycerol-resistant, non-fruiting (Gly^r, Fru^-). Plasmid pHMC8 did appear to complement both the *glrA1* (DK440) and *glrA2* (DK510) mutations as 60% and 59%, respectively, of transductants were glycerol-sensitive. Again the fruiting mutations were not complemented. In each case approximately 40% of transductants remained glycerol-resistant. These transductants were presumed to be homozygous merodiploids containing two mutant copies of the region as a result of gene conversion. The observation that pHMC8 complemented both the *glrA1* (DK440) and *glrA2* (DK510) mutations while pHMC6 did not complement either mutation narrowed the location of the *glrA* gene(s) to a 9.1kb *Eco*RI *Eco*RV fragment, as shown in Figure 6.2. However, as pKIA9, which is 2.3kb smaller than pKIA4, also complemented both mutations the location of the gene(s) can be narrowed down even further to a 6.8kb *Bam*HI *Eco*RV fragment (Figure 6.2). The plasmids pHMC40, pHMC42 and pHMC44 all contain DNA subcloned from this 6.8kb *Bam*HI *Eco*RV fragment as shown in Figure 5.7. All three plasmids were transduced

Donor	Recipient	Transductant Phenotype	
		$Gly^{r}Fru^{-}$	$Gly^{s}Fru^{-}$
pKIA4	DK440 (glrA1, Fru-)	22%	78%
	DK510 (glrA2, Fru-)	23%	77%
pKIA9	DK440	17%	83%
	DK510	28%	72%
pHMC6	DK440	100%	0%
	DK510	100%	0%
pHMC8	DK440	40%	60%
	DK510	41%	59%

Table 6.4 Complementation Analysis Using the glrA Region Subclones pHMC6 and pHMC8.

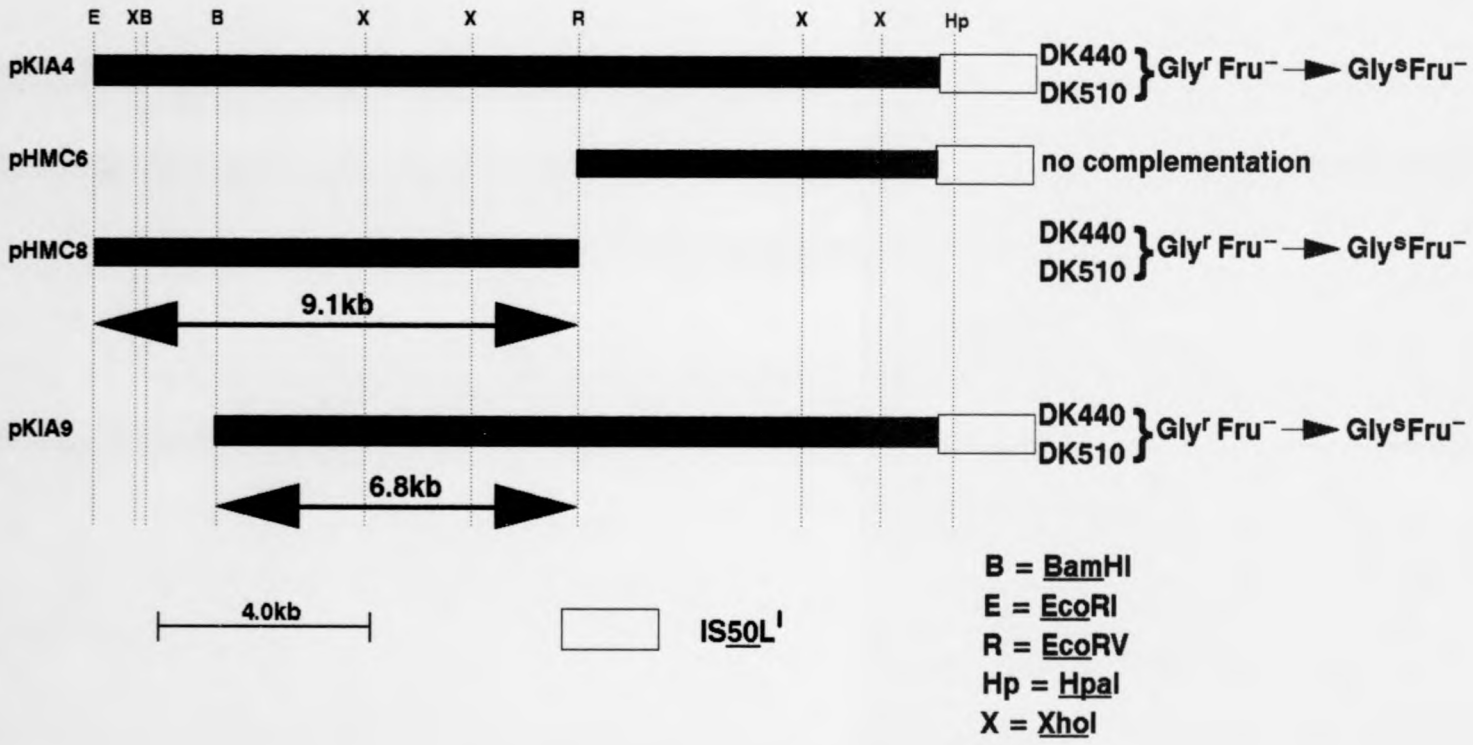


Fig. 6.2 Localization of the glrA1 and glrA2 Mutations.

into the wild-type, DK101, and the mutants DK440 (*glrA1*, *bsg-440*) and DK510 (*glrA2*, Fru$^-$). Kanamycin transductant merodiploids were selected for and 200 transductant colonies of each host/plasmid combination were screened for glycerol-sensitivity and fruiting phenotypes on DCY agar containing 1.0M glycerol and CF agar. The results are shown in Table 6.5. The plasmids pHMC40 and pHMC44 did not complement either the *glrA1* (DK440) or *glrA2* (DK510) mutations. Plasmid pHMC42, however, did appear to complement the *glrA1* (DK440) mutation. Seventy eight percent of the transductant colonies were glycerol sensitive indicating that the DNA cloned in pHMC42 complemented the mutation responsible for the glycerol-resistant phenotype of the host DK440 (*glrA1*, *bsg-440*). The fruiting mutation was not complemented. Twenty two percent of the transductants remained glycerol-resistant and were presumably homozygous merodiploids arising from gene conversion. The *glrA2* (DK510) mutation was not complemented by the 2.0kb *Xho*I fragment carried in pHMC42.

All the subclones derived from the *glrA* region were also transduced into DK3516 (*glrB1*, Fru$^-$) and DK3551 (*glrB2*, Fru$^-$). As expected no complementation was evident.

6.3.2 *Examination of Promoter Activity in the glrA Region*

The plasmid pHMC40 contains a 2.8kb *Bam*HI *Xho*I region of *M. xanthus* DNA cloned into the multiple cloning site of the vector pDAH283. The vector pDAH283 (Figure

Donor	Recipient	Transductant Phenotype	
		Gly^{r} Fru^{-}	Gly^{s} Fru^{-}
pKIA4	DK440 (glrA1, Fru^{-})	22%	78%
	DK510 (glrA2, Fru^{-})	23%	77%
pHMC40	DK440	100%	0%
	DK510	100%	0%
pHMC42	DK440	22%	78%
	DK510	100%	0%
pHMC44	DK440	100%	0%
	DK510	100%	0%

Table 6.5 Complementation Analysis Using the glrA Region Subclones pHMC40, pHMC42 and pHMC44.

5.10) contains a promoterless lacZ gene with a multiple cloning site upstream of it so that fragments can be easily cloned into the plasmid. If the DNA fragment contains an exogenous promoter in the correct orientation it can direct transcription of the lacZ gene. Plasmid pHMC40 was constructed such that the BamHI end of 2.8kb fragment was adjacent to the lacZ gene (see Figure 6.3). A dense 5ml culture (A_{600} = 0.7) of DK101[pHMC40] (the wild-type strain containing the integrated plasmid pHMC40) cells was centrifuged at 4,500rpm in a Wifug Labor-50M bench-top centrifuge and resuspended in 2.5ml TM buffer (Section 2.3.2) and 20μl aliquots were inoculated onto DCY agar, DCY agar containing 1.0M glycerol, A1 minimal agar (Section 2.3.3) and TM agar (Section 2.3.3). A1 minimal agar was used as a control as it just allows the growth of M. xanthus without starvation occurring. After two days incubation at 33^{o}C the plates were assayed for ß-galactosidase expression using 4-MUG (Section 2.12). Duplicate plates were also set up containing 40μg/ml X-gal as an indicator for ß-galactosidase expression. Using 4-MUG ß-galactosidase expression was evident on all plates suggesting that the gene in question is expressed constitutively (i.e. it is expressed during vegetative growth, glycerol-induced sporulation and fruiting body sporulation). The plates containing X-gal as an indicator for ß-galactosidase activity showed clear activity in vegetative cells and during fruiting body sporulation as the cells on DCY, A1 and TM agar were blue in colour. The cells on DCY agar

containing 1.0M glycerol were yellow with a very slight green tinge, suggesting that there was very little ß-galactosidase expression. The contradictory results for ß-galactosidase expression from the X-gal and 4-MUG assays suggests that glycerol-induced spores may not take up X-gal as efficiently as vegetative cells and fruiting body spores. 4-MUG was used as the indicator for ß-galactosidase activity in all future qualitative assays.

A 500ml culture of DK101[pHMC40] cells was grown in DCY broth (Section 2.3.3) to a density of 9.0×10^7 cells/ml ($A_{600} = 0.3$). A t=0 sample was withdrawn and the culture was divided into two samples. To one half of the culture 50% glycerol was added to a final concentration of 1.0M. The other sample was left unmodified as a vegetative control. The cultures were shaken at 33°C and samples were withdrawn 1, 2, 3, 4, 5, 10, 15, 20, and 25 hours after the addition of glycerol. Samples were collected and stored as described in Section 2.11. Phase-contrast microscopy revealed that in the sample to which glycerol had been added the rod shaped cells converted to spherical spores 1 - 3 hours after the addition of glycerol. ß-Galactosidase specific activity was determined (Section 2.12, 2.13) after disruption of the cells by sonication (Section 2.11). ß-Galactosidase expression remained at a moderately constant level of 50 - 70 units/mg protein throughout both vegetative growth and glycerol-induced sporulation. This suggests that the gene in question is a constitutive gene.

A summary of the results obtained from analysis of

the glrA region is shown in Figure 6.3.

6.4 Analysis of the glrB Region

6.4.1 Complementation Analysis Using Subclones Derived from pKIA2

The complementation analysis with the pKIA plasmids suggested that pKIA2 (pKIA7) may complement the glrB2 (DK3551) mutation. Therefore, subclones were constructed from the insert in pKIA2. The plasmids pHMC2, pHMC4, pHMC30, pHMC32, pHMC34 and pHMC36 all contain DNA subcloned from the insert in pKIA2, as shown in Figure 5.8. The construction of these plasmids is discussed in Chapter 5 (Section 5.3). All the plasmids were constructed so that they contain a kanamycin resistance determinant in the vector DNA. Each of the subclones was transduced into the wild-type strain DK101, DK3516 (glrB1, Fru^-) and DK3551 (glrB2, Fru^-) using coliphage P1 (Section 2.14). Kanamycin resistant merodiploids were selected for. Two hundred transductant colonies were screened for glycerol-resistance and fruiting phenotype on DCY agar containing 1.0M glycerol and CF agar.

The plasmids pHMC2 and pHMC4 were constructed and assessed for complementation analysis first. Neither subclone complemented either the glrB1 (DK3516) or the glrB2 (DK3551) mutation. All transductants showed the glycerol-resistant, non-fruiting (Gly^r, Fru^-) phenotype of the parental strains. The plasmids pHMC2 and pHMC4 each contain approximately half of the DNA cloned in the plasmid pKIA2, splitting the DNA at the unique SalI site

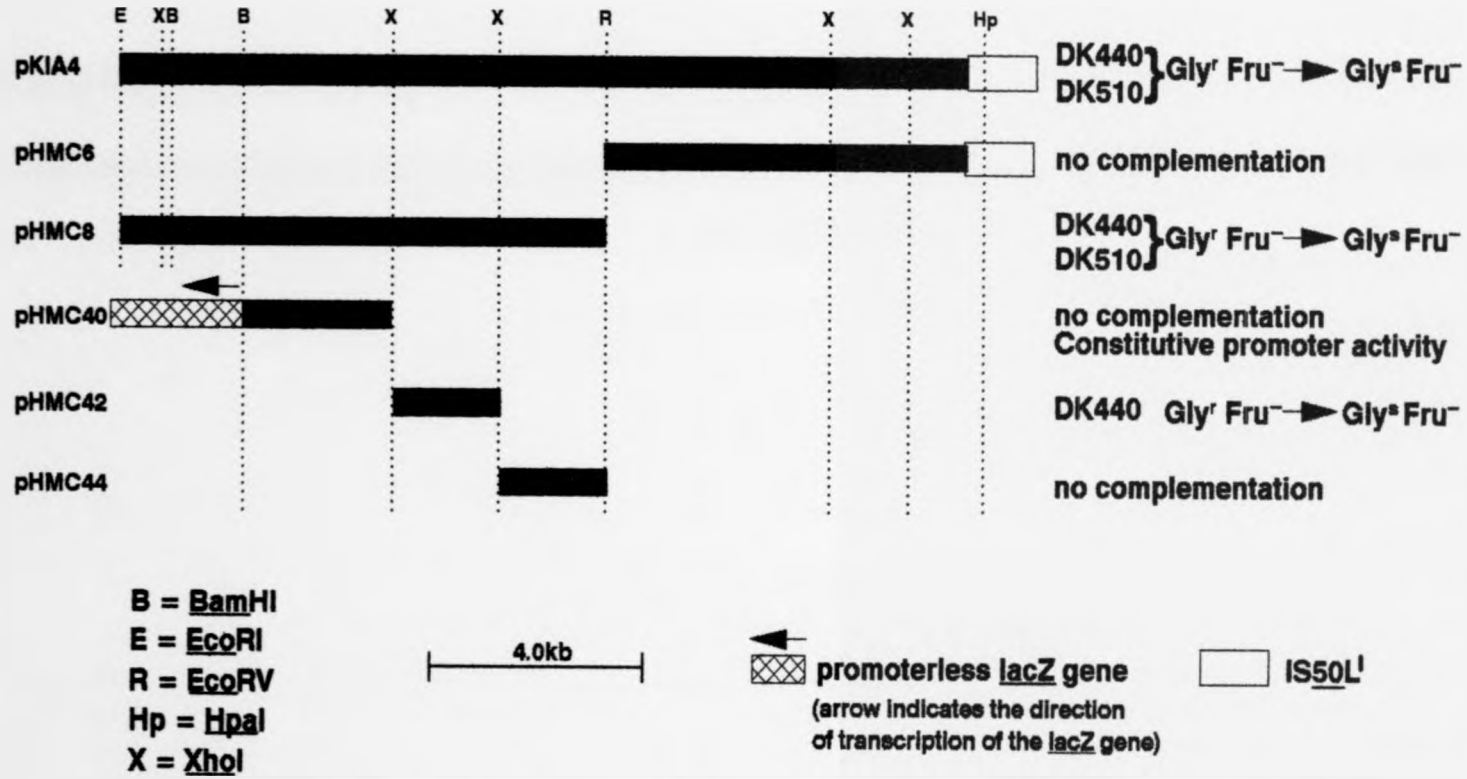


Fig. 6.3 Summary of Observations from the glrA Region.

(See Figure 5.8). Hence, if pKIA2 does contain the necessary DNA to complement the glrB2 (DK3551) mutation, but neither of the two subclones complement the mutation it could be suggested that the glrB2 mutation may be very close to the SalI site within the pKIA2 clone which was used to clone the fragment into two subclones. For this reason two other subclones pHMC30 and pHMC32 were constructed which contain DNA subcloned from across the SalI site, as shown in Figure 5.8. These subclones were used in complementation analysis and were found not to complement either the glrB1 (DK3516) or the glrB2 (DK3551) mutations.

Complementation analysis with pKIA2 (pKIA7) had suggested that it contained DNA capable of complementing the glycerol-resistant mutant HMC194 (glrB3, Fru^+). In view of this pHMC2, pHMC4, pHMC30 and pHMC32 were also transduced into the mutant HMC194. The mutation in glycerol-induced sporulation of this mutant was not complemented by any of the subclones derived from pKIA2.

All the subclones derived from the glrB region were also transduced into DK440 (glrA1, bsg-440) and DK510 (glrA2, Fru^-). As expected no complementation was evident.

6.4.1 Examination of Promoter Activity in the glrB Region

Two other plasmids were constructed which contained subcloned DNA from the glrB region. The plasmids pHMC34 and pHMC36 contain DNA fragments (Figure 5.8) cloned into the vector pDAH283 (Figure 5.10). The construction of

these plasmids is discussed in Chapter 5 Section 5.3. The fragments were ligated into restriction sites in the multiple cloning site in pDAH283 upstream of the promoterless *lacZ*. The plasmids pHMC34 and pHMC36 were transduced into the wild-type, DK101, background and kanamycin resistant transductants were selected for. The transductant colonies were screened for fruiting and glycerol-resistance on CF agar and DCY agar containing 1.0M glycerol respectively. All transductant colonies remained glycerol-sensitive, fruiting (Gly^S, Fru^+). Dense (A_{600} = 0.7) 5ml cultures of DK101[pHMC34] and DK101[pHMC36] cells were precipitated at 4,500rpm in a Wifug Labor-50M bench-top centrifuge and resuspended in 2.5ml TM buffer and inoculated in 20µl aliquots onto DCY agar, DCY agar containing 1.0M glycerol, A1 minimal agar and TM agar. After two days incubation at 33°C the plates were assessed for ß-galactosidase specific activity using 4-MUG. DK101[pHMC34] showed no evidence of ß-galactosidase expression under any conditions. Therefore, presumably the fragment does not contain a promoter in the correct orientation to transcribe the *lacZ* gene. DK101[pHMC36] cells, however, showed considerable ß-galactosidase expression during vegetative growth and during both glycerol-induced sporulation and fruiting body sporulation. Hence, the 2.6kb *Xho*I *Sal*I fragment cloned in pHMC36 appears to contain a promoter in the appropriate orientation to transcribe the *lacZ* gene. The cloned fragment is orientated such that the *Sal*I end of the fragment is adjacent to the *lacZ* gene. A

500ml culture of DK101[pHMC36] cells was grown in DCY broth to a density of 9.0 x 10^7 cells/ml (A_{600} = 0.3) and samples were collected during glycerol-induced sporulation and vegetative growth, in the manner described for DK101[pHMC40] in Section 6.3.2, and assayed for ß-galactosidase specific activity (Section 2.12, 2.13). The results are shown in Figure 6.4. The promoter in question was expressed at elevated levels during vegetative growth as ß-galactosidase expression increased approximately five-fold during 25 hours of growth. A similar pattern of ß-galactosidase expression was seen during glycerol-induced sporulation, although expression dropped off sharply 20 - 25 hours after the addition of glycerol. Hence, expression from the promoter is not sporulation-specific, but may have an interesting relationship to stationary phase since ß-galactosidase activity in vegetative cells continues to increase even when the cells enter stationary phase.

6.4.3 Insertion Mutagenesis of the glrB Region

In order to further analyse this gene the plasmid pHMC38 was constructed which contained a tetracycline resistance determinant, derived from ColE1 Tn*5*-132 (Rothstein *et al.*, 1980) inserted at the *Sal*I site of pKIA2, as shown in Figure 6.5. The tetracycline cassette was derived from Tn*10* and expression of the tetracycline resistance is inducible by subinhibitory levels of the antibiotic (Wray *et al.*, 1981; Bertrand *et al.*, 1983). A 4.84kb *Xho*I fragment from ColE1 Tn*5*-132 containing the

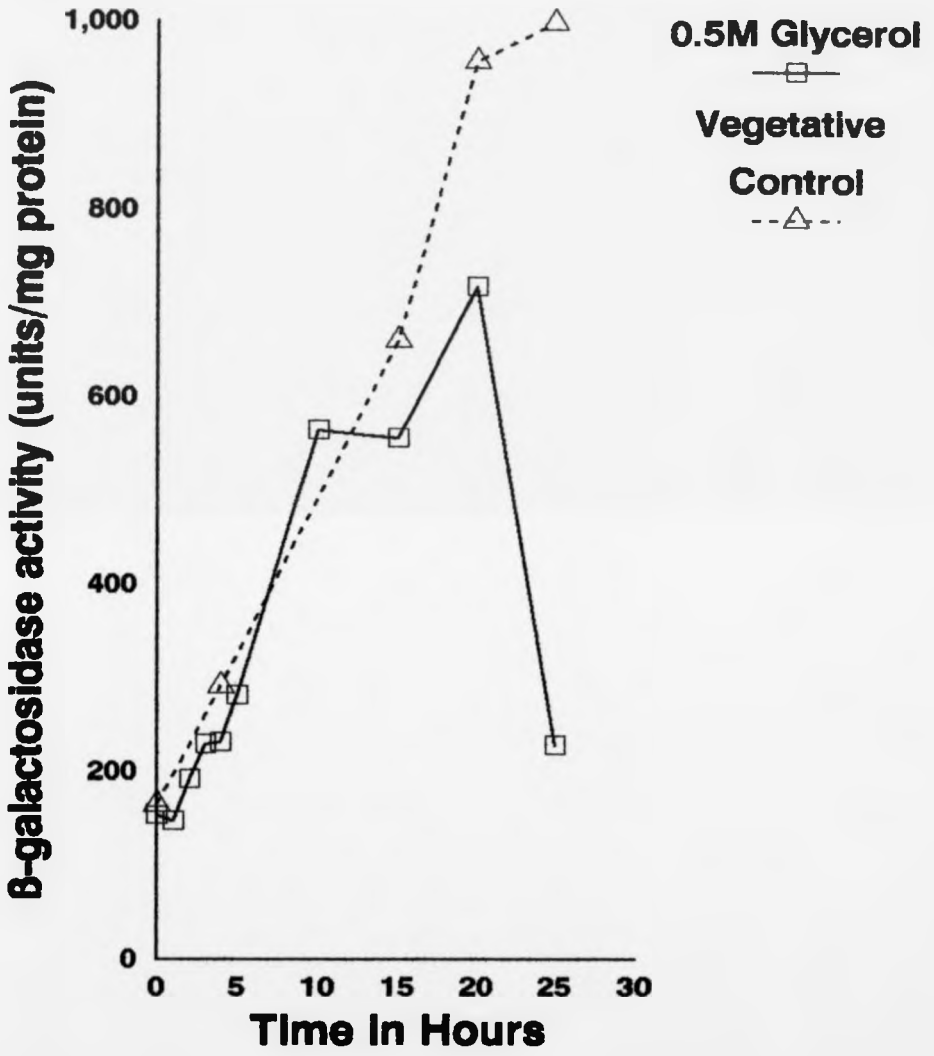


Fig. 6.4 ß-Galactosidase Expression During Glycerol-Induced Sporulation and Vegetative Growth of DK101[pHMC36].

tetracycline resistance cassette was inserted into the SalI site in the centre of the pKIA2 cloned DNA (Figure 6.5). This disrupts the clone at the point where promoter activity was recorded. Recombinant clones were obtained in E. coli MC1061 as described in Section 2.21. Transformants were selected for on LB agar (Section 2.3.3) containing 50μg/ml kanamycin and a gradient of tetracycline from 0 - 10μg/ml. The gradient of tetracycline allows the induction of the tetracycline cassette. After 16 hours incubation at 37°C confluent growth was apparent at low levels of tetracycline while single colonies were apparent on agar containing higher levels of tetracycline. These single colonies were picked onto fresh LB agar containing 50μg/ml kanamycin and 10μg/ml tetracycline to confirm the antibiotic resistances. The insertion of the tetracycline resistance determinant at the expected site was confirmed by restriction analysis.

