Molecular characterisation of behavioural functions in Agrobacterium tumefaciens

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Molecular Characterisation of Behavioural Functions in *Agrobacterium tumefaciens*

A thesis submitted to the Department of Biological Sciences, University of Durham in accordance with the requirements for the degree of Doctor of Philosophy.

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Declaration

This thesis, submitted for the degree of Doctor of Philosophy, entitled "Molecular Characterisation of Behavioural Functions in *Agrobacterium tumefaciens*" is based upon experiments conducted by the author during the period between 1988 and 1991. All work recorded in this thesis is my own original work, except where due reference is made to co-workers and none has been submitted for a degree at this or any other university.

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Abbreviations

Amp	-	Ampicillin
Km	-	Kanamycin
Rif	-	Rifampicin
Tc	-	Tetracycline
bp	-	Base pair
kb	-	Kilobase pair
Mb	-	Megabase pair
kDa	-	Kilodalton
A_{260}	-	Absorbance at 260nm
A_{280}	-	Absorbance at 280nm
UV	-	Ultraviolet
LMP	-	Low melting point
SDS	-	Sodium dodecyl sulphate
DTT	-	Dithiothreitol
X-gal	-	5-bromo-4-chloro-3-indoyl-β-D-galactoside
leu	-	Leucine
met	-	Methionine
dATP	-	Deoxyadenosine triphosphate
dCTP	-	Deoxycytosine triphosphate
MCP	-	Methyl-accepting chemotaxis protein
GBP	-	Galactose binding protein
nod	-	Nodulation gene

Abstract

Tn5 insertion behavioural mutants of *A. tumefaciens* C58C^1 were available. Cloning of the kanamycin resistance gene allowed isolation of Tn5 flanking sequences from a number of the mutants. Flanking sequences from five mutants were used to isolate cosmids, overlapping the Tn5 insertion sites of the mutantsfrom a C58C^1 library. Two cosmids, pDUB1900 and 1905 have been characterised.

pDUB1900 contains the insertion sites of eight motility mutants, with another immediately adjacent. The pDUB1905 insert overlaps sequences interrupted in another three mutants. Behavioural genes in *Agrobacterium* are clustered together on the chromosome, as in other motile bacteria. Restriction maps of the isolated cosmids show that none of motility mutants analysed was the result of inactivation of *pscA* or *chvB* which would lead to an altered behavioural phenotype.

Flanking sequences from three of the mutants hybridised to *R. meliloti* chromosomal DNA, but not to DNA from other motile genera. DNA adjacent to the insertion site of *fla-11* hybridised to the insert of pRZ