The plasmid pHMC38 was transduced into the M. xanthus wild-type strain DK101, as described in Section 2.14 except that the transductants were selected for on DCY agar containing a gradient of oxytetracycline from 0 - 12.5μg/ml to allow induction of the tetracycline cassette. The kanamycin resistance determinant carried on the vector DNA was not selected for. After 4 - 5 days incubation at 33°C confluent growth of M. xanthus cells occurred at low levels of oxytetracycline. However, single colonies were observed on agar containing higher levels of oxytetracycline. These colonies were picked

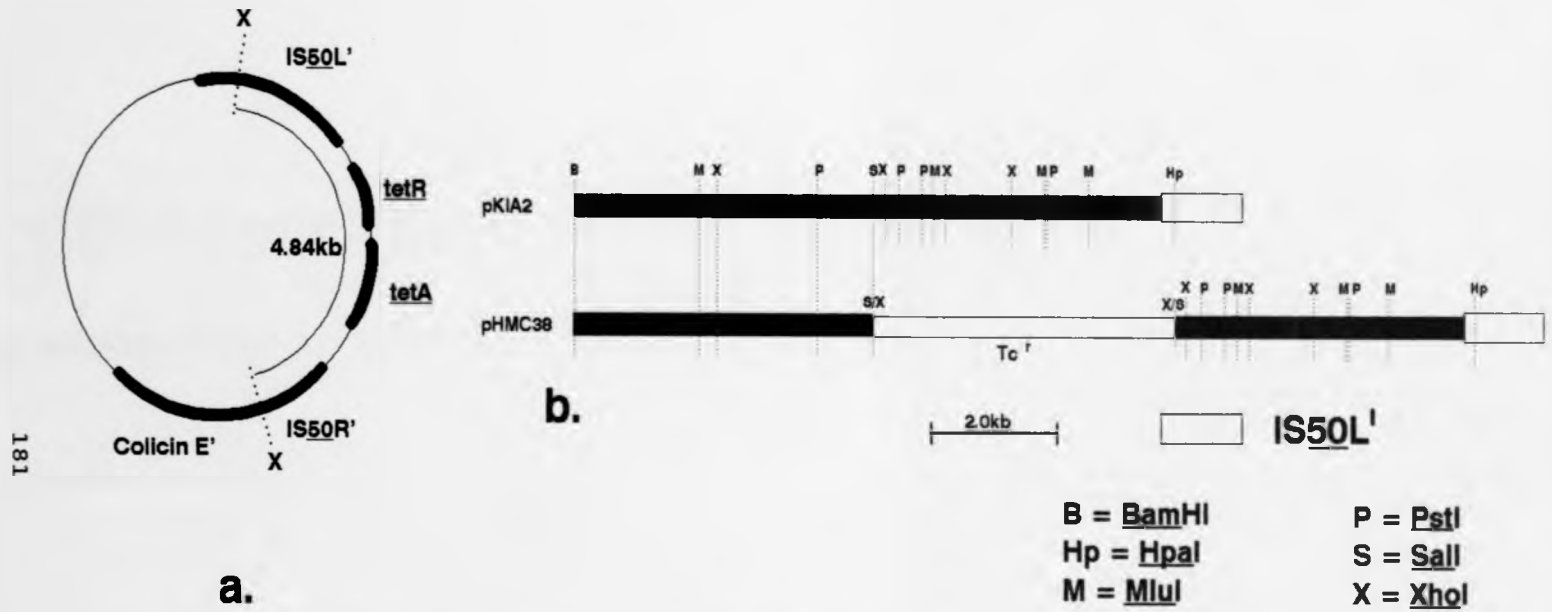


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Fig. 6.5 Construction of pHMC38, a Derivative of pKIA2 in which a Tetracycline Resistance Determinant has been Inserted into the SalI Site of pKIA2.

a. Col E1 Tn5-132

b. pKIA2 and the derivative pHMC38 in which a 4.84kb XhoI fragment containing the tetracycline resistance determinant was inserted at the unique SalI site within the M. xanthus DNA cloned in pKIA2.

onto fresh DCY agar containing 12.5μg/ml oxytetracycline to confirm the presence of the tetracycline resistance determinant in the M. xanthus chromosome. Eighty four tetracycline resistant transductants were obtained. Introduction of the plasmid into M. xanthus without selection for the antibiotic resistance on the vector portion of the plasmid could have either of two possible outcomes as shown in Figure 6.6; (1) A single crossover event results in the introduction of plasmid DNA and a second disrupted version of the region into the M. xanthus chromosome; (2) A double crossover event results in the replacement of the intact copy of the region with the disrupted version. Transductants in which a single crossover event had occurred would still carry the kanamycin resistance determinant on the integrated plasmid DNA. Transductants in which a double crossover event had occurred would be kanamycin sensitive. Transductant colonies were, therefore, screened for kanamycin sensitivity. Seventy two out of eighty four DK101[pHMC38] colonies were kanamycin sensitive, showing that a double crossover event had occurred. The other twelve transductants were kanamycin resistant, indicating that a single crossover event had occurred. Tetracycline resistant, kanamycin sensitive transductants, in which the intact copy of the region had been replaced by the disrupted version, were screened on CF agar and DCY agar containing 1.0M glycerol to test for glycerol-resistance and fruiting phenotypes. All 72 tetracycline resistant, kanamycin sensitive transductants

Fig. 6.6 Possible Outcomes of Introduction of pHMC38 into M. xanthus Without Selection for the Kanamycin Determinant in the Plasmid Vector Region.

A. A Single Crossover Event.

B. A Double Crossover Event.

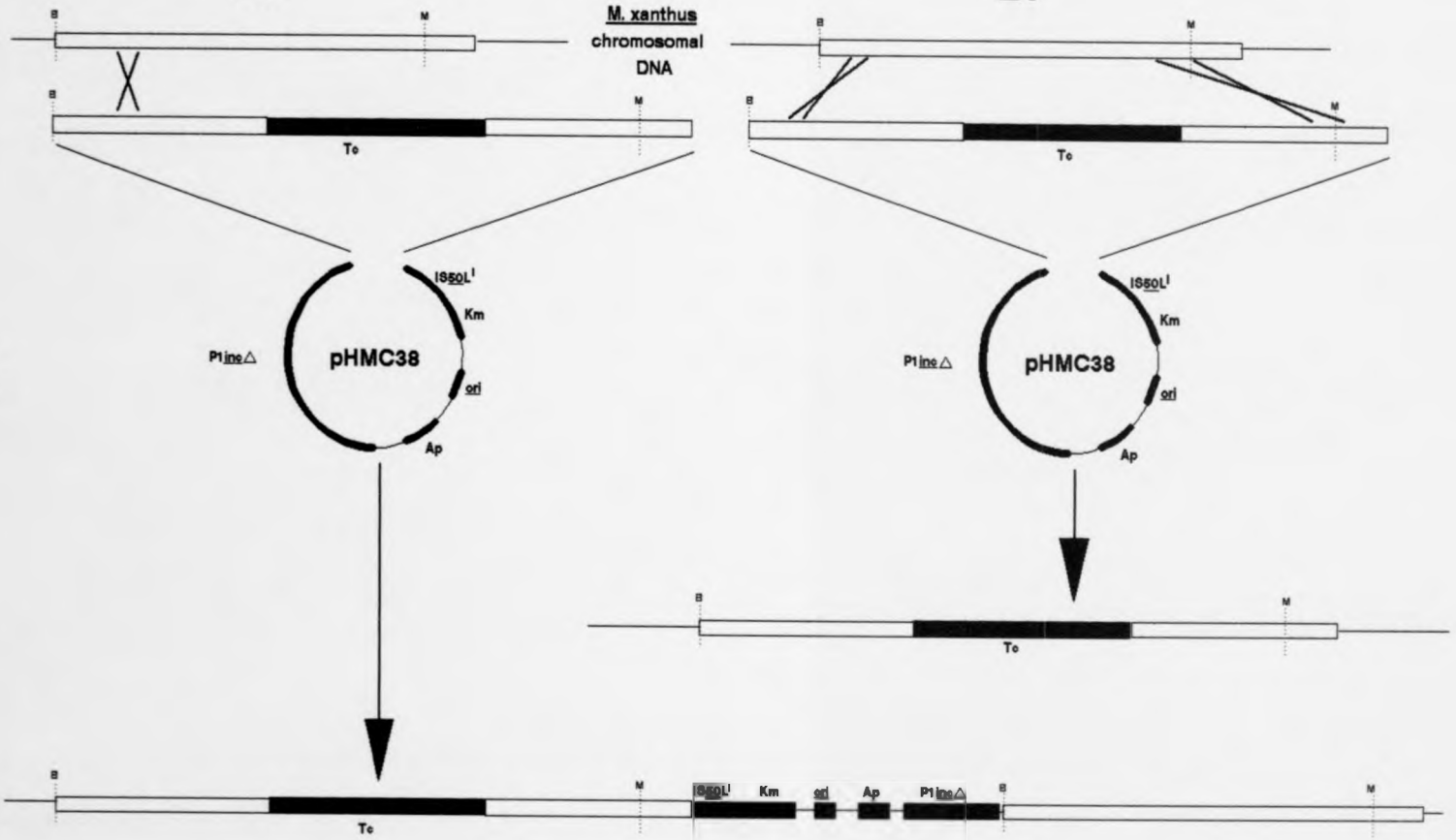

A.
B.
M. xanthus
chromosomal
DNA
B
M
Tc
IS50L
Km
ori
Ap
P1 ine△
pHMC38

Fig. 6.6

were glycerol-sensitive and fruited normally (i.e. wild-type phenotype). These results suggest that the SalI site occurs in a region that is not essential for vegetative growth, glycerol-induced sporulation or fruiting body sporulation.

A summary of the results observed for the glrB region is shown in Figure 6.7.

6.5 Discussion

Complementation analysis with the pKIA plasmids allowed the following observations: DK440 (glrA1, bsg-440) and DK510 (glrA2, Fru^-) were complemented from glycerol-resistant, non-fruiting to glycerol-sensitive, non-fruiting by pKIA3, pKIA4 and pKIA9. DK3551 (glrB2, Fru^-) was complemented from glycerol-resistant, non-fruiting to glycerol-sensitive, non-fruiting by the plasmid pKIA2 (pKIA7) and DK3516 (glrB1, Fru^-) was complemented from glycerol-resistant, non-fruiting to glycerol-resistant, fruiting by pKIA1. It was, therefore, suggested that the plasmids pKIA3, pKIA4 and pKIA9 contain the $glrA^+$ gene and pKIA2 (pKIA7) contains the $glrB^+$ gene. This is consistent with Southern blot analysis of the pKIA plasmids (Chapter 4). That in all cases only one of the two mutant phenotypes was complemented suggested that DK440, DK510, DK3516 and DK3551 may all contain two independent mutations at separate sites within the chromosome, one mutation being responsible for the block in glycerol-induced sporulation and the other mutation being responsible for the block in

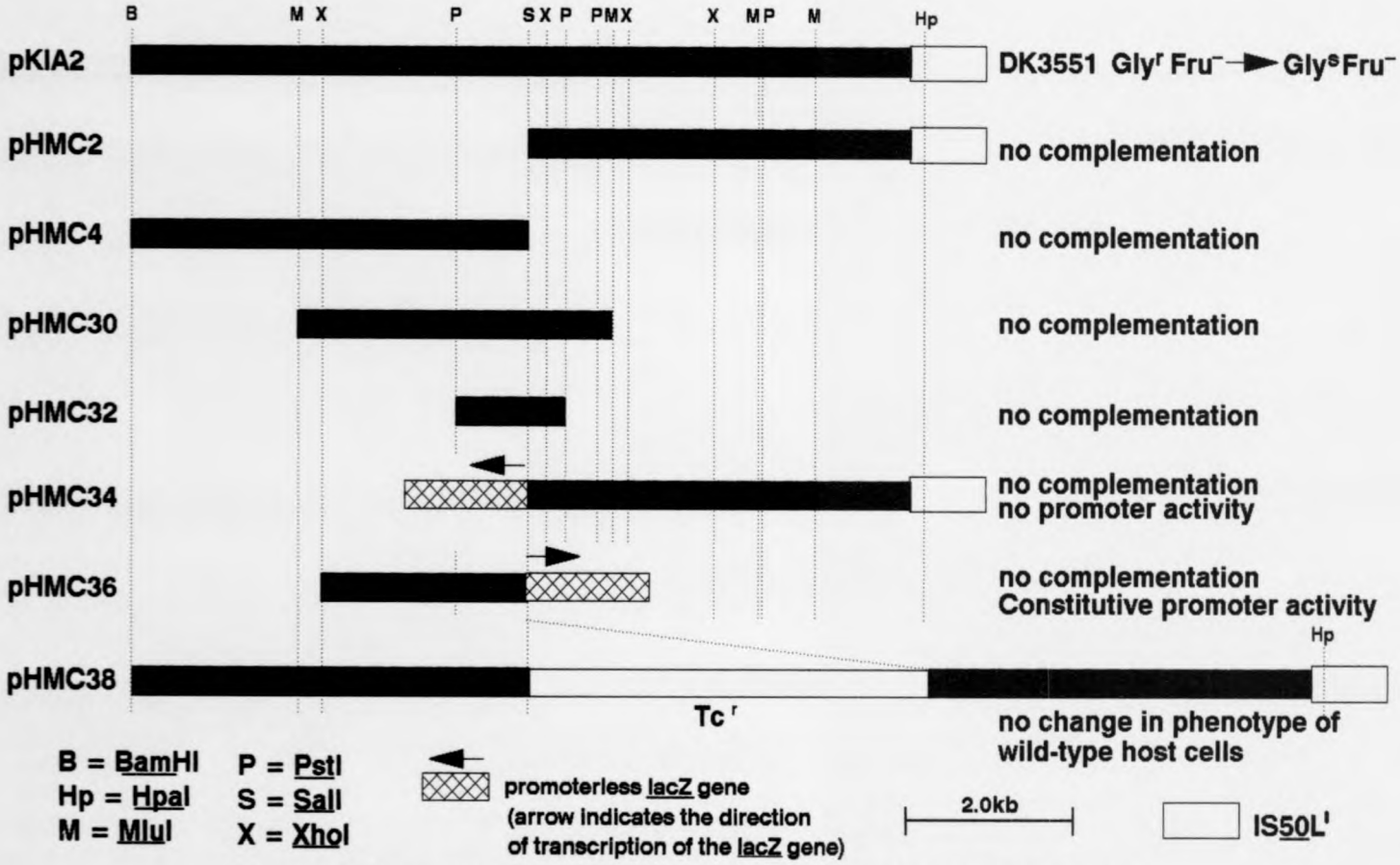


Fig. 6.7 Summary of Observations from the glrB Region.

fruiting body sporulation.

The plasmids, pKIA3 (*glrA*$^+$) and pKIA2 (*glrB*$^+$) were also transduced into a further 43 glycerol-resistant mutants. Of these mutants 30 fruited normally while the remaining 13 glycerol-resistant mutants were also blocked in fruiting body sporulation. One mutant, HMC194 (Gly^r, Fru$^+$) appeared to be complemented to glycerol-sensitivity by pKIA2. The mutation in this mutant was, therefore, designated *glrB3*. Another mutant HMC105 (Gly^r, Fru$^+$) showed an unexpected result. Sixty six percent of transductants obtained after introduction of pKIA3 (*glrA*$^+$) and 72% of transductants obtained after introduction of pKIA2 (*glrB*$^+$) were glycerol-sensitive and, hence, showed the wild-type phenotype. As pKIA2 (*glrB*$^+$) and pKIA3 (*glrA*$^+$) share no regions of homology (See Southern blot analysis, Chapter 4 and Restriction analysis, Chapter 5) it is highly unlikely that both plasmids would complement the same mutation. A more likely explanation is that transduction of vector DNA into HMC105 results in a high frequency of reversion. This hypothesis could be tested by introducing a plasmid containing the same vector sequences but an unrelated region of *M. xanthus* chromosomal DNA, e.g. genes involved in the regulation of carotenogenesis. When pKIA3 was transduced into HMC107 (Gly^r, Fru$^+$) and HMC109 (Gly^r, Fru$^+$) 8% and 4% respectively of the resultant transductants were glycerol-sensitive. As the frequency of glycerol-sensitive transductants was very low it is thought that they are the result of reversion events. A

similar observation was noted when pKIA2 was introduced into HMC108 (Gly^r, Fru^+).

As complementation analysis revealed that pKIA3, pKIA4 and pKIA9 contained the *glrA*$^+$ gene, subclones derived from pKIA4 were constructed and assessed for complementation ability. The results are summarized in Figure 6.3. Both the *glrA1* (DK440) and *glrA2* (DK510) mutations appear to be located within a 6.8kb *Bam*HI *Eco*RV (Figure 6.2) fragment as indicated by the observation that both mutations are complemented by pHMC8 but not by pHMC6 (Table 6.4). The subclones derived from this 6.8kb fragment allowed further localization of the mutations. Plasmid pHMC42 complemented DK440 (*glrA1*, *bsg-440*) but did not complement DK510 (*glrA2*, Fru^-). Plasmids pHMC40 and pHMC44 did not complement either the *glrA1* (DK440) or *glrA2* (DK510) mutations. Hence, it appears that the *glrA1* (DK440) locus is located within the 2.0kb *Xho*I fragment cloned in pHMC42. As the *glrA2* (DK510) mutation is not complemented by pHMC40, pHMC42 or pHMC44 it may be that the *glrA2* (DK510) mutation is located very close to one of the two *Xho*I sites used in constructing these three subclones, and, hence, is probably located within approximately 2.0kb of the *glrA1* (DK440) mutation. This suggests that both the *glrA1* (DK440) and *glrA2* (DK510) mutations are located between 9.1kb and 11.3kb away from the Tn*5* insertion ΩDK3554, and may effect different genes. Mx*8* transductions suggested that the *glrA1* (DK440) mutation was located 18.0kb away from the ΩDK3554 Tn*5* insertion while the *glrA2* (DK510) mutation was

located 15.0kb from ΩDK3554 (Andreasson, Blea and Kaiser, personal communication).

Plasmid pHMC40 contains a 2.8kb BamHI XhoI fragment orientated so that the BamHI end of the fragment is adjacent to the promoterless lacZ gene of pDAH283. Promoter activity was detected by ß-galactosidase assay when pHMC40 was in the wild-type background. The promoter in question was expressed at relatively constant levels of 50 - 70 unit/mg protein throughout both vegetative growth and glycerol-induced sporulation. Qualitative assays using 4-MUG to detect ß-galactosidase expression suggested that the promoter was also expressed during fruiting body sporulation. Hence, the gene in question is expressed constitutively. However, there is no evidence that the gene in question is involved in glycerol-induced sporulation. It would be of interest to assess promoter activity of this 2.8kb BamHI XhoI fragment in the opposite orientation. The promoter activity of the 2.0kb XhoI fragment cloned in pHMC42 should also be assessed in both orientations. However, before this can be achieved the PstI and SmaI restriction sites within this 2.0kb XhoI fragment will have to be fully mapped so that the two orientations of the fragment are distinguishable.

Complementation analysis suggested that pKIA2 (pKIA7) contained the glrB gene. This observation is inconsistent with the map of the glrB region obtained from Mx8 co-transduction frequencies (Figure 1.5). Mx8 co-transduction frequencies suggest that the glrB2

(DK3551) mutation is located 25.0kb away from the ΩDK3517 Tn*5* insertion and the *glrB1* (DK3516) mutation is located 13.0kb away from ΩDK3517. As pKIA2 (pKIA7) contains only 9.4kb of cloned DNA, starting immediately adjacent to the Tn*5* insertion, one would not expect this plasmid to complement either the *glrB1* (DK3516) or *glrB2* (DK3551) mutations. Our complementation data for the *glrB* region is also inconsistent with preliminary complementation analysis by Blea (personal communication) which suggested that pKIA2, pKIA6, pKIA7 all complement DK3516 (*glrB1*, Fru^-) to glycerol-sensitivity. The plasmid pKIA7 also appeared to complement DK3551 (*glrB2*, Fru^-) to glycerol-sensitivity, while pKIA2 and pKIA6 were not tested in DK3551. The data is clearly inconsistent. The co-transduction frequencies of the *glrB1* (DK3516) and *glrB2* (DK3551) mutations and the Tn*5* insertion ΩDK3517 should be reassessed using Mx*8*. Further complementation analysis should also be carried out in order to clarify the situation.

Subclones were derived from pKIA2 (Figure 5.8) and assessed for their ability to complement DK3551 (*glrB2*, Fru^-). A summary of the results observed for the *glrB* region is shown in Figure 6.7. The plasmids pHMC2 and pHMC4 each contained approximately half of the DNA cloned in pKIA2, splitting the clone in two at the unique *Sal*I site. However, neither plasmid complemented the *glrB2* (DK3551) mutation. It was thought that may be the gene in question had been split in half. Plasmids pHMC30 and pHMC32 were, therefore, constructed which contained DNA

cloned from across the SalI site. These two plasmids also did not complement DK3551 (glrB2, Fru$^-$). Hence, it was decided to construct two plasmids, pHMC34 and pHMC36, which would allow DNA around the SalI site to be assessed for promoter activity. Fragments were cloned into the multiple cloning site of pDAH283 which is located upstream of a promoterless lacZ gene. DK101[pHMC34] cells did not express ß-galactosidase and, hence, the cloned fragment did not contain a promoter in the correct orientation to direct transcription of the lacZ gene. In DK101[pHMC36] cells significant ß-galactosidase expression was observed. ß-Galactosidase specific activity increased five-fold during 25 hours of vegetative growth. A similar pattern of expression was observed during glycerol-induced sporulation except that the levels of ß-galactosidase specific activity dropped off sharply 20 - 25 hours after the addition of glycerol. Qualitative assays using 4-MUG as an indicator for ß-galactosidase suggested that the gene in question is also expressed during fruiting body sporulation. Hence, expression of the gene is not sporulation-specific. However, the gene in question could be a glr gene, as a gene which results in a glycerol-resistant phenotype if mutated, need not be glycerol-inducible. The genes required for production of the glycerol "receptor" on the cell surface, and genes involved in the initiation of the glycerol-induced sporulation pathway might well be expressed constitutively. In order to ascertain whether this region of DNA is essential for glycerol-induced

sporulation a tetracycline resistance determinant was inserted into the SalI site of the DNA cloned in pKIA2. This disrupted version was designated pHMC38. This plasmid was transduced into the wild-type background and transductants where the wild-type copy had been replaced with the disrupted version were obtained. The disruption of the gene did not result in a change of phenotype of the host cells. All transductants remained glycerol-sensitive and fruited normally (i.e. showed the wild-type phenotype). Hence, either the gene is not essential for glycerol-induced sporulation as disruption does not result in a change of phenotype, or the entire gene does not contain the SalI site. In this case introduction of the tetracycline resistance determinant at the SalI site would have no effect.

Overall the data for the glrB region suggest that pKIA2 (pKIA7) does indeed contain a glrB gene, since both pKIA2 and pKIA7 complement DK3551 (glrB2, Fru^-) to glycerol-sensitivity. However, the observation that none of the subclones derived from pKIA2 complemented DK3551 (glrB2) suggests that the glrB region may be a large operon containing several genes. The glycerol-resistant phenotype of DK3551 may be the result of either a deletion affecting two or more glr genes or the result of two independent mutations in separate glr genes. Hence, subclones derived from pKIA2 may not contain sufficient DNA to complement all the mutated glr genes. Transposon mutagenesis of the insert of pKIA2 (pKIA7) and subsequent assessment of Tn5-disrupted copies of the pKIA2 insert

for the ability to complement DK3551 (*glrB2*) and HMC194 (*glrB3*) may allow the location of the *glr* gene(s) within this cloned region to be determined and could indicate whether the *glrB* region is a large operon containing several genes. The observation that pKIA6, which contains the entire region of DNA cloned in pKIA2 and pKIA7 and an additional 8.0 - 10.0kb of DNA, did not complement DK3551 (*glrB2*) is peculiar and needs fruther examination. This plasmid is known to contain a deletion of a region of the vector pREG429 DNA, but this is not thought to affect the region of homology shared with pKIA2 (pKIA7). However, complete restriction mapping of pKIA6 was not possible due to its large size, and was further complicated by the deletion of vector DNA.

Chapter 7

Cloning and Analysis of the isgB Region

7.1 Introduction

Using a homology based promoter probe a promoter has been identified which showed a spectacular increase (up to 80-fold) in ß-galactosidase expression during glycerol-induced sporulation (Hartree, 1989). ß-Galactosidase expression increased only two-fold in spores during fruiting body sporulation. However, a 15-fold increase in ß-galactosidase expression occurred during starvation-induced sporulation in the non-motile strain DK306 (*mgl*), which was unable aggregate. Kim and Kaiser (1990a) reported that on starvation of non-motile cells spores were still formed despite the absence of fruiting bodies. An intermediate level of expression occurred in DK101, in which fruiting body sporulation was delayed due to a partial motility defect. This suggested that the gene in question was not required for fruiting body sporulation but might have a role when spores are forced to form in the absence of fruiting bodies. The region was designated *isgB* (Hartree, 1989).

A gene fusion contains only part of the transcript unit of interest and, thus, it was desirable to clone the intact copy of the gene. Several previous attempts to clone the gene using the plasmid rescue strategy shown in Figure 1.4 were unsuccessful. One explanation of this failure could have been the absence of nearby sites in the chromosome for the restriction enzymes used. The resulting plasmid would then have been very large, particularly if dimers or multimers were formed. While there is no theoretical limit to the size of a plasmid

generated by circularisation of chromosomal fragments, large plasmids transform *E. coli* with low efficiency. The transformation probability P_p of a plasmid decreases linearly with increasing plasmid size (Hanahan, 1983). Hence, a plasmid of 3.2kb has a transformation probability of 3.5×10^{-3}, while a plasmid 66kb in size has a transformation probability of 0.15×10^{-3}. An alternative explanation was that the gene product was lethal to *E. coli*. This can be a problem when cloning certain prokaryotic genes in *E. coli*. These include genes for structural surface proteins such as *ompA* in *E. coli* (Beck and Bremer, 1980) and genes for enzymes important in cell metabolism such as *polA* in *E. coli* (Murray and Kelley, 1979).

Some deleted variants of pUWM5 were obtained during the previous cloning attempts. These might have arisen from plasmid that had been excised from the chromosome by homologous recombination. When such excised plasmids were digested, size fractionated and ligated a plasmid equivalent to pUWM5 would be reisolated upon transformation of *E. coli*. Alternatively, if dimer insertions of pUWM5 exist in the chromosome, digestion and ligation would result in isolation of a variant of the original plasmid. This second possibility could be avoided by the insertion of a *cer* region into pUWM5, which would then resolve dimers to monomers before integration into the chromosome.

Two methods were employed in attempts to clone the complete *isgB* gene: (1) to insert a *cer* region into the

promoter fusion plasmid and then use this plasmid in attempts to isolate the *isgB* gene using the strategy shown in Figure 1.4; (2) to use bacteriophage lambda as a vector for a DNA library of *M. xanthus* chromosomal DNA. The chromosomal insert in pUWM5 was then used to screen the library for the appropriate region of DNA.

7.2 *Cloning the cer Region into pUWM5*

Studies of ColE1 show that ColE1 and related plasmids are partitioned randomly at cell division and that plasmid stability is correlated inversely with plasmid multimerization. ColE1 is stable because it encodes a determinant that is necessary for *recA*-, *recF*- and *recE*-independent recombination events that efficiently convert multimers to monomers (Summers and Sherratt, 1984). The monomerizing and plasmid stability determinants of ColE1 were localized to within a 0.38kb region (the *cer* region) that, when cloned into plasmid vectors, greatly increases their stability (Summers and Sherratt, 1984). This *cer* region could only act in *cis* and functions as the locus for site-specific recombination. Later analysis revealed that this region contained a recombination site designated *cerS* and also showed that ColE1 encoded a monomerization function designated *cerP* (Sherratt *et al.*, 1984). A similar system, *ckr*, has been found in ColK (Summers *et al.*, 1985).

The *cer* region was cloned into the glycerol-inducible promoter fusion plasmid pUWM5 in order to

ensure that multimers were resolved to monomers. The cer region was obtained on a 0.32kb fragment from pDAH212 by EcoRV SmaI digestion and ligated into pUWM5 linearized with ScaI as shown in Figure 7.1. The ligation mixture was then used to transform E. coli MC1061 (Section 2.21). Kanamycin resistant, ampicillin sensitive transformant colonies were identified, implying that the cer region had been ligated into the ScaI site in the ampicillin resistance gene of pUWM5. DNA was isolated from several transformants and digested with several restriction enzymes to confirm the presence of the cer region. Digests confirmed that a fragment of DNA of approximately 300bp had inserted into the ampicillin resistance gene of pUWM5. However, several restriction sites had been deleted from either end of the cer region, while other restriction sites had been generated suggesting that some rearrangement had occurred. To be sure that the insert was the cer fragment DNA from the transformants was probed with the cer region. Potential recombinant plasmid was digested with various restriction enzymes, run on an 0.7% agarose gel and transferred to nitrocellulose membrane by Southern transfer (Section 2.29). Plasmid pUWM5 DNA was used as a negative control. The cer fragment was obtained from pDAH212 by EcoRV SmaI digestion as before and radiolabelled by nick translation (Section 2.28). Hybridization was carried out as described in Section 2.30. The results confirmed that the cer region has been cloned into the ampicillin gene of pUWM5. A typical recombinant plasmid was chosen and

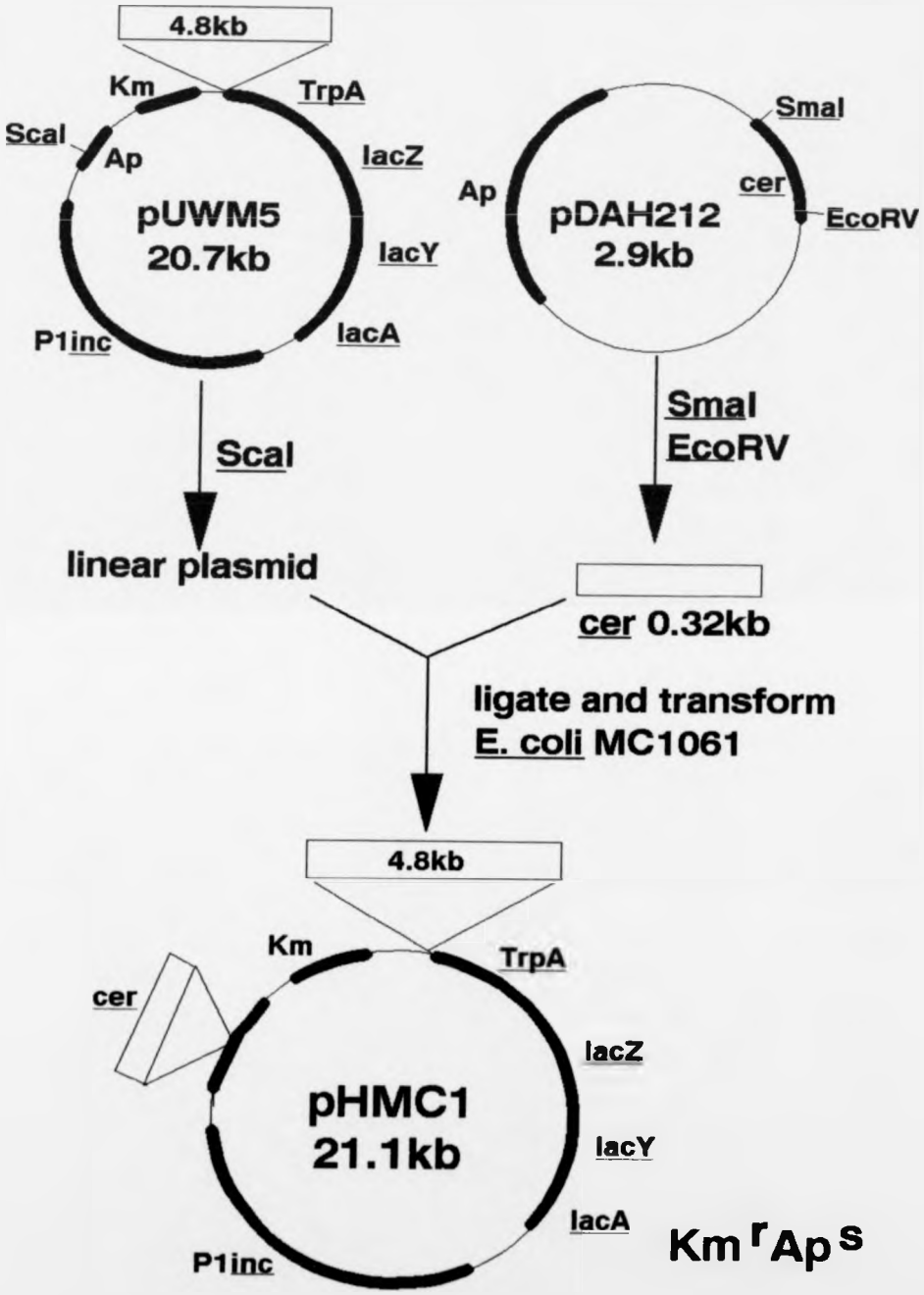


Fig. 7.1 Cloning the cer Region into pUWM5.

designated pHMC1 and a restriction map is shown in Figure 7.2.

7.3 Cloning Strategy Using the Promoter Fusion Plasmid

The strategy employed for cloning is shown in Figure 1.4. The plasmid pHMC1 was, therefore, transduced into the wild-type strain (DK101) using the bacteriophage P1 as described in Section 2.14. Kanamycin resistant transductants were obtained. Fifty transductant colonies were screened for glycerol-resistance and fruiting phenotypes on DCY agar containing 1.0M glycerol (Section 2.3.3) and CF agar (Section 2.3.3). All 50 transductants were glycerol-sensitive, fruiting-positive indicating that the introduction of the plasmid had no effect on the phenotype of the parental strain.

Chromosomal DNA from a transductant colony, designated DK101[pHMC1], was isolated as described in Section 2.19. The enzyme to be used for cloning was one which cut within the plasmid but not within the chromosomal DNA already present in pUWM5. BglII, EcoRV and MluI were used to digest the chromosomal DNA. The strategy is shown for BglII in Figure 7.3. The cer, ori and kanamycin resistance cassette from the plasmid vector should be maintained in the resultant plasmid. The digested chromosomal DNA was size fractionated by agarose gel electrophoresis and the region of the gel containing fragments greater than 12.0kb in length was subject to electroelution. This was necessary in order to exclude fragments too small to contain both a plasmid origin and

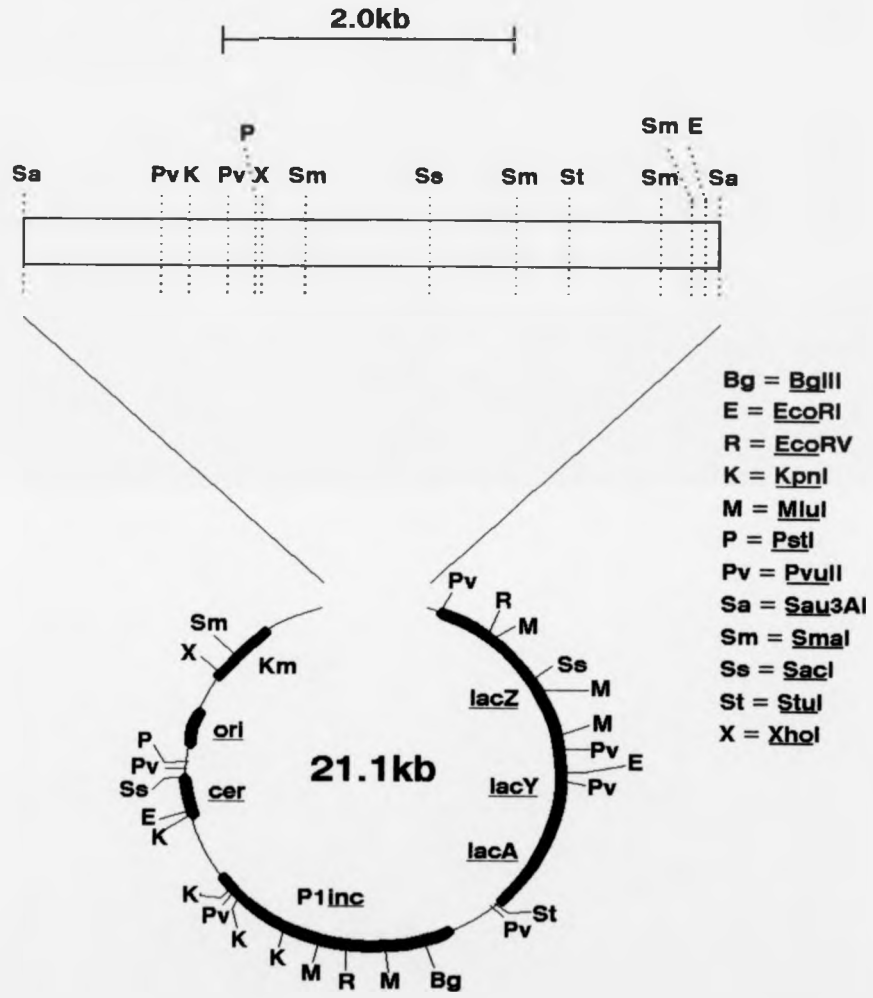


Fig. 7.2 Restriction Map of pHMC1.

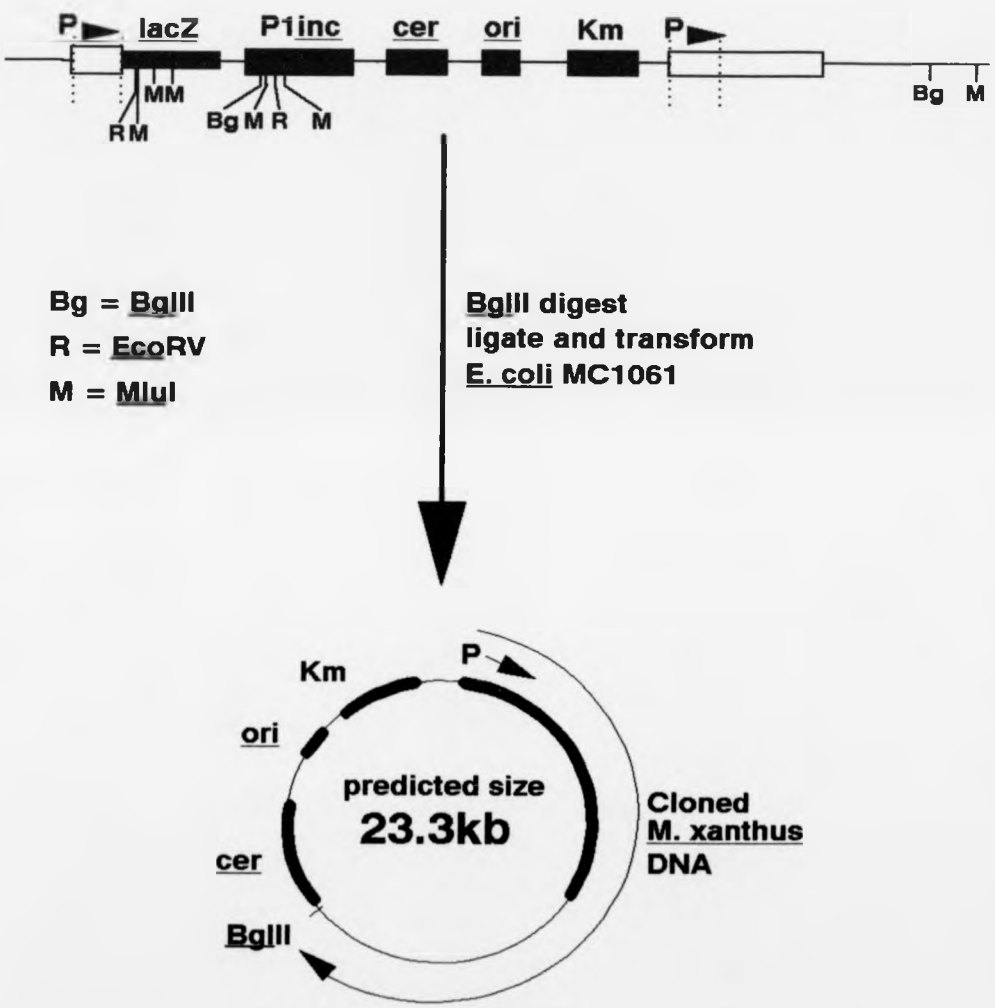


Fig. 7.3 Cloning the Gene for Which a Gene Fusion has been Identified.

chromosomal sequences of greater length than those already obtained in pUWM5. The DNA was ligated and used to transform E. coli MC1061 as described in Section 2.21. Plasmids prepared from kanamycin resistant recombinant clones were compared by restriction analysis with pUWM5 and pHMC1. One clone, designated pHMC20, was obtained, from BglII digested chromosomal DNA, which was 23.4kb in size and appeared to contain the M. xanthus DNA sequences present in pUWM5 plus an additional 10.0 - 11.0kb of downstream DNA. Attempts to clone the downstream DNA using EcoRV and MluI digested chromosomal DNA were unsuccessful. No kanamycin resistant transformants were ever obtained.

7.4 Cloning Strategy Using Lambda as a Cloning Vector

If the gene product(s) of the isgB region was (were) detrimental to E. coli, one strategy for cloning the complete gene would be to use lambda as a cloning vector. Prior to beginning this work chromosomal DNA from the wild-type strain DK1622 was probed with the pUWM5 insert to identify suitable enzymes to use for creating a random library. Chromosomal DNA was isolated from DK1622 as described in Section 2.19. Samples of chromosomal DNA were digested with BamHI, EcoRI, MluI, PstI, PvuII and XhoI, subject to electrophoresis on a 0.5% agarose gel and transferred to nitrocellulose membrane by Southern transfer (Section 2.29). The insert of pUWM5 was isolated and radiolabelled as described in Section 4.2, and used to probe the nitrocellulose membrane using the

hybridization conditions described in Section 2.30. The fragments hybridized by the pUWM5 insert are shown in Figure 7.4. The results suggested that EcoRI and BamHI or Sau3AI would be suitable enzymes to use to create a random library of fragments in lambda.

Lambda EMBL3 BamHI and EMBL4 EcoRI cloning vectors were obtained from Stratagene and their maps are shown in Figure 7.5. EMBL3 and EMBL4 are replacement vectors capable of accepting DNA fragments ranging in size from 9.0 to 23.0kb. The arms are prepared by double digestion with BamHI and EcoRI. This leaves the arms and stuffer fragment with incompatible ends so there is no need to physically separate them. EMBL3 and EMBL4 take advantage of the Spi^+ (sensitive to P2 inhibition) selection. Lambda phage which contain active red^+ and gam^+ genes are unable to grow on host strains which are P2 phage lysogens. Lambda phage without these genes are able to grow on strains such as P2392, a P2 lysogen of LE392. The red and gam genes in EMBL3 and EMBL4 are located on the stuffer fragment; therefore, wild-type EMBL3 or EMBL4 cannot grow on P2392. When the stuffer fragment is replaced by an insert, the recombinant becomes red^- and gam^- and is able to grow on P2392. Therefore, by plating the library on P2392 only recombinant phage will be able to grow.

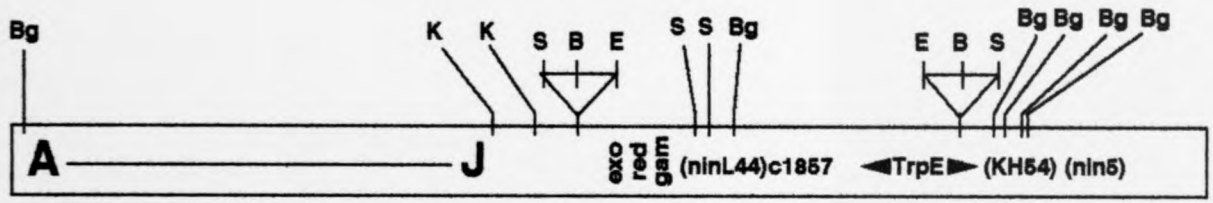
Chromosomal DNA was isolated from the wild-type M. xanthus strain DK1622 as described in Section 2.19. A partial Sau3AI digest was performed on 100μg of DNA using 0.25units/μg of DNA. The restriction enzyme was removed

BamHI	EcoRI	MluI
8.8kb	11.0kb	18.0kb

PstI	PvuII	XhoI
5.0kb	4.7kb	7.0kb
1.65kb	3.8kb	1.7kb

Fig. 7.4 Fragments Hybridized when Wild-Type Chromosomal DNA, Digested with a Variety of Enzymes, was Probed with the Insert of pUWM5.

Lambda EMBL3



B = BamHI
Bg = BglII
E = EcoRI
K = KpnI
S = SalI

Lambda EMBL4

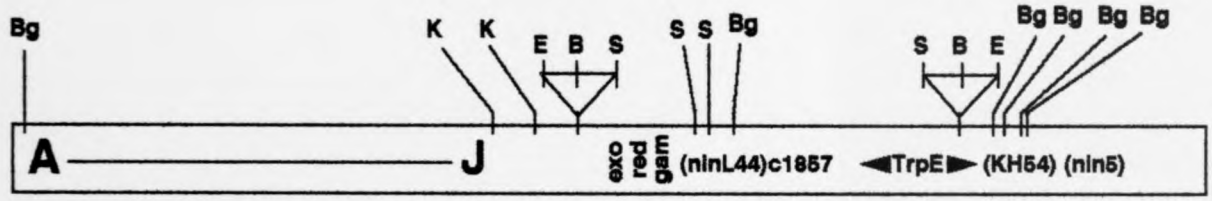


Fig. 7.5 The Lambda EMBL3 and EMBL4 Cloning Vectors.

by phenol extraction and the DNA was precipitated with ethanol. The chromosomal fragments were ligated into lambda EMBL3 BamHI in the manner recommended by the suppliers. Chromosomal DNA was also digested completely with EcoRI and ligated into lambda EMBL4 EcoRI. The lambda libraries were packaged using the Gigapack II Gold Packaging Extract in the manner recommended by the suppliers and cultured on P2392.

Samples of the resultant lambda libraries were immobilized on nitrocellulose membrane in the manner described in Maniatis et al. (1982). The filters were probed with the 4.8kb Sau3AI chromosomal insert of pUWM5. The probe fragment was isolated and labelled as described in Section 4.2 and the hybridization conditions are described in Section 2.30. Hybridizing plaques were picked into SM buffer (Section 2.3.2) and further rounds of plaque hybridizations were carried out until pure plaques, which showed hybridization to the pUWM5 insert, were identified. Four positive plaques were identified for the lambda EMBL4 EcoRI library but no positive plaques were obtained for the lambda EMBL3 library. DNA was prepared from the four positive plaques from the lambda EMBL4 EcoRI library, as described in Section 2.18, and all four lambda clones were found to contain an 11.0kb EcoRI fragment. This 11.0kb fragment was subcloned into the unique EcoRI site in the chloramphenicol resistance gene of pBR329 (Covarrubias and Bolivar, 1982). Tetracycline and ampicillin resistant transformants of E. coli MC1061 were obtained

as described in Section 2.21 and were screened for sensitivity to chloramphenicol. The insertion of the 11.0kb fragment into the chloramphenicol gene of pBR329 was then confirmed by restriction analysis. One such plasmid was selected and designated pHMC10.

7.5 Restriction Mapping of pHMC10 and pHMC20

In order to ascertain whether the fragments cloned in pHMC10 and pHMC20 contained the remainder of the *isgB* region the sites for 19 restriction enzymes, with six base pair recognition sequences, were determined for both plasmids. Restriction sites were also determined for *Sau*3AI. Preliminary map comparison with the chromosomal insert in pUWM5 suggested that pHMC20 contained the pUWM5 insert and 10.13kb of additional downstream DNA which should, therefore, include the remainder of the transcript unit of interest (Figure 7.6). Further restriction mapping of pUWM5 revealed a previously unrecorded *Eco*RI site 100bp from the *lacZ* gene proximal end. pHMC10 contained most of the fragment already cloned from this *Eco*RI site and an additional 6.3kb of DNA from the upstream region as shown in Figure 7.6. Therefore, pHMC10 did not contain the remainder of the *isgB* transcript unit and so was not fully restriction mapped. The restriction map of pHMC20 is shown in Figure 7.7.

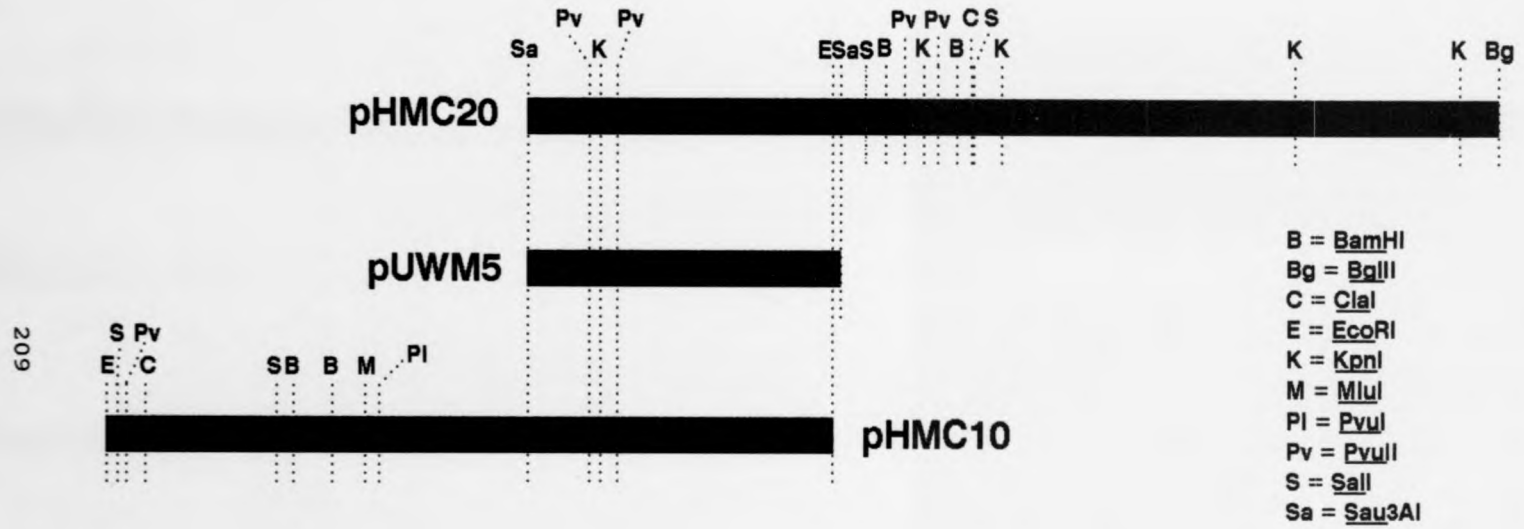
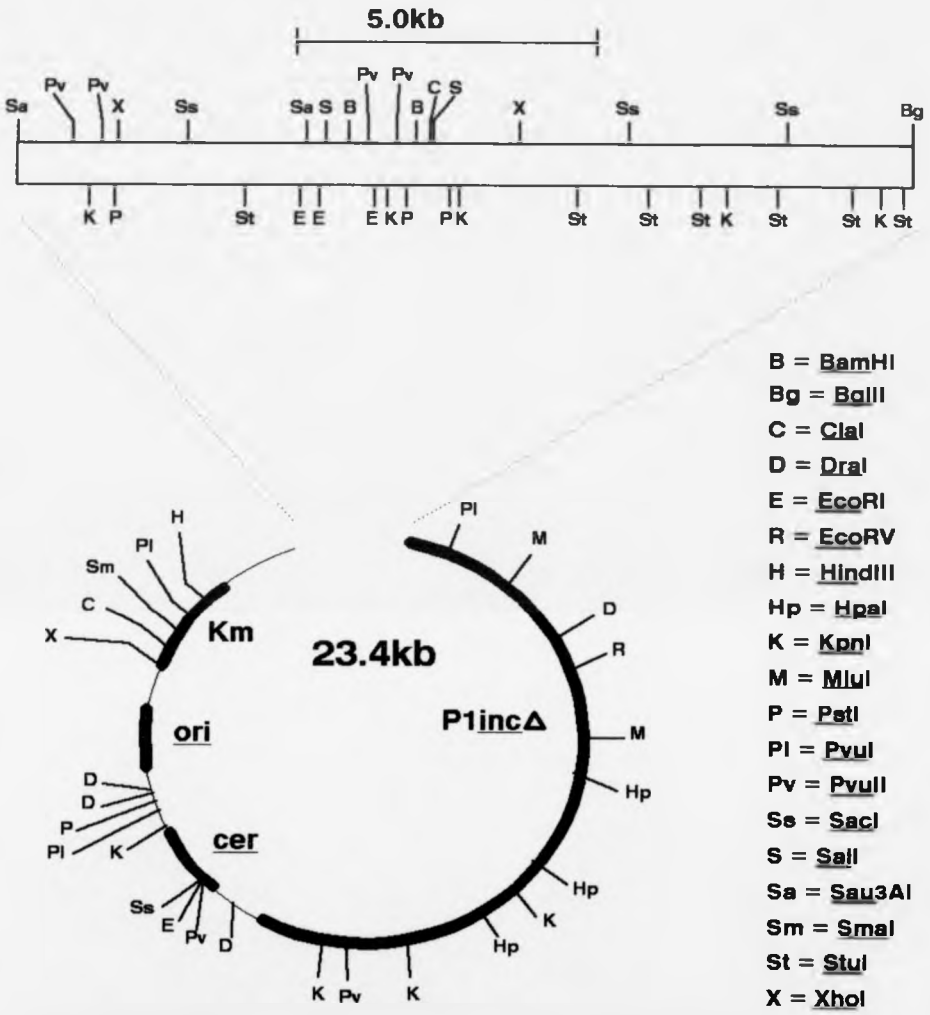


Fig. 7.6 The Overlapping Regions Between pUWM5, pHMC10 and pHMC20.



The cloned region of M. xanthus DNA contained no sites for the following restriction enzymes:-

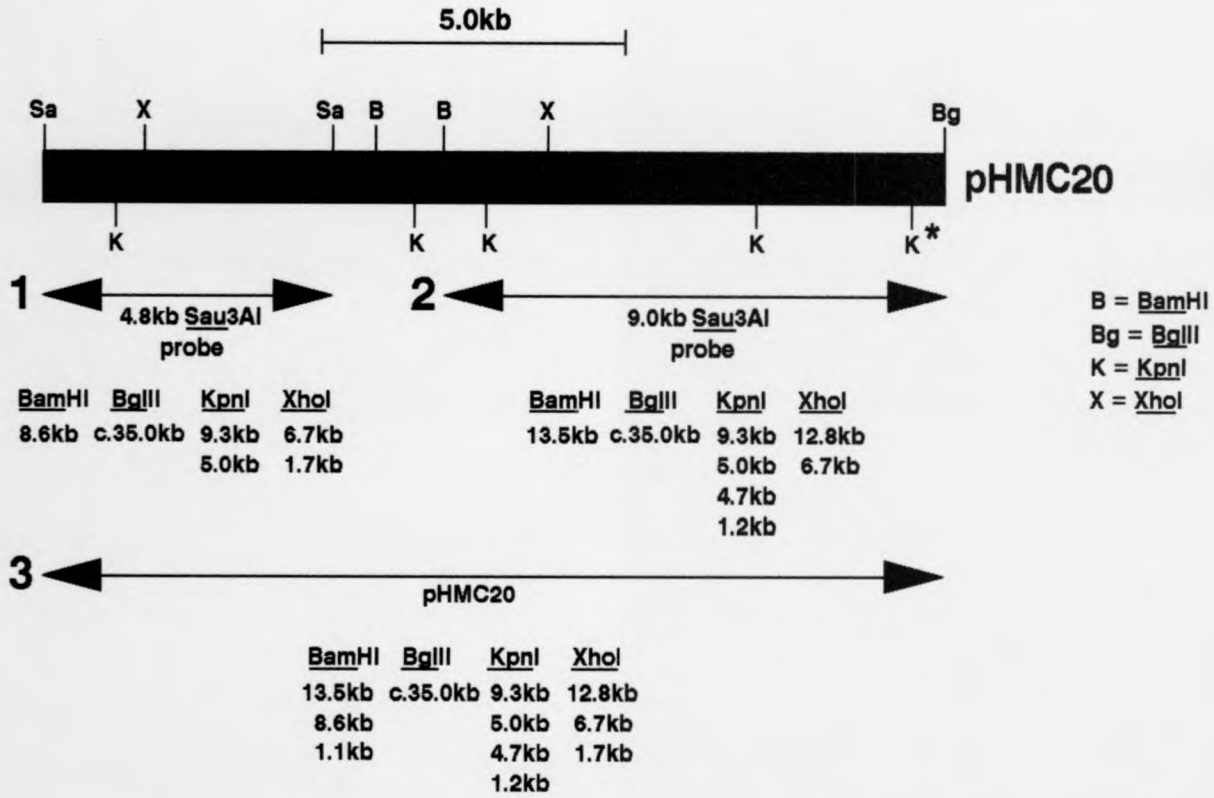
DraI
EcoRV
HindIII
HpaI
MluI
PvuI
ScaI

There were too many SmaI sites within the cloned region to map.

Fig. 7.7 Restriction Map of pHMC20.

7.6 Probing The Chromosome of M. xanthus

To confirm that pHMC20 did indeed contain DNA from the isgB region M. xanthus, chromosomal DNA was probed with pHMC20 and two Sau3AI fragments derived from it. Chromosomal DNA was isolated from M. xanthus DK1622, as described in Section 2.19, digested with BamHI, BglII, KpnI and XhoI, subject to electrophoresis on a 0.4% agarose gel and transferred to nitrocellulose membrane by Southern transfer (Section 2.29). Sau3AI cuts infrequently in M. xanthus DNA, but cuts frequently in E. coli vector DNA. Therefore, Sau3AI digestion of a plasmid containing M. xanthus DNA results in large fragments of M. xanthus DNA while the vector DNA is digested into small fragments. Sau3AI digestion of pHMC20 released two large fragments; a 4.8kb fragment, which correspond to the fragment cloned in pUWM5, and an 9.0kb fragment. These two Sau3AI fragments and the plasmid pHMC20 were each independently radiolabelled by nick translation (Section 2.28) and used to probe M. xanthus chromosomal DNA. Hybridization conditions used are described in Section 2.30. The fragments hybridized are shown in Figure 7.8. These results confirm that pHMC20 does contain DNA from the isgB region. The fragments hybridized in the BamHI, BglII and XhoI digests are consistent with the restriction map. However, there is a discrepancy in the data for KpnI which suggests that an additional KpnI site may have been generated during cloning of the M. xanthus DNA into pHMC20, as shown in Figure 7.8.



* = Additional site which may have been generated during cloning

Fig. 7.8 Chromosomal Fragments Hybridized by Probes from the isgB Region.

7.7 Gene Replacement Studies

In order to localise the isgB gene, and ascertain its function, gene replacement studies were carried out. Two constructs, derived from pHMC20, were made in which fragments of DNA were replaced with a tetracycline cassette derived from ColE1 Tn5-132 (Rothstein et al., 1981). The tetracycline cassette is derived from Tn10 and expression of tetracycline resistance is inducible by subinhibitory levels of the antibiotic (Wray et al., 1981; Bertrand et al., 1983). In pHMC22 a 1.13kb BamHI fragment was replaced by a 2.78kb BglII fragment from ColE1 Tn5-132 containing the tetracycline cassette, as shown in Figure 7.9. This disrupts the isgB region 0.7kb downstream of the promoter fragment present in pUWM5. In pHMC24 a 1.75kb SalI fragment was replaced with a 4.84kb XhoI fragment from ColE1 Tn5-132 containing the tetracycline resistance cassette, as shown in Figure 7.9. This disrupts the isgB region 0.3kb downstream of the promoter fragment present in pUWM5. Recombinant clones were obtained in E. coli MC1061 as described in Section 2.21. Transformants were selected for on LB agar containing 50μg/ml kanamycin and a gradient of tetracycline from 0 - 10μg/ml. The gradient of tetracycline allows the induction of the tetracycline cassette. After 16 hours incubation at 37°C confluent growth was apparent at low levels of tetracycline while several single colonies were apparent at higher levels of tetracycline. These single colonies were picked onto fresh LB agar containing 50μg/ml kanamycin and 10μg/ml

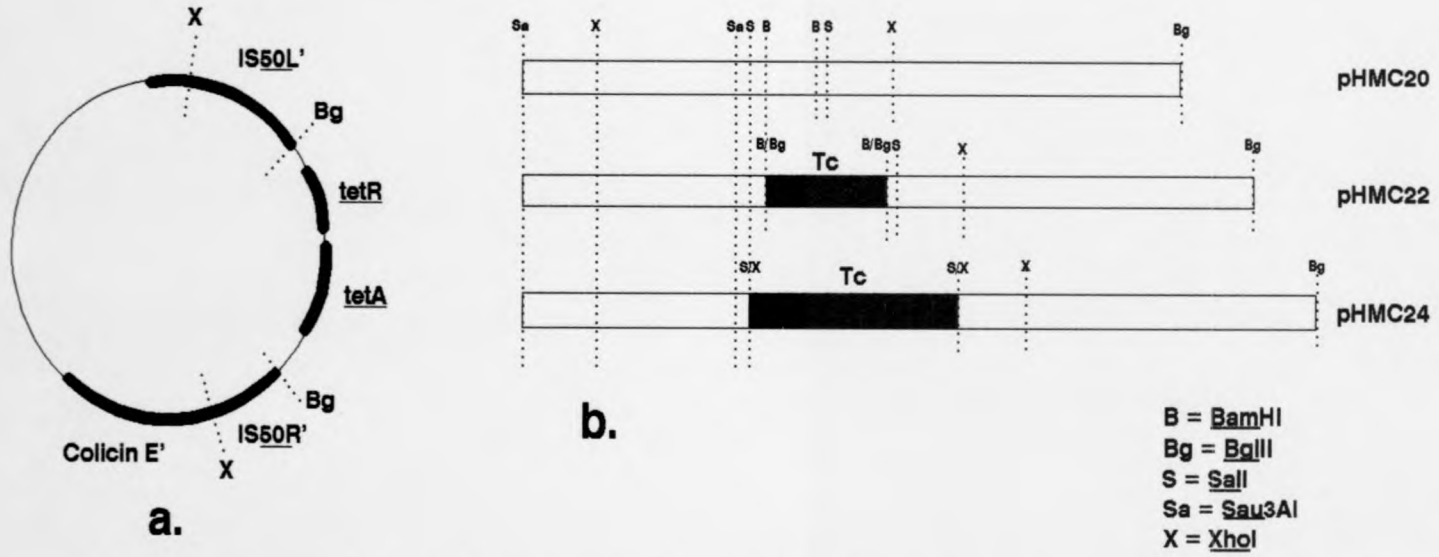


Fig. 7.9 Preparation of Constructs in Which a Portion of the isgB Region has been Replaced with a Tetracycline Cassette.

a. Col E1 Tn5-132

b. pHMC20 and derivatives in which DNA has been replaced by a tetracycline cassette. In pHMC22 a 1.13kb BamHI fragment has been replaced with a 2.78kb BglII fragment containing the tetracycline cassette. In pHMC24 a 1.75kb SalI fragment has been replaced with a 4.84kb XhoI fragment containing the tetracycline cassette.

tetracycline to confirm the antibiotic resistances. The insertion of the tetracycline resistance determinant in the expected sites was then confirmed by restriction analysis.

Plasmids pHMC22 and pHMC24 were transduced into the M. xanthus wild-type strain DK101, as described in Section 2.14 except that the transductants were selected for on DCY agar containing a gradient of oxytetracycline from 0 - 12.5μg/ml. The kanamycin resistance determinant carried on the plasmid vector was not selected for. After 4 - 5 days incubation at 33°C confluent growth of M. xanthus cells occurred at low levels of oxytetracycline. However, single transductant colonies were observed on the agar containing higher levels of oxytetracycline. These colonies were picked onto DCY agar containing 12.5μg/ml oxytetracycline to confirm the presence of the tetracycline cassette in the M. xanthus chromosome. Two hundred and six DK101 tetracycline resistant transductants derived from pHMC22 and 151 DK101 transductants derived from pHMC24 were obtained.

Introduction of the plasmid into M. xanthus without selection for the antibiotic resistance on the vector portion of the plasmid could have either of two possible outcomes, as shown for pHMC38 in Figure 6.6; (1) A single crossover event results in the introduction of plasmid DNA and a second disrupted version of the region into the M. xanthus chromosome; (2) A double crossover event results in the replacement of the intact copy of the region with the disrupted version. Transductants in

which a single crossover event had occurred would still carry the kanamycin resistance determinant on the promoter probe vector. Transductants in which a double crossover event had occurred would be kanamycin sensitive. Transductant colonies were, therefore, screened for kanamycin sensitivity. Thirty seven out of 206 pHMC22 transductant colonies were kanamycin sensitive, showing that a double crossover event had occurred resulting in replacement of the intact copy of the region with the disrupted version. The other 169 transductants were kanamycin resistant, indicating that a single crossover event had occurred. For pHMC24 transductants 130 out of 151 transductant colonies were kanamycin sensitive, indicating that a double crossover event had occurred, while the remaining 21 transductants were kanamycin resistant and, hence, were the result of a single crossover event. It was confirmed that replacement of the isgB region had occurred in six pHMC22 kanamycin sensitive transductants by isolating chromosomal DNA and probing SalI digests with the tetracycline resistance cassette and pDAH122. No fragments hybridized when the chromosomal DNA was probed with pDAH122 showing that the vector DNA had not been introduced into the chromosome. One 3.45kb fragment hybridized in all six samples of transductant DNA confirming that the tetracycline cassette had been incorporated into the chromosomal DNA.

Tetracycline resistant, kanamycin sensitive transductants, in which the intact copy of the gene had

been replaced by the disrupted copy, were picked onto CF and DCY agar containing 1.0M glycerol to test for glycerol-resistance and fruiting phenotypes. All 37 pHMC22 transductant colonies and 130 pHMC24 transductant colonies were glycerol sensitive and fruited normally. These results suggest that isgB is not an essential gene for vegetative growth, glycerol-induced sporulation or fruiting body sporulation as disruption of the region has no effect on the phenotype of the host strain. An alternative explanation is that the entire gene is located before the SalI site at which the tetracycline resistance determinant is inserted in pHMC24. However, analysis of the promoter region (Section 7.8) implies that this is not the case.

7.8 Analysis of the Promoter Region

Two fragments derived from pHMC20 were cloned into a promoter probe vector, pDAH283, in order to look at promoter activity of the isgB region. The fragments are shown in Figure 7.10. pDAH283 (Figure 5.10) contains a promoterless lacZ gene with a multiple cloning site upstream of it so that fragments can easily be cloned into the plasmid in the correct orientation for an exogenous promoter to direct transcription of lacZ.

Plasmid pHMC12 contains a 4.7kb EcoRI Sau3AI fragment ligated into the BamHI EcoRI sites in the multiple cloning site upstream of the promoterless lacZ of pDAH283. The 4.7kb EcoRI Sau3AI fragment is essentially the fragment cloned in pUWM5 with 80 - 100

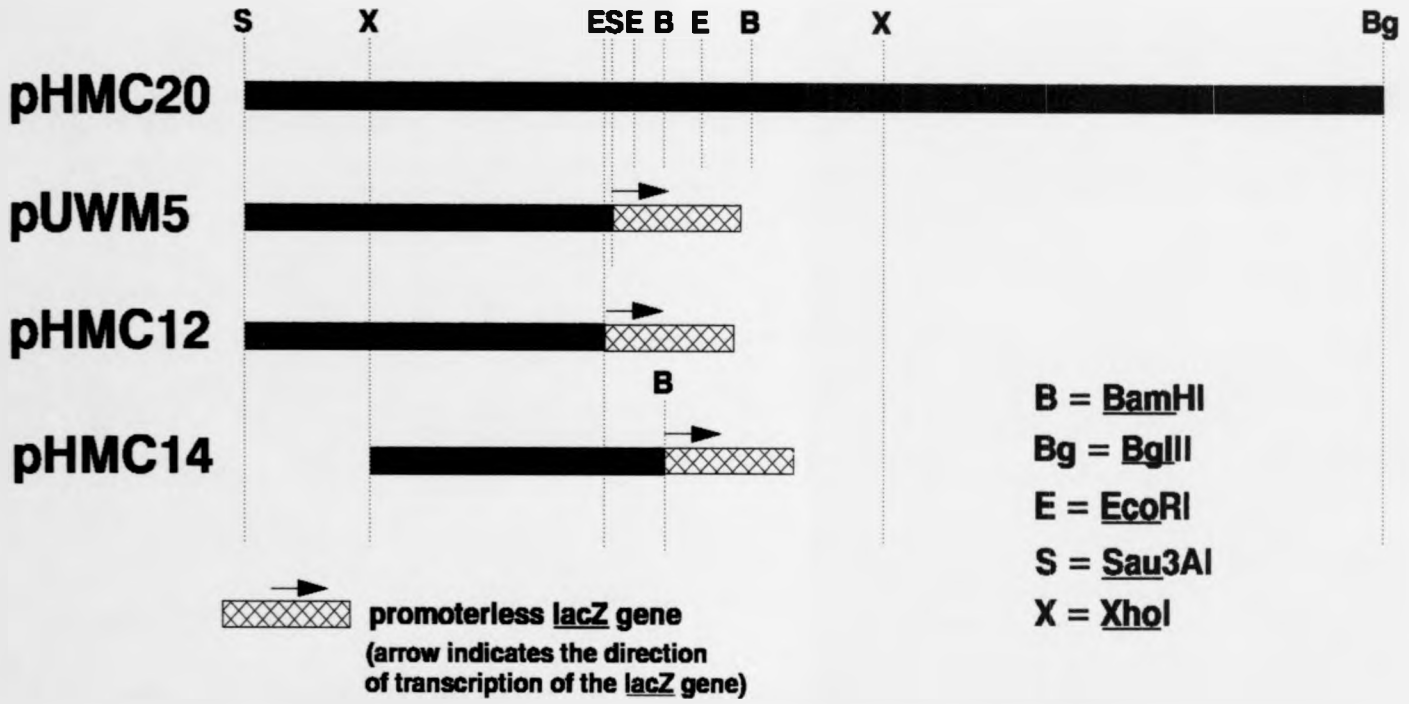


Fig. 7.10 The Fragments from the isgB Region Which were Subcloned into the pDAH283 Promoter Probe Vector in Order to Analyse the Promoter Region.

base pairs deleted from the 3' end. Plasmid pHMC14, contains a 3.85kb BamHI XhoI fragment cloned into the BamHI SalI sites of the multiple cloning site of pDAH283. The 3.85kb fragment contains an additional 0.7kb of DNA at the 3' end than previously cloned in pUWM5.

Plasmids pUWM5, pHMC12 and pHMC14 were all transduced into the wild-type (DK101) background using the coliphage P1 as described in Section 2.14. Fifty kanamycin resistant transductant colonies of each strain were screened on DCY agar containing 1.0M glycerol and CF agar for their glycerol-resistance and fruiting phenotypes. DK101[pUWM5], DK101[pHMC12] and DK101[pHMC14] transductants remained glycerol-sensitive and fruited normally, indicating that introduction of the plasmid into the chromosome had no effect on the phenotype of the parental strain. All three strains showed some expression of ß-galactosidase on glycerol and fruiting media as well as on DCY and A1 vegetative control plates, using 4-MUG as an indicator for ß-galactosidase activity.

Cultures of DK101[pUWM5], DK101[pHMC12] and DK101[pHMC14] were grown to a density of 9 x 10^7 cells/ml (A_{600} = 0.3) and glycerol was added to a final concentration of 1.0M. Samples were taken out, as described in Section 2.11, at t=0 (the time of glycerol addition) and 1, 2, 3, 4, 5, 10, 15, 20 and 25 hours after glycerol addition. Phase-contrast microscopy revealed that the rod-shaped vegetative cells converted to spores 1 - 3 hours after the addition of glycerol. ß-

Galactosidase activity was determined (Sections 2.12 and 2.13) after disruption of the spores by sonication (Section 2.11). The results are shown in Figure 7.11. In strain DK101[pHMC14] a peak in ß-galactosidase specific activity of 682 units/mg protein was reached ten hours after the addition of glycerol. In DK101[pUWM5] a peak in specific activity of 800 units/mg protein was reached at 20 hours, while there was no increase in ß-galactosidase expression in DK101[pHMC12]. There was no increase in ß-galactosidase expression in vegetative cultures of DK101[pUWM5], DK101[pHMC12] or DK101[pHMC14]. These results suggest that the glycerol-inducible promoter, or at least the start site, is contained in a 100 base pair EcoRI Sau3AI region. That the timing of expression was different during glycerol-induced sporulation of DK101[pHMC14] and DK101[pUWM5] may merely be a result of differences in the kinetics of the two fusions, or the additional 0.7kb region in pHMC14 may contain a regulatory region of some kind. Alternatively, the fusion in pHMC14 may represent a different glycerol-inducible promoter from that in pUWM5.

7.9 Discussion

A summary of the observations for the *isgB* region is shown in Figure 7.12.

The *isgB* region was cloned in pHMC20 using the promoter fusion to clone additional downstream DNA. The plasmid was restriction mapped and used to probe the *M. xanthus* chromosome and the data is consistent with the

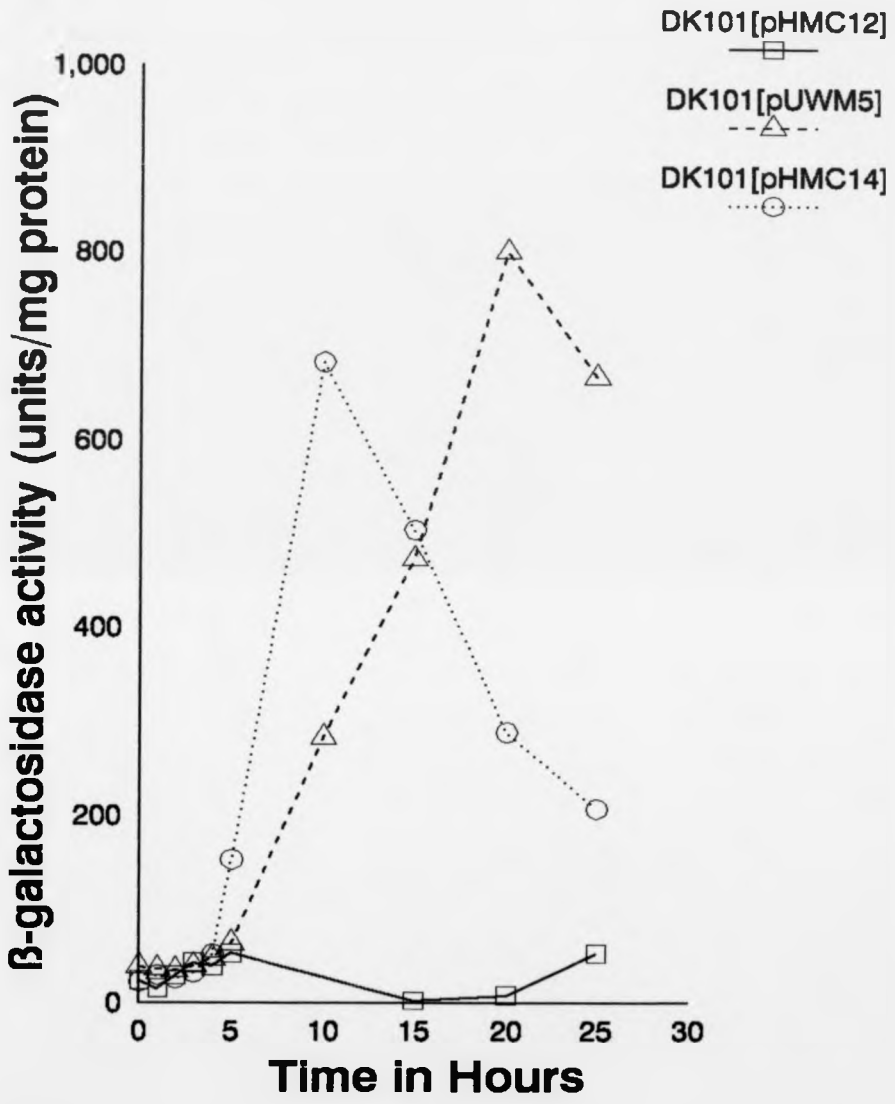


Fig. 7.11 ß-Galactosidase Expression During Glycerol-Induced Sporulation of the Strains DK101[pUWM5], DK101[pHMC12] and DK101[pHMC14].

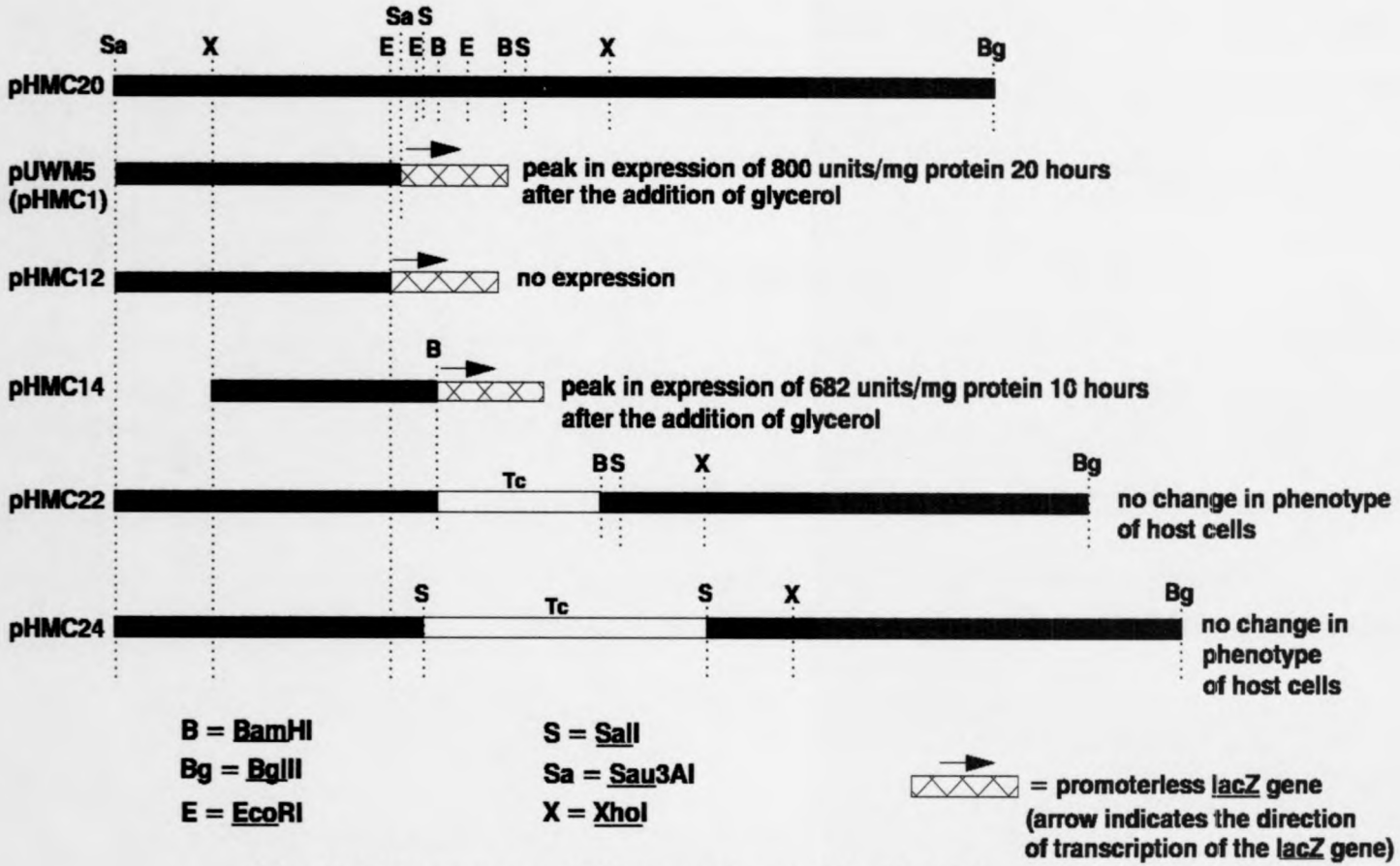


Fig. 7.12 Summary of Observations of the isgB Region.

plasmid, pHMC20, containing DNA from the isgB region. The additional 10.13kb region cloned contained no restriction sites for EcoRV and MluI which is consistent with Southern analysis of the isgB region which suggests that the nearest downstream MluI site is at least 20.0kb away from the fusion point, in the intact copy of the gene (Hartree, 1989).

A second plasmid, pHMC10, contained an 11.0kb EcoRI fragment, originally cloned in lambda EMBL4. This 11.0kb fragment hybridized to the 4.8kb promoter fragment cloned in pUWM5. However, the fragment was found to contain 4.7kb of the fragment cloned previously and an additional 6.3kb of upstream DNA. It could not, therefore, contain the isgB transcript unit.

Disruption of the isgB region by the integration of a tetracycline cassette had no effect on the glycerol or fruiting phenotypes of the host strain suggesting that correct expression of the isgB gene product is not required for either glycerol-induced sporulation or fruiting body sporulation. The isgB region, therefore, contains a non-essential gene. An alternative explanation is that the entire gene is located upstream of the SalI site at which the tetracycline resistance determinant was inserted in pHMC24. However, analysis of the promoter region suggested that this is not the case.

The promoter region was analysed by subcloning two fragments into a promoter probe vector and looking at the levels of ß-galactosidase specific activity. The results suggest that the isgB glycerol-inducible promoter, or at

least the start site, is located within a 100bp EcoRI Sau3AI fragment. Further deletion studies and sequencing of the promoter region would be of interest to elucidate how gene expression is induced by glycerol and DMSO. Hartree (1989) showed that the isgB gene showed a similar pattern of ß-galactosidase activity during glycerol-induced sporulation and DMSO-induced sporulation.

Overall the data for the isgB region suggests that the cloned region of M. xanthus DNA in pHMC20 is likely to contain the complete transcript of the isgB gene. It would, therefore, be of interest to sequence this region. Sequence analysis of the isgB gene may give some indication of the role of the isgB gene in glycerol-induced sporulation and DMSO-induced sporulation.

Chapter 8

Epistasis Analysis of the isgB Region

8.1 Introduction

Epistasis is the masking of the phenotype caused by one mutation by the phenotype of another mutation. It is often used to test whether two genes function in the same genetic pathway. If two genes are part of the same pathway, then a strain containing two mutations with distinguishable phenotypes will have the phenotype of the mutation occurring earlier in the pathway (Gill and Shimkets, 1993). This principle has been applied to analysis of the developmental pathway of *M. xanthus* by using strains containing both a mutation that disrupts development and a reporter for a developmental gene. For example, when 21 developmentally regulated transcriptional *lacZ* fusions were transferred into a strain carrying an *asg* mutation, ß-galactosidase activity was reduced or eliminated in 18 strains (Kuspa *et al*., 1986). These results suggest that the block imposed by the *asg* mutation precedes the expression of 85% of the developmentally regulated genes and is consistent with results obtained with biochemical markers that suggest that *asg* mutants are blocked early in development (LaRossa *et al*., 1983). While the results of this approach have not been extended to very many developmental mutations, a pathway is apparent with the few that have been tested. The *asg* mutation appears to block developmental gene expression at about two hours after initiation and *csgA* at about six hours (Kroos and Kaiser, 1987).

The aim of this work was to use epistasis to analyse

expression from the *isgB*>*lacZ* fusion in different genetic backgrounds during both glycerol-induced sporulation and fruiting body sporulation. Previous work with the *isgB*>*lacZ* fusion has shown that ß-galactosidase expression increased as much as 80-fold during glycerol-induced sporulation but only increased two-fold during fruiting body sporulation in the wild-type (DK1622) background (Hartree, 1989). DK306, which is non-motile due to a mutation in the *mgl* locus, is unable to aggregate into fruiting bodies. However, spores are formed on starvation, despite the absence of fruiting bodies (Kim and Kaiser, 1990a). In these spores a 15-fold increase in ß-galactosidase expression occurred. In DK101, where fruiting body sporulation is delayed due to a partial motility defect in the social motility system, an intermediate level of ß-galactosidase expression was seen. These results suggested that the *isgB* gene product is not required in the normal fruiting body sporulation pathway but is required in spores that form outside of fruiting bodies. This led to the proposal that there are two pathways of starvation-induced sporulation (Figure 1.6): (1) fruiting body sporulation and (2) fruiting-independent sporulation i.e, sporulation outside of fruiting bodies. It is believed that both these pathways can occur simultaneously during sporulation of wild-type cells.

ß-Galactosidase expression from the *isgB*>*lacZ* fusion was analysed in *asg*, *csg* and *dsg* mutant backgrounds during starvation conditions. In an *asg* mutant

background there was a slight increase in ß-galactosidase expression but only during days two and three. This can be readily explained as asg mutants are blocked early in sporulation and, therefore, there is no significant increase in expression. The level of isgB>lacZ expression in a csg background decreased during the course of starvation. The csg gene is proposed to be active at a relatively late stage in sporulation (Kroos and Kaiser, 1987). This suggests that the isgB gene is a gene expressed at a late stage of development. This correlates well with the observed time of expression in DK306 where the increase in ß-galactosidase expression is not apparent until 72 hours after the onset of starvation. In a dsg mutant the enzyme activity was significantly greater than in the other strains tested at the onset of the experiment and expression increased still further in cells placed under starvation conditions. The peak of expression was seen much earlier than in sporulating strains. It may be that the block in the sporulation process results in a gene normally expressed in spores being expressed in vegetative cells. It is known that the Dsg phenotype arises from a leaky mutation in an otherwise essential gene (Cheng and Kaiser, 1989a,b).

8.2 Epistasis During Glycerol-Induced Sporulation

It was of interest to analyse expression from the isgB promoter upon the addition of glycerol in several glycerol-resistant mutant backgrounds. DK101 was used as

a positive control for *isgB* expression. DZF1281 which carries a *frz* mutation was also used. A mutation in a *frz* gene results in the strain being unable to control the frequency of reversal of the cells, i.e, the cells are fully motile but they have lost the chemotaxis response (Zusman *et al.*, 1990).

The plasmid pUWM5 containing the *isgB*>*lacZ* fusion was transduced into the following strains using coliphage P1 (Section 2.14); DK101 (wild-type), DK440 (*glrA1*, *bsg-440*) (LaRossa *et al.*, 1983), DK510 (*glrA2*, Fru^-) (Hagen *et al.*, 1978), DK3516 (*glrB1*, Fru^-), DK3551 (*glrB2*, Fru^-) and DZF1281 (*frz*). Fifty transductant colonies of each strain were screened for glycerol-sensitivity and fruiting phenotype. No change in Fru^-, Gly^r or Frz^- phenotype occurred in any of the transductant strains. Cultures of the transductants containing the *isgB*>*lacZ* fusion were grown in DCY broth (Section 2.3.3) to a density of 9.0×10^7 cells/ml (A_{600} = 0.3) and 50% glycerol was added to a concentration of 1.0M. Samples were taken out of the cultures as described in Section 2.11, at t=0 (the time of glycerol addition) and 1, 2, 3, 4, 5, 10, 15, 20, and 25 hours after glycerol addition. Phase-contrast microscopy revealed that DK101[pUWM5] and DZF1281[pUWM5] vegetative cells converted to spherical spores 1 - 3 hours after the addition of glycerol, while DK440[pUWM5], DK510[pUWM5], DK3516[pUWM5] and DK3551[pUWM5] cells were resistant to glycerol and remained as rod-shaped vegetative cells. ß-Galactosidase specific activity was determined (Sections 2.12 and 2.13)

after disruption of the cells by sonication (Section 2.11). The results for DK101[pUWM5] and DK3516[pUWM5] are shown in Figure 8.1. In DK101[pUWM5] a peak in ß-galactosidase specific activity of 800 units/mg protein was reached 20 hours after the addition of glycerol. A similar pattern of ß-galactosidase expression was seen in DZF1281[pUWM5] which also underwent glycerol-induced sporulation quite normally. In DK440[pUWM5], DK510[pUWM5], DK3516[pUWM5] and DK3551[pUWM5] there was no increase in ß-galactosidase expression at all. Expression from the isgB promoter was blocked by the mutations in these strains which resulted in glycerol-resistance.

The plasmid pUWM5 was then transduced as before into a further twenty glycerol-resistant mutants generated using EMS, NTG and UV. Eight out of 20 of these mutants were glycerol-resistant but underwent fruiting body sporulation quite normally, while the remaining twelve also had defects in fruiting body sporulation. Cultures of the transductants were grown on DCY broth and glycerol was added to a final concentration of 1.0M as before. However, this time samples were only taken out at the time of glycerol addition and at 20 hours after glycerol addition which is when the peak in isgB>lacZ expression should occur. In all 20 glycerol-resistant mutant backgrounds ß-galactosidase expression was either significantly reduced or eliminated, as shown in Figure 8.2. That ß-galactosidase expression from the isgB promoter was blocked in 24 different glycerol-resistant

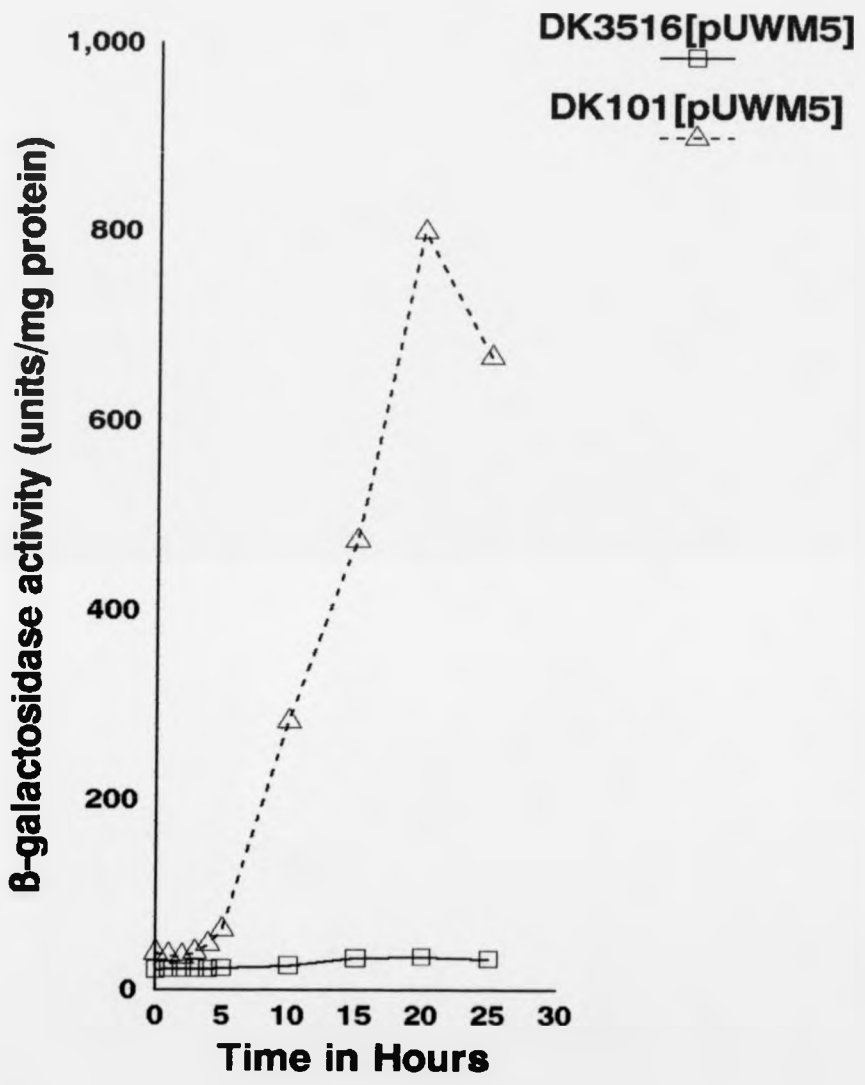


Fig. 8.1 ß-Galactosidase Expression During Glycerol-Induced Sporulation of Strains DK101[pUWM5] and DK3516[pUWM5].

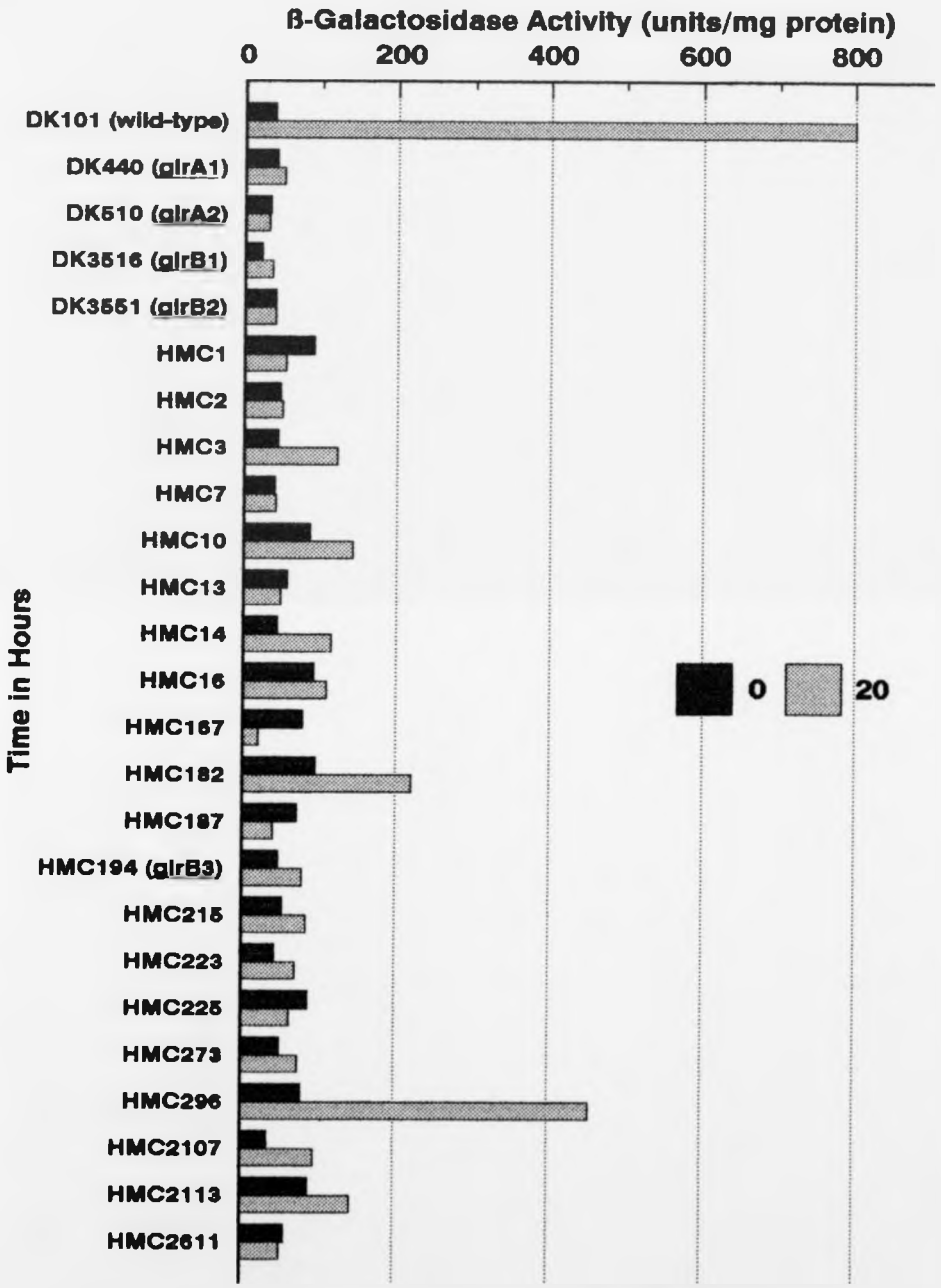


Fig. 8.2 Expression from the *isgB* Gene in Several Glycerol-Resistant Mutants After the Addition of Glycerol.

mutants suggests that the isgB gene product is expressed after the genes required for induction of the pathway. This is consistent with expression during glycerol-induced sporulation of the wild-type strain where the increase in ß-galactosidase expression did not begin until five hours after the addition of glycerol and reached a peak after 20 hours. Glycerol spores are morphologically fully formed approximately 2 - 3 hours after the addition of glycerol, but are not fully "mature". Resistance to sonic vibration, UV irradiation and heat develop during a maturation period lasting several hours (Sudo and Dworkin, 1969).

8.3 Epistasis During Starvation-Induced Sporulation

It was also of interest to see whether the isgB promoter was expressed during starvation conditions in a range of mutant genetic backgrounds. Before starting this work we felt it was necessary to repeat some of the experiments carried out previously to confirm the results and become familiar with the procedure. DK306[pUWM5] cells were grown in DCY broth (Section 2.3.3) to exponential phase and sedimented at 4,500rpm in an Wifug Labor-50M bench-top centrifuge. The cells were washed and resuspended in TM buffer (Section 2.3.2) and inoculated in 20µl aliquots onto TMP agar (Section 2.3.3) at a concentration of 5.0×10^9 cells/ml. Samples were scraped off the agar, as described in Section 2.11, at intervals over the next seven days. No increase in ß-galactosidase activity was detectable in the spores.

However, the amount of sporulation detected was very low. Only approximately 50 spores/5.0 x 10^9 of the original cells applied were detected in six independent attempts.

It has been shown that the alignment of cells is important for the intercellular transmission of C factor (Kim and Kaiser, 1990a). Non-motile cells failed to sporulate and express C factor-dependent genes but both of these defects could be rescued by orientation of the cells in parallel groups. Grooves were scored in the agar with 5 - 10μm aluminium oxide abrasive paper and cell suspensions were placed onto them. The cells settled in the grooves with their long axis oriented parallel to the direction of the groove. Cells aligned in this manner sporulated more than unaligned cells, achieving 16% of wild-type values (Figure 8.3). If non-motile cell density was increased by sedimentation, non-motile cell sporulation rose to 1% of wild-type values (Kim and Kaiser, 1990a) (Figure 8.3). In view of these observations TM agar was scored with fine grain aluminium oxide abrasive paper (obtained from B & Q) and DK306[pUWM5] cells were spotted on in 20μl aliquots at 5.0 x 10^9 cells/ml. Again sporulation was poor, only 100 spores/5.0 x 10^9 of the original cells applied was seen. Such poor sporulation was probably a result of the grooves being too wide. The fine grain aluminium abrasive paper gave grooves of 10 - 15μm and examination of the cells in the grooves using a plate microscope suggested that they may not effectively force the cells to align. Inadequate washing of the cells before

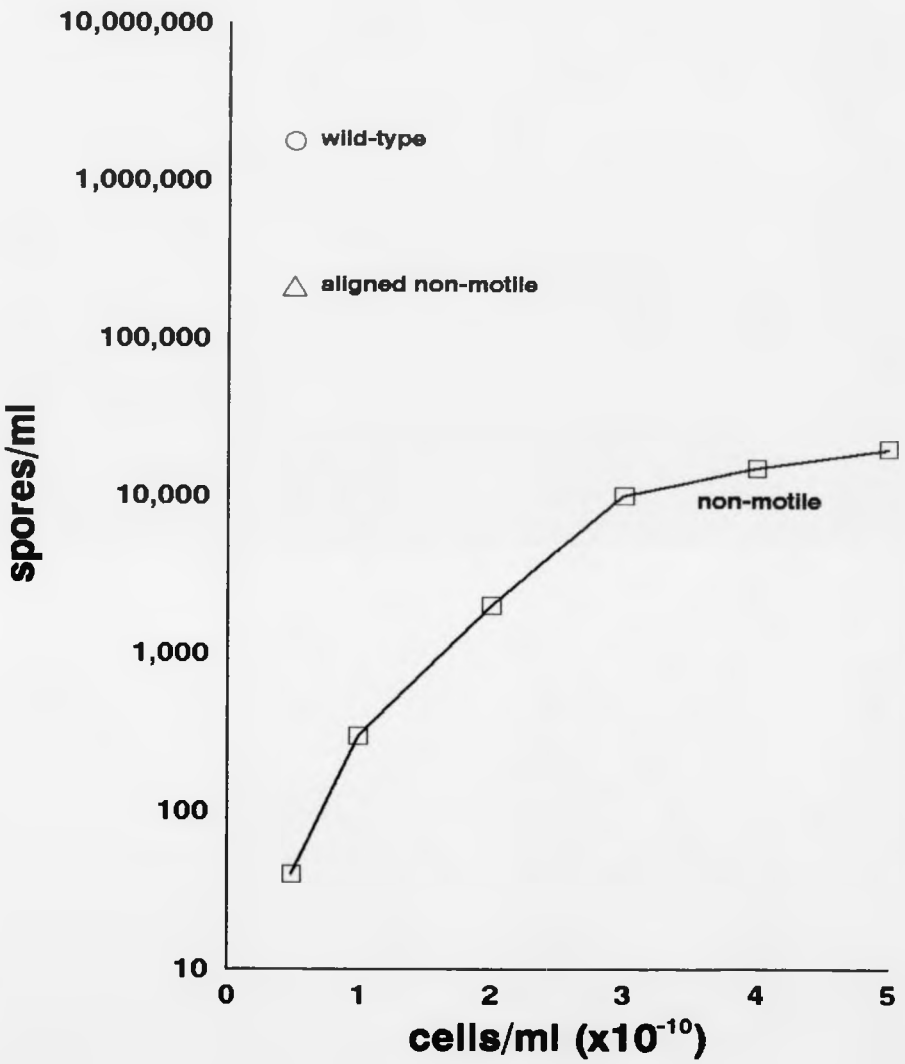


Fig. 8.3 Effect of Cell Density and Position on Spore Formation in the Non-motile Strain, DK306 (Redrawn from Kim and Kaiser, 1990a).

spotting onto starvation media may also have resulted in poor sporulation.

Although we were unable to repeat the control experiments, attempts were made to analyse expression from the *isgB>lacZ* fusion during starvation-induced sporulation in some mutant genetic backgrounds. Cultures of the following strains were grown up on DCY broth and inoculated onto TMP agar in 20µl aliquots at 5.0×10^9 cells/ml; DK440[pUWM5], DK3516[pUWM5] and DK370[pUWM5]. DK370 carries a *cglF* mutation (Hodgkin and Kaiser, 1979a) and, hence, is defective in the adventurous motility system. Two samples from each starved culture were collected daily for seven days as described in Section 2.11. The samples were assayed for ß-galactosidase activity after sonication. Spores were assayed separately from the vegetative cells. This distinction is possible due to the different sonication resistances of the two cell types. Fruiting body spores are 19.3 times more resistant to sonic vibration than vegetative cells (Sudo and Dworkin, 1969). In DK370[pUWM5] sporulation was observed at a frequency of 2.0×10^5 spores/5.0×10^9 of original cells, which is approximately 10% of the documented wild-type values (Kim and Kaiser, 1990a). In DK3516[pUWM5] sporulation occurred at a frequency of 1.6×10^6 spores/5.0×10^9 of original cells, which corresponds to approximately 80% of documented wild-type values. However, there was no increase in ß-galactosidase expression in either the spores or vegetative cells of either strain. In

DK440[pUWM5] a massive increase in ß-galactosidase expression was seen in the vegetative cells as shown in Figure 8.4. Sporulation was observed at a frequency of 1.5×10^6 spores/5.0×10^9 of original cells. This corresponds to approximately 75% of documented wild-type levels. Some increase in ß-galactosidase expression was also seen in the spores. However, one cannot rule out the possibility that this increase in expression was due to carry over from the vegetative cells. It would be of interest to repeat the experiment with adequate washing of the spores after the vegetative cells have been sonicated to ensure that there is no carry over of ß-galactosidase from the vegetative cells to the spores. That the *isgB* gene was expressed in the vegetative cells during starvation-induced sporulation of DK440[pUWM5] is of interest as it is the only example of elevated levels of *isgB* expression in vegetative cells. DK440 was originally isolated because it showed the *bsg-440* mutant phenotype, i.e. it could only form fruiting bodies in the presence of Bsg^+ cells, and it was also found to be glycerol-resistant. Hence, it could be the *bsg-440* mutation or the *glrA1* mutation which allows expression from the *isgB* promoter in the vegetative cells. Expression from the *isgB* promoter has not yet been examined in a B signalling mutant background. In order to elucidate whether it is the *glrA* mutation or the *bsg-440* mutation which allows expression of the *isgB* gene in the vegetative cells of DK440 under starvation conditions, it would be necessary to study expression of

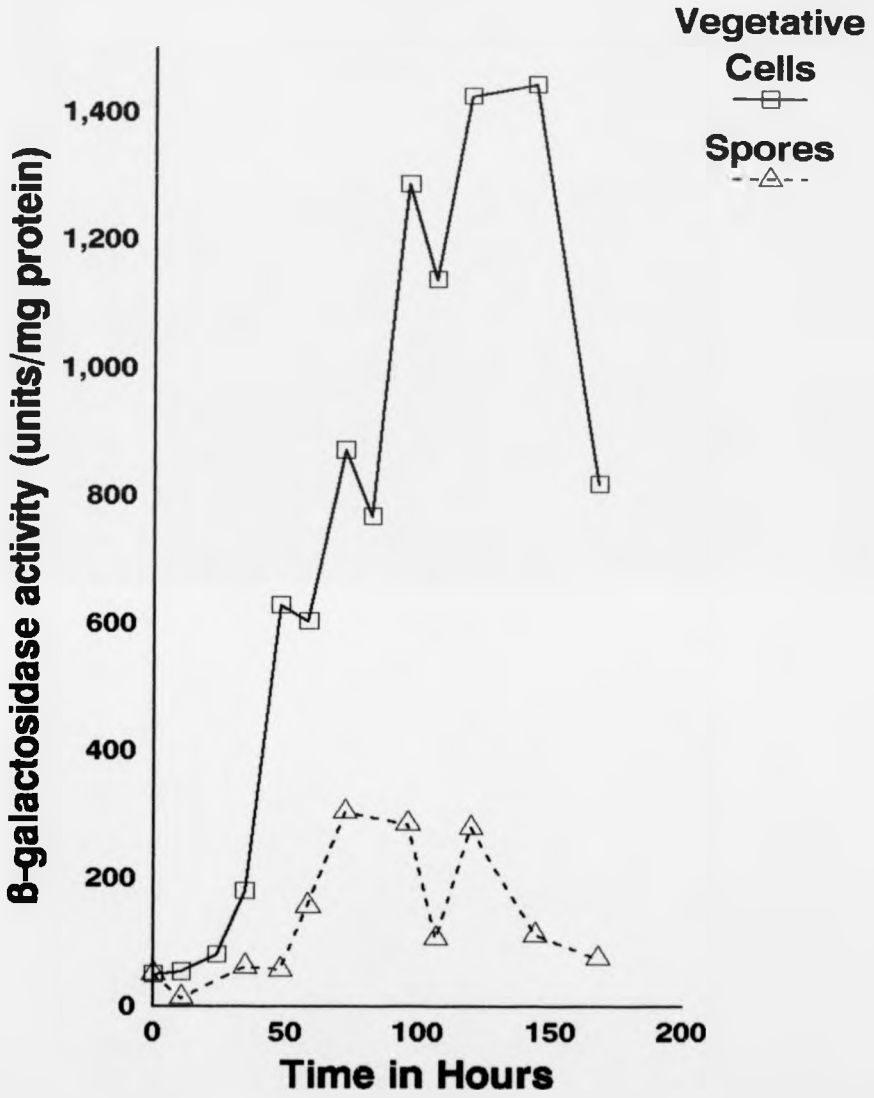


Fig. 8.4 ß-Galactosidase Expression from isgB During Starvation-Induced Sporulation in the Strain DK440[pUWM5] (glrA1, Fru⁻).

the *isgB* gene during starvation in the following mutant backgrounds; (1) *bsg-440*, (2) *glrA1* and (3) DK510 (*glrA2*, Fru$^-$). However, in order to attain the *glrA1* mutation in a fruiting background and the *bsg-440* mutation in a glycerol-sensitive background, it would be necessary to independently transduce the *glrA1* and *bsg-440* mutations into the wild-type background. However, no conclusions can be drawn from these observations for starvation-induced sporulation because the control experiments were unsuccessful.

8.4 Discussion

The *isgB* gene appears to be expressed late in the glycerol-induced sporulation pathway as shown by the time of expression. In the wild-type strain expression did not begin until approximately five hours after the addition of glycerol and reached a peak in expression 20 hours after the addition of glycerol. Phase-contrast microscopy revealed that glycerol-induced spores were morphologically fully formed 2 - 3 hours after the addition of glycerol. Although morphologically fully formed 2 - 3 hours after the addition of glycerol, resistance to sonic vibration, UV irradiation and heat develop over a period of several hours. There was no increase in ß-galactosidase expression from the *isgB>lacZ* fusion after addition of glycerol to 24 different glycerol-resistant mutants. This is consistent with the expression of the *isgB* gene occurring late in the glycerol-induced sporulation pathway. Alternatively, all

the glycerol-resistant mutants may be blocked very early in the pathway of glycerol-induced sporulation.

Studying the expression from the *isgB* gene was harder during starvation conditions as it seemed difficult to achieve high enough levels of sporulation to get accurate results of expression within the spores. Hartree (1989) showed that the *isgB*>*lacZ* fusion is expressed at 15-fold levels during starvation-induced sporulation in the non-motile (*mgl*) strain DK306. Attempts to repeat the observation were unsuccessful. Only very poor levels of sporulation were obtained in repeated attempts. Hartree (1989) also reported poor sporulation of DK306[pUWM5]. Adequate sporulation for ß-galactosidase determination occurred on the first attempt, but on all subsequent attempts only very low levels of sporulation occurred. It has been shown that alignment of cells is important for transmission of C factor (Kim and Kaiser, 1990a). Non-motile cells failed to sporulate and express C factor dependent genes, but both these defects could be rescued by orientation of the cells in parallel groups in grooves formed by scoring the agar with 5 - 10μm aluminium oxide abrasive paper. Cells aligned in this manner sporulated more than unaligned cells achieving 16% of wild-type values (Kim and Kaiser, 1990a). In view of these observations DK306[pUWM5] cells were starved on TMP agar (Section 2.3.3) scored with fine grain aluminium oxide abrasive paper. Although this did double sporulation compared with starvation on ungrooved agar, sporulation was still very poor compared with wild-

type levels and levels of sporulation documented for non-motile strains by Kim and Kaiser (1990a). Such poor sporulation was probably the result of the grooves being too wide. Examination of cells on grooved agar using a plate microscope suggested that the grooves may not effectively force the cells to align.

Although attempts to repeat the control experiments were unsuccessful, preliminary experiments during starvation-induced sporulation were carried out on the strains DK370[pUWM5], DK440[pUWM5] and DK3516[pUWM5]. Sporulation occurred at approximately 10%, 75% and 80% of documented wild-type values respectively (Kim and Kaiser, 1990a). No expression was observed from the *isgB*>*lacZ* fusion in DK370[pUWM5] or DK3516[pUWM5]. However, the *isgB*>*lacZ* fusion was expressed at elevated levels in vegetative cells of DK440[pUWM5]. There was also some ß-galactosidase activity in the spore fraction, but this could have been carry over from the vegetative cell fraction. No conclusions can be drawn from these observations, however, because the control experiments were unsuccessful.

That the *isgB* gene is expressed in the vegetative cells of DK440 is interesting, however, and needs further examination as it is the only example of expression of the *isgB* gene in vegetative cells. It is not clear whether the observed expression in vegetative cells was the result of the *glrA1* mutation or the *bsg-440* mutation, as expression from the *isgB* gene has not been examined in a B signalling mutant background. However, the ΩDK4530

Tn*5* *lac* fusion, which also shows increased expression during glycerol-induced sporulation, is known to show reduced expression during fruiting body sporulation in a *bsgA* background (Kroos and Kaiser, 1987). The ΩDK4530 locus has been shown to represent an independent locus to the *isgB* fusion.

Chapter 9

Analysis of the ΩDK4530 Region

9.1 Introduction

Strain DK4530 contains a Tn*5* *lac* fusion [ΩDK4530] in the wild-type background (Kroos and Kaiser, 1987). Tn*5* *lac* is approximately 12kb in size and contains a promoterless *trp-lac* fusion fragment inserted near one end of the bacterial transposon Tn*5* in the correct orientation to fuse *lacZ* gene expression to promoters outside Tn*5* (Kroos and Kaiser, 1984). There are translational stop signals in all three reading frames ahead of the *lacZ* gene. Hence, transcriptional fusions, but not protein fusions are possible. The Tn*5* *lac* construct can be introduced into *M. xanthus* using bacteriophage P1::Tn*5* *lac* resulting in stable kanamycin-resistant transductants that contain no P1 DNA but have Tn*5* *lac* inserted randomly in the *M. xanthus* chromosome (Kroos and Kaiser, 1984). Of the 2374 Tn*5* *lac* insertion-containing strains of *M. xanthus* generated, 36 were identified that specifically increase ß-galactosidase expression at some particular time during development (Kroos *et al.*, 1986). For Tn*5* *lac* ΩDK4530 ß-galactosidase expression begins to increase approximately four hours after the onset of development, reaches a peak at 24 to 36 hours and then starts to decline (Kroos *et al.*, 1986; Fig.4b). ß-Galactosidase expression was found to be reduced during development in B signalling mutants, which are blocked early in development (Kroos and Kaiser, 1987). Increased ß-galactosidase expression from ΩDK4530 also occurred during glycerol-induced sporulation (Kroos, 1986). Expression reaches the same peak specific

activity three hours after the addition of glycerol that is reached at 24 - 36 hours during fruiting body development on plates. Increased ß-galactosidase expression must occur before the cells become sonication resistant because ß-galactosidase specific activity can be detected by sonication without glass beads. Thirteen other Tn*5* *lac* containing strains that increase in ß-galactosidase expression during fruiting body formation showed no change in ß-galactosidase specific activity during glycerol-induced sporulation (Kroos, 1986).

9.2 ß-Galactosidase Expression in DK4530 During Glycerol-Induced Sporulation

DK4530 cultures were grown to a density of 2.0×10^8 cells/ml (A_{600} = 0.7). The t=0 sample was withdrawn and 50% glycerol was added to the media to a final concentration of 0.5M. After the addition of glycerol, cultures were shaken at 33°C and samples were withdrawn after 2, 5 and 8 hours. Samples were collected and stored as indicated in Section 2.11. Phase-contrast microscopy revealed that rod-shaped cells converted to spherical spores 1 - 3 hours after the addition of glycerol. ß-Galactosidase specific activity was determined (Sections 2.12, 2.13) after disruption of the glycerol spores by sonication with glass beads (Section 2.11).

Initially DK4530 cells were grown in DCY broth (Section 2.3.3), but no significant increase in ß-galactosidase specific activity was detected. When the

fusion was assayed previously for ß-galactosidase expression (Kroos, 1986) the cells had been grown in CTT broth (Section 2.3.3). Therefore, it seemed that a component of DCY may be suppressing glycerol-induced promoter activity of the ΩDK4530 fusion. DCY broth contains an additional 10g/l casitone and 2g/l yeast extract in comparison to CTT broth. DK4530 cells were, therefore, grown up on the following variants of media; (1) CTT broth, (2) CTT broth containing an additional 10g/l casitone, (3) CTT broth containing 2g/l yeast extract and (4) DCY broth. These cultures were then induced to sporulate by the addition of glycerol in the manner described above. The samples collected were subsequently assayed for ß-galactosidase specific activity. The results are shown in Figure 9.1. Cells grown and induced to sporulate in CTT reached a peak of ß-galactosidase specific activity of 64 units/mg protein five hours after the addition of glycerol. Cells grown on CTT broth with 2g/l yeast extract showed reduced expression reaching a peak of 33 units/mg protein five hours after the addition of glycerol. However, cells grown on DCY broth or CTT broth containing an additional 10g/l casitone showed no increase in ß-galactosidase specific activity during glycerol-induced sporulation. Therefore, it would seem that expression from the promoter in question is suppressed by amino acids or other components of casitone. Presumably, expression from the fusion is reduced in CTT broth with 2g/l yeast extract because yeast extract contains a low

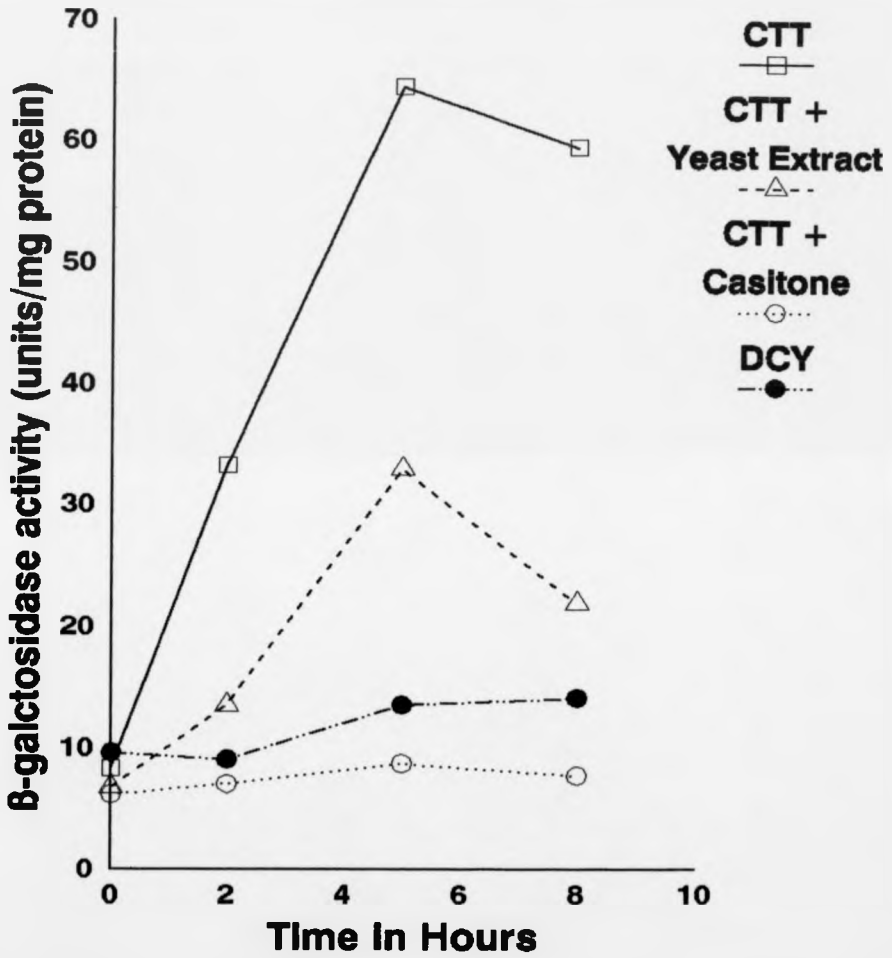


Fig. 9.1 ß-Galactosidase Expression During Glycerol-Induced Sporulation of Strain DK4530 Grown on a Variety of Media.

concentration of amino acids. Subsequently cultures for assay of the ΩDK4530 fusion were grown on CTT broth. The peak in expression was observed later than previously reported (Kroos, 1986). This may be due to slight differences in the way cells were prepared for glycerol-induced sporulation. Kroos grew cultures to exponential phase and then sedimented the cells and resuspended them at 2.5 x 10^8 cells/ml in fresh media to which glycerol was then added.

Dimethylsulphoxide (DMSO) can also be used to induce sporulation of M. xanthus cells and appears to act in the same manner as glycerol (Bacon and Rosenberg, 1967). Hence, DK4530 cells were also induced to sporulate by the addition of DMSO to a final concentration of 0.7M. DK4530 cells to which no inducer had been added were also assayed for ß-galactosidase expression. The results are shown in Figure 9.2. Cells induced to sporulate with DMSO showed a similar pattern of ß-galactosidase activity to that seen during glycerol-induced sporulation, while there was no increase in ß-galactosidase expression in the vegetative control.

9.3 Expression of ΩDK4530 in a Glycerol-Resistant Background

It was of interest to see if the ΩDK4530 fusion would be expressed in a glycerol-resistant background after the addition of glycerol. It was, therefore necessary to transfer the fusion into several glycerol-resistant mutants. This was done by myxophage

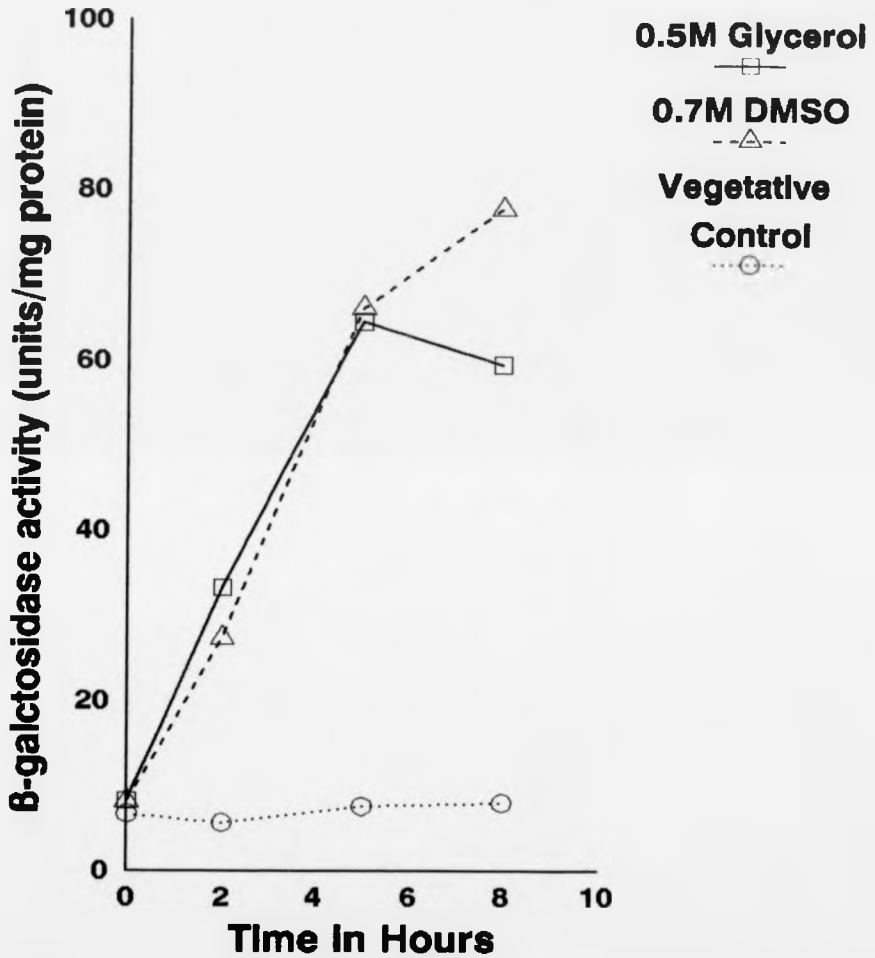


Fig. 9.2 ß-Galactosidase Expression During Glycerol-Induced Sporulation, DMSO-Induced Sporulation and Vegetative Growth of DK4530.

transduction. DK4530 was first tested for sensitivity to Mx4 and Mx8 by spotting 10μl aliquots of the two phage stocks onto an overlay of DK4530 cells on DCY agar. Zones of clearance around the Mx4 spots indicated DK4530 was sensitive to Mx4 and that plaques had been formed. No zones of clearance appeared around the Mx8 spots indicating that DK4530 is Mx8 resistant. An Mx4 lysate of DK4530 was made as described in Section 2.15.1. This Mx4::ΩDK4530 lysate was used to introduce the ΩDK4530 fusion into the following glycerol-resistant backgrounds:- (1) DK440 (glrA1, bsg-440) (LaRossa et al., 1983), (2) DK510 (glrA2, Fru^-) (Hagen et al., 1978), (3) DK3551 (glrB2, Fru^-) and (4) HMC194 (glrB3, Fru^+) as described in Section 2.15.2. Kanamycin resistant transductants were obtained indicating that the Tn5 lac fusion had been transduced into the relevant glycerol-resistant backgrounds. Fifty transductant colonies for each strain were screened for glycerol-resistance and fruiting phenotypes. Introduction of the Tn5 lac fusion had no effect on the glycerol or fruiting phenotypes of any of the parental strains. HMC400 (glrA1, bsg-440, ΩDK4530), HMC401 (glrA2, Fru^-, ΩDK4530), HMC402 (glrB2, Fru^-, ΩDK4530) and HMC403 (glrB3, Fru^-, ΩDK4530) cultures were grown to a density of 2.0×10^8 cells/ml (A_{600} = 0.7) and glycerol was added to a final concentration of 0.5M. Samples were taken at 0, 2, 5 and 8 hours as before. Phase-contrast microscopy revealed that the cells remained rod-shaped as expected for glycerol-resistant strains. ß-Galactosidase specific activity was

assayed and the results for HMC400 (glrA1, bsg-440, ΩDK4530) are shown in Figure 9.3. There was no increase in ß-galactosidase expression in HMC400 (glrA1, bsg-440, ΩDK4530), HMC401 (glrA2, Fru$^-$, ΩDK4530), HMC402 (glrB2, Fru$^-$, ΩDK4530) or HMC403 (glrB3, Fru$^-$, ΩDK4530) suggesting that expression from ΩDK4530 is blocked by the mutation in the parental strain which results in glycerol-resistance.

9.4 Probing the Chromosome of DK4530

In order to ascertain if ΩDK4530 represented a different locus from the glrA and glrB loci, defined by Mx8 mapping of glycerol-resistant mutations (Andreasson, Blea and Kaiser, personal communication) (Chapters 4, 5 and 6), and the isgB locus, identified as a glycerol-inducible gene fusion (Hartree, 1989) (Chapters 7 and 8), chromosomal DNA from DK4530, and the wild-type strain DK1622, were probed with clones representing each of the aforementioned loci.

Chromosomal DNA was isolated from strains DK1622 and DK4530 as described in Section 2.19. SacI digested DNA was transferred to nitrocellulose membrane by Southern transfer (Section 2.29). Probes were prepared, as described in Section 4.2, from the following plasmids:(1) pKIA3 (glrA$^+$), (2) pKIA2 (glrB$^+$) and (3) pUWM5 (isgB), and the hybridization conditions used are described in Section 2.30. If one of the probes was homologous to the ΩDK4530 locus one more hybridizing fragment would be seen in the DK4530 track, than in the wild-type track, as Tn5

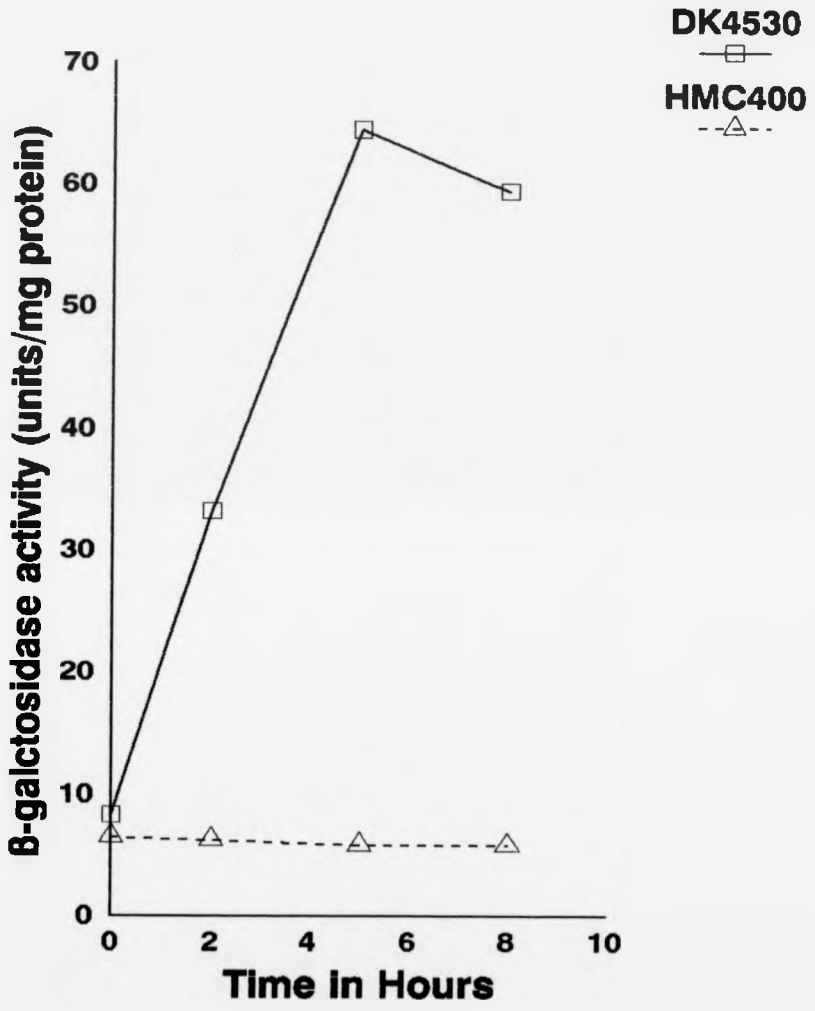


Fig. 9.3 ß-Galactosidase Expression During Glycerol-Induced Sporulation of Strains DK4530 and HMC400 (glrA1, bsg-440, ΩDK4530).

lac has a *Sac*I site in the *lacZ* region. The total size of the hybridizing fragments would be 12kb longer for DK4530, corresponding to the Tn*5* *lac* insertion. For all three probes used the hybridizing fragments were identical in both the wild-type and DK4530 tracks. The fragments hybridized by each probe are shown for the wild-type, DK1622, in Chapter 4, Table 4.1. These results show that ΩDK4530 represents an independent locus from *glrA*, *glrB* and *isgB*.

9.5 Discussion

DK4530 contains a Tn*5* *lac* insertion. Expression from the exogenous promoter is increased during fruiting body development. Expression is also increased during chemical-induced sporulation with either 0.5M glycerol or 0.7M DMSO reaching a peak five hours after the addition of the inducer. However, the fusion is not expressed in vegetative cells. Hence, the fusion represents a development specific promoter which is induced during both starvation-induced fruiting body sporulation and glycerol-induced sporulation, reaching the same peak in specific activity. However, it should be noted that this fusion shows 10-fold lower expression during glycerol-induced sporulation than the glycerol-inducible promoter representing the *isgB* region.

Expression from the promoter during glycerol-induced sporulation seems to be suppressed by amino acids as shown by the reduced expression caused by the addition of yeast extract to the growth media and the abolition of

induction of expression caused by additional casitone in the growth media. Amino acids do not suppress expression from the *isgB* fusion which was first identified in cells grown on DCY media.

That the ΩDK4530 fusion was not expressed in *glrA* or *glrB* glycerol-resistant mutants suggests that the gene it represents is expressed later in the glycerol sporulation pathway than the gene products of either *glrA* or *glrB*.

Southern analysis confirmed that ΩDK4530 represented an independent locus from the *glrA*, *glrB* or *isgB* regions.

Presumably, the insertion of ΩDK4530 Tn*5* *lac* would have destroyed the gene. Hence, like the *isgB* gene, the region downstream of the glycerol-inducible promoter is not essential for glycerol-induced sporulation or fruiting body sporulation.

The ΩDK4530 fusion shows reduced expression during fruiting body development in a B signalling (*bsgA*) mutant (Kroos and Kaiser, 1987). It would be of interest to see if a *bsgA*$^-$ background would have an effect on expression of ΩDK4530 during glycerol-induced sporulation. The other signalling factors may also be necessary for correct expression of the ΩDK4530 fusion. It would, therefore, be interesting to look at expression from the ΩDK4530 fusion during fruiting body and glycerol-induced sporulation in *asg*, *csg*, *dsg* and *esg* mutant backgrounds. It would also be of interest to assess the effect of a glycerol-resistant background on expression of the ΩDK4530 *lacZ* fusion during fruiting body sporulation.

Although it is known that the ΩDK4530 fusion is

expressed in fruiting body sporulation and glycerol-induced sporulation, it is not known whether the fusion is expressed during starvation-induced, fruiting-independent sporulation. For this reason the ΩDK4530 fusion should be transduced by Mx4 transduction into the non-motile strain DK306, which is unable to aggregate into fruiting bodies. Kim and Kaiser (1990a) reported that spores were formed on starvation of non-motile cells, despite the absence of fruiting bodies. The number of spores formed can be increased by aligning the cells in parallel grooves formed by scoring the agar with 5 - 10μm aluminium oxide paper. Aligning the cells allows the transmission of C factor which is required for sporulation. These spores are presumed to have been formed by the starvation-induced, fruiting-independent sporulation pathway (Hartree, 1989). Assaying the expression from the ΩDK4530 lacZ fusion in spores formed on starvation of DK306 cells would indicate whether the ΩDK4530 fusion was expressed in starvation-induced, fruiting-independent sporulation. However, several attempts to obtain spores from starved DK306 cells in order to assay expression of the isgB fusion have been unsuccessful (See Chapter 8).

Chapter 10

Discussion

10.1 Chemical-Induced Sporulation: The Nature of the Signal

Sporulation in M. xanthus can be induced by adding high concentrations of low molecular weight compounds such as glycerol (Dworkin and Gibson, 1964) or DMSO (Bacon and Rosenberg, 1967) to exponential phase cultures growing in complex liquid medium. The best inducers of sporulation are fully saturated aliphatic compounds containing two to four carbons with at least one primary or secondary alcohol group (Sadler and Dworkin, 1966). It is thought that the inducer interacts with a "receptor" on the cell surface. This is based on the observations that little, if any, of the inducer is incorporated into the cell (Sadler and Dworkin, 1966) and that the removal of the inducer prior to the appearance of non-refractile spores results in an exact reversal of the sequence of morphological events leading to spore formation (Dworkin and Sadler, 1966). The receptor(s) and genes involved in the production of the receptor are likely to be expressed constitutively. A mutation in one of these genes would lead to mutants which are not induced to sporulate by the addition of glycerol. Genes involved in the initiation of the chemical-induced sporulation pathway might also be constitutively expressed.

DMSO and ethylene glycol are thought to induce sporulation by the same mechanism as glycerol, since mutants isolated for resistance to induction of sporulation by glycerol are also resistant to DMSO and

ethylene glycol. Phenylethyl alcohol is thought to induce sporulation by a different mechanism or interact with a different receptor on the cell surface, since mutants resistant to glycerol still formed spores at a low frequency in response to phenylethyl alcohol (Burchard and Parish, 1975).

The pathway of chemical-induced sporulation could be complex involving many genes. The pathway might be expected to share common genes with the starvation-induced sporulation pathways; fruiting body sporulation and fruiting-independent sporulation. It is to be expected that the structural genes for assembly of the physical structures and for enclassing the spores with their physical properties will also be common between these three spore types and probably the "peripheral rods" (Section 1.2.2.4).

10.2 Glycerol-Resistance

Burchard and Parish (1975) reported that mutants could be isolated which were not induced to sporulate by the addition of glycerol to exponential phase cultures. These mutants were described as glycerol-resistant. They were also found to be resistant to DMSO and ethylene glycol, although spores were still formed in response to phenylethyl alcohol. In this study glycerol-resistant mutants were isolated, from the wild-type (DK1622) and a non-motile strain (DK306), by inoculating EMS, NTG and UV mutagenized cultures onto complex media containing 1.0M glycerol. Mutants resistant to glycerol arose at a

frequency of 1.5×10^{-6} - 3.5×10^{-5} and appeared to be stable without maintaining constant selection pressure. Spontaneous glycerol-resistant mutants arose at a frequency of 2×10^{-7}. This was a lower frequency of spontaneous mutation than recorded by Burchard and Parish (1975). However, this is probably primarily because they selected for glycerol-resistant mutants on media containing 0.75M glycerol as oppose to the 1.0M used in this study. The glycerol-resistant mutants were not tested for resistance to any of the alternative inducers. It would be of interest to assess the response of some of the glycerol-resistant mutants isolated to the alternative inducers DMSO, ethylene glycol and phenylethyl alcohol in order to confirm the observations of Burchard and Parish (1975) that glycerol-resistant mutants are also resistant to DMSO and ethylene glycol but are not resistant to phenylethyl alcohol.

Several glycerol-resistant mutants, which were also unable to undergo normal fruiting body sporulation, have been isolated by EMS and UV mutagenesis. The mutations in these mutants were mapped by Mx8 transduction to two unlinked clusters, glrA and glrB (Figure 1.5). These clusters were cloned into two series of plasmids (the pKIA plasmids) (Andreasson, Blea and Kaiser, personal communication). Southern blot analysis (Chapter 4) and restriction mapping (Chapter 5) confirmed that these plasmids contained DNA from two unlinked loci. The plasmids could be grouped into three families: (1) pKIA3, pKIA4 and pKIA9, (2) pKIA2, pKIA6 and pKIA7 and (3)

pKIA1. The first of these families are plasmids which contain DNA from adjacent to the Tn*5* insertion ΩDK3554 which is co-transducible with the *glrA* loci, while the other two families are plasmids where the cloned DNA comes from adjacent to the Tn*5* insertion ΩDK3517 which is co-transducible with the *glrB* loci.

Complementation showed that the plasmids pKIA3, pKIA4 and pKIA9 complemented both DK440 (*glrA1*, *bsg-440*) and DK510 (*glrA2*, Fru^-) to glycerol-sensitive, non-fruiting (Gly^S, Fru^-) (Table 6.1). In each case 18 - 28% of the transductants were glycerol-resistant, non-fruiting (Gly^r, Fru^-) and were presumably the result of gene conversion events. These observations confirmed that these three plasmids shared considerable regions of homology and all contained DNA from the *glrA* region. The data suggests that both the DK440 (*glrA1*) and DK510 (*glrA2*) mutations are located within 13.6kb from the ΩDK3554 Tn*5* insertion as the smallest plasmid pKIA9 contains only 13.6kb of cloned *M. xanthus* DNA and complements both mutations. Mx*8* transduction mapping of the *glrA* mutations had indicated that the *glrA1* (DK440) mutation was situated 18.0kb away from the ΩDK3554 Tn*5* insertion and that the *glrA2* (DK510) mutation was situated 15.0kb away from the ΩDK3554 Tn*5* insertion (See Figure 1.5) (Andreasson, Blea and Kaiser, personal communication).

The plasmids pKIA2 and pKIA7 complemented DK3551 (*glrB2*, Fru^-) to glycerol-sensitive, non-fruiting (Gly^S, Fru^-) (Table 6.1). Again a small minority (39 - 42%) of

glycerol-resistant, non-fruiting (Gly^r, Fru^-) transductants were obtained which were presumed to be homozygotes arising from gene conversion. The plasmid pKIA6, which was shown by Southern blot analysis to share considerable homology with pKIA2 and pKIA7, did not appear to complement the DK3551 (*glrB2*) mutation. Plasmid pKIA6 is known to contain a deletion from the vector pREG429 DNA. This is not thought to effect the region of homology shared with pKIA2 (pKIA7). However, complete restriction mapping of pKIA6 was not possible due to the large size of the plasmid and was further complicated by the deletion. These observations are inconsistent with the map of the *glrB* region obtained by Mx*8* co-transduction frequencies. Mx*8* co-transduction frequencies suggested that the *glrB1* (DK3516) and *glrB2* (DK3551) mutations were located 13.0kb and 25.0kb away from the Tn*5* insertion ΩDK3517 respectively (Andreasson, Blea and Kaiser, personal communication). As pKIA2 (pKIA7) contains only 9.4kb of cloned DNA, from immediately adjacent to the Tn*5* insertion, one would not expect this plasmid to complement either the *glrB1* (DK3516) or *glrB2* (DK3551) mutations. Our complementation data for the *glrB* region is also inconsistent with preliminary complementation analysis by Blea (personal communication) which suggested that pKIA2, pKIA6 and pKIA7 all complemented DK3516 (*glrB1*, Fru^-) to glycerol-sensitivity. The plasmid pKIA7 also appeared to complement DK3551 (*glrB2*, Fru^-) to glycerol-sensitivity, while pKIA2 and pKIA6 were not tested in DK3551.

The plasmid pKIA1, which contains DNA from the opposite side of the Tn*5* insertion ΩDK3517 than pKIA2 (pKIA7) and pKIA6, did not complement the glycerol phenotype of any of the glycerol-resistant mutants.

The plasmid pKIA4 was taken as a representative of the *glrA* region and subclones derived from pKIA4 were constructed (Section 5.3.1) and assessed for ability to complement the mutants DK440 (*glrA1*, *bsg-440*) and DK510 (*glrA2*, Fru$^-$) (Section 6.3). The results of analysis of the *glrA* locus are summarized in Figure 6.3. The cloned DNA in pKIA4 was first subcloned into two plasmids pHMC6 and pHMC8. pHMC8 complemented both the *glrA1* (DK440) and *glrA2* (DK510) mutations but pHMC6 did not complement either mutation. Hence, both mutations must be located in the region of DNA cloned in pHMC8. Three further subclones, pHMC40, pHMC42 and pHMC44 were derived from this region. Plasmids pHMC40 and pHMC44 did not complement either the *glrA1* (DK440) or *glrA2* (DK510) mutations. Plasmid pHMC42 complemented the *glrA1* (DK440) mutation but did not complement the *glrA2* (DK510) mutation. Hence, it appears that the *glrA1* (DK440) mutation is located within the 2.0kb *Xho*I fragment subcloned in pHMC42. As the *glrA2* (DK510) mutation was complemented by pHMC8 but was not complemented by pHMC40, pHMC42 or pHMC44 it would seem that the *glrA2* (DK510) mutation is either located very close to one of the two *Xho*I sites used in constructing the subclones, pHMC40, pHMC42 and pHMC44, or is a deletion mutation. This suggests that both the *glrA1* (DK440) and *glrA2* (DK510)

mutations are located between approximately 9.1kb and 11.3kb away from the Tn*5* insertion ΩDK3554. Promoter activity was detected from the 2.8kb *Bam*HI *Xho*I fragment cloned in pHMC40. The fragment was orientated so that the *Bam*HI end of the fragment was adjacent to the promoterless *lacZ* of the vector pDAH283. Quantitative ß-galactosidase assays showed that the promoter in question was expressed at relatively constant levels of 50 - 70 unit/mg protein throughout both vegetative growth and glycerol-induced sporulation. Qualitative assays using 4-MUG to detect ß-galactosidase expression suggested that the promoter in question is also expressed during fruiting body sporulation. Hence, the gene in question is expressed constitutively. However, there is no evidence that the gene in question is involved in glycerol-induced sporulation. It would be of interest to assess promoter activity of this 2.8kb *Bam*HI *Xho*I fragment in the opposite orientation. The promoter activity of the 2.0kb *Xho*I fragment cloned in pHMC42 should also be assessed in both orientations. However, before this can be achieved the *Pst*I and *Sma*I restriction sites within the 2.0kb *Xho*I fragment will have to be fully mapped so that the two orientations of the fragment are distinguishable.

Little has been determined about the nature of the *glrA* gene product(s). However, it is known that the *glrA* gene or genes are required early in the pathway of induction of glycerol-induced sporulation as neither the *isgB*>*lacZ* fusion nor the ΩDK4530 Tn*5* *lacZ* fusion were

expressed in a glrA mutant background.

Complementation analysis suggested that pKIA2 contained the glrB$^+$ loci. Hence, subclones derived from pKIA2 were constructed (Section 5.3.2) and assessed for their ability to complement DK3551 (glrB2) (Section 6.4). The results of analysis of the glrB region are summarized in Figure 6.7. The plasmids pHMC2 and pHMC4 each contained approximately half of the cloned M. xanthus DNA in pKIA2, splitting the cloned DNA at the unique SalI site. However, neither plasmid complemented the glrB2 (DK3551) mutation. It was thought that the gene of interest may have been split in half. Plasmids pHMC30 and pHMC32 were, therefore, constructed which contained DNA cloned from across the SalI site. These plasmids also did not complement DK3551 (glrB2, Fru$^-$). Therefore, the plasmids pHMC34 and pHMC36 were constructed which allowed DNA from around the SalI site to be assessed for promoter activity. DK101[pHMC34] cells did not express ß-galactosidase and, hence, the cloned fragment did not contain a promoter in the correct orientation to direct transcription of the lacZ gene. In DK101[pHMC36] cells significant ß-galactosidase expression was observed. ß-Galactosidase specific activity increased five-fold during 25 hours of vegetative growth (Figure 6.4). A similar pattern of expression was observed during glycerol-induced sporulation except that levels of ß-galactosidase specific activity dropped off sharply 20 - 25 hours after the addition of glycerol (Figure 6.4). Qualitative assays using 4-MUG as an indicator for ß-

galactosidase suggested that the gene in question is also expressed during fruiting body sporulation. Hence, expression of the gene is not sporulation-specific. Although there is no evidence that the promoter is accessing a gene for glycerol-resistance the constitutive expression observed is an expected property of the "receptor" for chemical-induced sporulation. The gene may, therefore, be the glycerol "receptor", or be involved in the production of the "receptor". Some genes involved in the initiation of glycerol-induced sporulation might also be constitutive. In order to ascertain whether this region of DNA was essential for glycerol-induced sporulation a tetracycline resistance determinant was inserted into the SalI site of the DNA cloned in pKIA2. This disrupted version was designated pHMC38. This plasmid was transduced into the wild-type background and transductants where the wild-type copy had been replaced with the disrupted version were obtained. The disruption of this region of DNA did not result in a change of phenotype of the parental cells. Hence, either the gene in question is not essential for glycerol-induced sporulation or the SalI site allows access to the gene promoter without disruption of the gene transcribed.

Overall the data for the glrB region suggest that pKIA2 (pKIA7) does indeed contain a glrB gene, since both pKIA2 and pKIA7 both complement DK3551 (glrB2, Fru$^-$) to glycerol-sensitivity. However, the observation that none of the subclones derived from pKIA2 complemented DK3551 (glrB2) suggests that the glrB region may be a large

operon containing several genes. The glycerol-resistant phenotype of DK3551 may be the result of either a deletion affecting two or more *glr* genes, or the result of two independent mutations in separate *glr* genes. Hence, subclones derived from pKIA2 may not contain sufficient DNA to complement all the mutated *glr* genes. The observation that pKIA6, which contains the entire region of DNA cloned in pKIA2 (pKIA7) and an additional 8.0 - 10.kb of DNA, did not complement the *glrB2* (DK3551) mutation is peculiar and needs further examination. This plasmid is known to contain a deletion in the vector pREG429 DNA, but this is not thought to effect the region of homologous DNA shared with pKIA2 and pKIA7. However, complete restriction mapping of this plasmid was not possible due to its large size.

Little is known about the nature of the *glrB* gene product(s). However, the gene (or genes) appears to be expressed early in the pathway of induction of glycerol-induced sporulation since neither the *isgB>lacZ* fusion or the ΩDK4530 Tn*5* *lacZ* fusion is expressed in a *glrB* mutant background.

The plasmids pKIA3 and pKIA2 were taken as representatives of the *glrA*$^+$ and *glrB*$^+$ regions respectively and transduced into a further 43 glycerol-resistant mutants, derived by EMS, NTG and UV mutagenesis, in order to look for complementation (Tables 6.2 and 6.3). The pKIA3 did not complement any of the glycerol-resistant mutants. The plasmid pKIA2 complemented the mutant HMC194 (Gly^r, Fru$^+$) to glycerol

sensitivity (Gly^S) (Table 6.3). This observation was confirmed using pKIA7 which is identical to pKIA2. Hence, the mutation in HMC194 was designated *glrB3*. The other 42 glycerol-resistant mutants were not complemented by pKIA2. The observation that expression from the glycerol-inducible *isgB>lacZ* fusion was switched off in several of these glycerol-resistant mutants implies that the genes responsible for the glycerol-resistant phenotype of these mutants are also early "receptor" genes. That only one out of 43 glycerol-resistant mutants was complemented by $glrB^+$ DNA and none of the mutants were complemented by $glrA^+$ DNA suggests that there are numerous genes involved in the reception of the signal for glycerol-induced sporulation.

10.3 The Interaction Between Glycerol-Induced Sporulation and Fruiting Body Sporulation

It has been suggested that glycerol acts directly upon the mechanism of myxospore formation that normally operates within fruiting bodies (Witkin and Rosenberg, 1970). However, previous data were limited to two strains which did not form glycerol-induced spores in shake culture, one of which did not form fruiting bodies and the other formed rudimentary fruiting bodies without myxospores (Dworkin and Sadler, 1966). More recently Burchard and Parish (1975) isolated 117 glycerol-resistant mutants of which only eight (6.84%) were unable to form fruiting bodies containing mature myxospores.

Of the 3742 glycerol-resistant mutants isolated in

this study, only 23 (0.61%) were unable to form fruiting bodies containing mature myxospores. A higher frequency of glycerol-resistant mutants which also had fruiting defects was observed from NTG mutagenized cultures, than from EMS or UV mutagenized cultures. This observation can be accounted for by the fact that NTG is known to generate a higher frequency of double site mutations than EMS or UV. Glycerol-sensitive colonies gave rise to a similar frequency of non-fruiting mutants. The observation that most of the glycerol-resistant mutants isolated were able to undergo normal fruiting body sporulation was consistent with the observations of Burchard and Parish (1975). It would appear, therefore, that the pathways of induction of glycerol-induced sporulation and fruiting body sporulation share few, if any, common genes.

Complementation analysis also suggested that mutations in glycerol-induced sporulation and fruiting body sporulation are not 100% linked. The plasmids pKIA3, pKIA4 and pKIA9 complemented both DK440 (*glrA1*, *bsg-440*) and DK510 (*glrA2*, Fru^-) to glycerol-sensitive, non-fruiting (Gly^S, Fru^-) and, hence, contained DNA from the *glrA* region. That the fruiting mutations in these strains were not also complemented suggests that the fruiting defect arises as a result of a mutation at a second locus which is co-transducible with ΩDK3554, but is independent of the *glrA* locus. The plasmids pKIA2 and pKIA7 complemented DK3551 (*glrB2*, Fru^-) to glycerol-sensitive, non-fruiting (Gly^S, Fru^-). Again the

observation that the fruiting defect was not complemented suggested that the defect in fruiting may be the result of a second mutation at a locus co-transducible with ΩDK3517, but independent of the glrB2 locus. The plasmid pKIA1, which contains DNA from the opposite side of the Tn5 insertion ΩDK3517 than pKIA2 (pKIA7) and pKIA6, did not complement the glycerol phenotype of any of the glycerol-resistant mutants. However, pKIA1 did appear to complement the fruiting mutation of DK3516 (glrB1, Fru^-). That the fruiting defect in this strain could be complemented without complementation of the mutation in glycerol-induced sporulation suggested that the two defects were the result of two independent mutations at separate loci, which are both co-transducible with ΩDK3517. That in all cases only one of the two mutant phenotypes was complemented suggested that DK440, DK510, DK3516 and DK3551 may all contain two independent mutations at separate sites within the chromosome, one mutation being responsible for the block in glycerol-induced sporulation and the other mutation being responsible for the block in fruiting body sporulation. In each case both mutant phenotypes were co-transducible. The observation that the fruiting and glycerol-resistant phenotypes in these mutants can be complemented independently is consistent with the observations of mutation analysis, which indicate that double glycerol-resistant, non-fruiting (Gly^r, Fru^-) mutants arise from double mutation events.

A Tn5 lac insertion has been identified (Kroos and

Kaiser, 1987) which shows increased activity during both glycerol-induced sporulation (Kroos, 1986) and fruiting body sporulation (Kroos et al., 1986). Expression from this fusion, designated ΩDK4530, begins to increase approximately four hours after the onset of starvation, reaches a peak at 24 to 36 hours and then starts to decline (Kroos et al., 1986). During glycerol-induced sporulation expression reaches the same peak specific activity three hours after the addition of glycerol that is reached 24 - 36 hours during fruiting body sporulation (Kroos, 1986). This fusion is discussed in detail in Section 10.5

10.4 The Interaction Between Glycerol-Induced Sporulation and Starvation-Induced, Fruiting-Independent Sporulation

It has been suggested that glycerol and other chemical inducers might "short circuit" the starvation-induced, fruiting-independent sporulation pathway. This hypothesis was based on the observation that the glycerol-inducible fusion, isgB>lacZ, is also expressed in spores formed outside of fruiting bodies on starvation of DK306 cells (Hartree, 1989). DK306 cells are non-motile and, hence, are unable to aggregate. Kim and Kaiser (1990a) reported that spores were still formed on starvation of DK306 cells, despite the absence of fruiting bodies (See Figure 8.3). The number of spores formed can be increased by aligning the cells in parallel groups which allows the transmission of C factor which is

required for sporulation (Figure 8.3). These spores formed in the absence of fruiting bodies are proposed to have been formed by the starvation-induced, fruiting-independent sporulation pathway (Figure 1.6).

Glycerol-resistant mutants of DK306 were isolated using UV mutagenesis and selection on complex media containing 1.0M glycerol. In order to assess these glycerol-resistant mutants for starvation-induced, fruiting-independent sporulation a method for screening for fruiting-independent sporulation was devised (Figure 3.2). Only one out of 2011 glycerol-resistant mutants derived from DK306 was also unable to undergo starvation-induced, fruiting-independent sporulation. This mutant was designated HMC300. The data suggests that there are very few common genes in the pathways of induction of glycerol-induced sporulation and starvation-induced, fruiting-independent sporulation. The pathway of induction of chemically-induced sporulation must, therefore, be independent of starvation-induced, fruiting-independent sporulation. As at least one gene (*isgB*) is induced by both chemical-induced sporulation and starvation-induced, fruiting-independent sporulation there must be at least two independent "receptors" for initiation of the pathway.

The mutant HMC300 was of interest because it was completely deficient in sporulation i.e. it was unable to undergo glycerol-induced sporulation, fruiting body sporulation or starvation-induced, fruiting-independent sporulation. However, HMC300 may only be deficient in

fruiting body sporulation as a result of the *mgl* motility defect which renders the cells unable to aggregate. It would be of interest to transduce a Tn*5*-linked *mgl*$^+$ gene into HMC300 which should restore motility. The resultant transductants should be assessed for fruiting body sporulation. This would allow one to determine whether HMC300 is deficient in fruiting body sporulation as a result of the motility defect, or is a true sporulation-deficient mutant. A second mutant, HMC301, was identified which was glycerol-sensitive but was unable to undergo starvation-induced, fruiting-independent sporulation. Glycerol-induced spore formation in liquid culture in this strain should be examined to determine if spores are indeed formed. If the mutation which results in the block in fruiting-independent sporulation was in a gene encoding for a structural or physical property of spores the mutant might be unable to form spores by any method. Such a mutation would not necessarily result in a glycerol-resistant phenotype, since the mutation may be in a gene which is not activated until after the glycerol signal has been recognised and the sporulation pathway initiated. HMC301 should also be transduced to *mgl*$^+$ using a Tn*5*-linked *mgl*$^+$ gene in order to ascertain whether fruiting body formation and sporulation is blocked by the mutation.

Sporulation in *M. xanthus* is difficult to study because fruiting body sporulation is very complex. Mutations in motility or aggregation can result in a non-fruiting phenotype, and will thus effect sporulation.

Starvation-induced sporulation in the absence of fruiting bodies may be equivalent to sporulation in Bacillus subtilis (Losick et al., 1989), where each single cell produces a spore. Hence, mutants which are deficient in starvation-induced, fruiting-independent sporulation could be true sporulation-deficient mutants equivalent to those identified in Bacillus subtilis. Analysing true sporulation-deficient mutants would greatly enhance the study of the pathways of spore formation in M. xanthus.

10.5 Glycerol-Inducible lacZ Fusions

The isgB locus was identified as a promoter fusion in a promoter probe vector. The promoter shows a spectacular increase in ß-galactosidase activity during glycerol-induced sporulation of the wild-type strain and is also expressed during starvation-induced, fruiting-independent sporulation of the non-motile strain DK306 (Hartree, 1989). The isgB gene appears to be expressed late in the glycerol-induced sporulation pathway, since expression in the wild-type background did not begin to increase until approximately five hours after the addition of glycerol and reached a peak in expression 20 hours after the addition of glycerol. A similar pattern of activity is observed during DMSO-induced sporulation (Hartree, 1989). This suggests that the isgB gene product is required for maturation of the spores as the spores are morphologically fully formed 2 - 3 hours after the addition of glycerol but are not fully resistant to UV irradiation, sonic vibration or heat for several

hours. Expression from the *isgB*>*lacZ* fusion was examined in several glycerol-resistant mutants. In 24 different glycerol-resistant mutants tested there was no increase in ß-galactosidase activity after the addition of glycerol. This suggested that the expression of the *isgB* gene occurs late in the glycerol-induced sporulation pathway or that all the glycerol-resistant mutants are blocked very early in the pathway of glycerol-induced sporulation.

Attempts to examine the expression from the *isgB*>*lacZ* fusion during starvation-induced sporulation were unsuccessful. The strain DK306, a non-motile *mgl* strain, was used as a control for expression as a 15-fold increase in ß-galactosidase expression during starvation-induced fruiting-independent sporulation had been reported. The *isgB* gene was expressed late during starvation-induced, fruiting-independent sporulation, as ß-galactosidase expression was not observed until 72 hours after the onset of starvation (Hartree, 1989). However, repeated attempts to obtain spores from this strain in order to repeat the expression studies were unsuccessful. This phenomenon had also been reported by Hartree (1989) who only obtained spores from this strain on the first attempt. On all subsequent attempts poor sporulation was observed.

Although control experiments were unsuccessful, expression from the *isgB*>*lacZ* fusion during starvation of DK440 (*glrA1*, *bsg-440*) was of interest. A massive increase in ß-galactosidase activity was observed in the

vegetative cells (Figure 8.3). Some increase in ß-galactosidase activity was also observed in spores. However, one cannot rule out the possibility that this increase in expression was due to carry over from the vegetative cells. It is not clear whether the observed expression in vegetative cells was the result of the glrA1 mutation or the bsg-440 mutation. It would be of interest to analyse expression from the isgB>lacZ fusion in the following mutant backgrounds; (1) bsg-440, (2) glrA1 and (3) DK510 (glrA2, Fru^-). However, in order to obtain the glrA1 mutation in a fruiting background, and the bsg-440 mutation in a glycerol-sensitive background, the glrA1 and bsg-440 mutations would have to be independently transduced into the wild-type background. Expression from the isgB>lacZ fusion during starvation-induced sporulation should also be examined in other glycerol-resistant backgrounds.

A gene fusion contains only part of the transcript unit of interest, thus, it was desirable to clone the intact copy of the gene. Two methods were employed in attempt to clone the complete isgB gene: (1) to insert a cer region into the promoter fusion plasmid, pUWM5, and then use this plasmid to isolate the isgB gene using a plasmid rescue strategy (Figure 7.3); (2) to use bacteriophage lambda as a vector for a DNA library of M. xanthus chromosomal DNA, and then use the chromosomal insert in pUWM5 to screen for the appropriate region of DNA. The first strategy yielded one clone, designated pHMC20 which was 23.4kb in size and appeared to contain

sequences present in pUWM5 plus an additional 10.0 - 11.0kb of downstream DNA. The second strategy using bacteriophage lambda as a vector identified an 11.0kb EcoRI fragment which hybridized with the insert of pUWM5. This 11.0kb fragment was subcloned into the unique EcoRI site in the chloramphenicol resistance gene of pBR329. One such plasmid was selected and designated pHMC10.

Restriction mapping of pHMC10 and pHMC20, in comparison with pUWM5 and pHMC1, confirmed that pHMC20 contained the pUWM5 insert and 10.13kb of additional downstream DNA (Figure 7.6) which should, therefore, include the remainder of the transcript unit of interest. pHMC10 contained most of the pUWM5 insert and an additional 6.3kb of DNA from the upstream region (Figure 7.6).

The pHMC20 insert and two Sau3AI fragments derived from it were used to probe chromosomal DNA from the wild-type strain, DK1622. The observations were consistent with pHMC20 containing DNA from the isgB region. Gene replacement studies in which part of the isgB region DNA was replaced by a tetracycline resistance determinant derived from ColE1 Tn5-132 (Rothstein et al., 1981) revealed that the isgB gene was not essential for vegetative growth, glycerol-induced sporulation or fruiting body sporulation since disruption of the region had no effect on the phenotype of the host cells.

The promoter region of the isgB gene was analysed by subcloning two fragments from pHMC20 (Figure 7.10) into the promoter probe vector pDAH283 which contains a

promoterless *lacZ* gene. The resultant plasmids pHMC12 and pHMC14, and pUWM5 were transduced into the wild-type, DK101, and ß-galactosidase expression from the promoter region of each fragment was assessed during glycerol-induced sporulation (Figure 7.11) and vegetative growth. The results suggested that the *isgB* glycerol-inducible promoter, or at least the start site, is located within a 100bp *Eco*RI *Sau*3AI region. Further deletion studies and sequencing of this promoter region should allow the exact location of the promoter to be determined. However, these observations show that the gene(s) expressed from the promoter must have been disrupted by the tetracycline resistance determinant because the glycerol-induced promoter expression from pHMC14 was similar to that of pUWM5 and was observed at points downstream of the tetracycline resistance determinant insertion sites (See Figure 7.12). Sequence analysis of the *isgB* region may give some indication to the nature of the *isgB* gene product(s).

In summary, the *isgB* gene is expressed during glycerol-induced sporulation and starvation-induced, fruiting-independent sporulation but is not thought to be required for fruiting body sporulation (Hartree, 1989). The *isgB* gene is activated late in the pathway of glycerol-induced sporulation, as judged by the time of expression of the *isgB*>*lacZ* fusion in the wild-type background and the observation that the *isgB* gene is not expressed in 24 different glycerol-resistant mutants after the addition of glycerol to exponential phase

cultures. The *isgB* gene is also expressed late during starvation-induced, fruiting-independent sporulation of the non-motile strain DK306, as ß-galactosidase expression was not observed until 72 hours after the onset of starvation (Hartree, 1989). The *isgB* gene does not appear to be essential for vegetative growth, fruiting body sporulation or glycerol-induced sporulation since disruption of the gene by the insertion of a tetracycline resistance determinant does not alter vegetative growth, fruiting body sporulation or glycerol-induced sporulation of the host strain.

A second glycerol-inducible *lacZ* fusion, ΩDK4530, has been identified by random Tn*5* *lac* insertion (Kroos and Kaiser, 1987). ß-Galactosidase specific activity from the fusion increases during both fruiting body sporulation (Kroos *et al.*, 1986) and glycerol-induced sporulation (Kroos, 1986), but is not expressed at elevated levels in vegetative cells. Hence, the fusion represents a development specific promoter which is induced during fruiting body sporulation and glycerol-induced sporulation, reaching the same peak in specific activity. However, it should be noted that the peak in ß-galactosidase expression from this fusion is approximately ten-fold lower during glycerol-induced sporulation than is observed for the *isgB* fusion.

Expression from the promoter during glycerol-induced sporulation seems to be suppressed by amino acids since expression from the promoter was observed in cells grown in CTT broth (Section 2.3.3) but not cells grown in DCY

broth (Section 2.3.3) (Figure 9.1). Addition of yeast extract to CTT broth reduced expression from the ΩDK4530 fusion while addition of casitone to CTT broth abolished expression from the fusion (Figure 9.1). Presumably, expression from the ΩDK4530 fusion is reduced in CTT broth containing additional yeast extract because yeast extract contains some amino acids. Amino acids do not suppress expression from the isgB fusion which has only ever been assayed in cells grown in DCY broth.

The ΩDK4530 fusion showed increased activity immediately after the addition of glycerol to exponential phase cells and reached a peak five hours after the addition of glycerol. Similar ß-galactosidase activity was observed during DMSO-induced sporulation. However, although the fusion is expressed relatively early during glycerol-induced sporulation, the gene product is dependent on the glrA and glrB gene products, since the ΩDK4530 fusion was not expressed in either glrA or glrB mutant backgrounds. Expression of the ΩDK4530 fusion after the addition of glycerol to exponential phase cultures should be examined in several other glycerol-resistant mutants. It would also be of interest to determine whether a glycerol-resistant background affects the expression of the ΩDK4530 fusion during fruiting body sporulation.

Presumably, the insertion of the ΩDK4530 Tn*5* *lac* would have destroyed the gene. Hence, like the isgB loci, the region downstream of the glycerol-inducible promoter is not essential for glycerol-induced

sporulation or fruiting body sporulation.

The ΩDK4530 fusion shows reduced expression during fruiting body sporulation in a B signalling (bsgA) mutant (Kroos and Kaiser, 1987). It would be of interest to see if a bsgA$^-$ background would have any effect on expression of ΩDK4530 during glycerol-induced sporulation. The other signalling factors may also be required for correct expression of the ΩDK4530 fusion. It would, therefore, be of interest to look at expression from the ΩDK4530 fusion during fruiting body sporulation and glycerol-induced sporulation in asg, csg, dsg and esg mutant backgrounds. Expression from the ΩDK4530 fusion during starvation-induced, fruiting-independent sporulation of the non-motile strain DK306 should also be examined, as it is not known whether this fusion is also induced during fruiting-independent sporulation.

It would be of interest to see if the isgB and ΩDK4530 lacZ fusions are expressed in other documented methods of sporulation, such as sporulation induced by glucosamine (Mueller and Dworkin, 1991) or sporulation in liquid buffers such as MCM buffer (10mM MOPS [morpholinopropanesulfonic acid], 2mM $CaCl_2$, 4mM $MgSO_4$ [pH 7.2]) (Rosenbluh and Rosenberg, 1989b).

10.6 Future Prospects in Understanding the Role of Chemical-Induced Sporulation and its Interaction With Fruiting Body Sporulation

Although it is unclear where M. xanthus cells would come across 0.5M glycerol, 0.75M DMSO or other short

chain alcohols in the natural environment, chemical-induced sporulation appears not to be a laboratory artefact since both chemical-resistant mutants and chemical-inducible promoters have been identified.

Identifying genes involved in chemical-induced sporulation and analysing the nature and time of expression of the gene product will eventually allow one to begin to elucidate a pathway for chemical-induced sporulation. This pathway is likely to be complex and interact to some extent with starvation-induced, fruiting body sporulation and fruiting-independent sporulation. However, mutagenesis experiments (Chapter 3) suggest that the pathway of induction of glycerol-induced sporulation shares few, if any, common genes with the pathways of induction of fruiting body sporulation or starvation-induced, fruiting-independent sporulation. Further elucidation of the chemical-induced sporulation pathway should involve the isolation of chemical-inducible promoters using a plasmid-based promoter probe or Tn*5* *lac*. However, CTT media should be used for the ß-galactosidase assays to avoid the possibility of amino acid suppression, as observed for ΩDK4530. Two *glr* loci, *glrA* and *glrB* have been identified. Forty two glycerol-resistant mutants were identified which were not complemented by either *glrA*$^+$ or *glrB*$^+$ DNA. These mutants may represent another *glr* locus (or loci). Mx*8* transduction mapping of these glycerol-resistant mutations should be carried out. Subsequently DNA from the loci identified should be cloned.

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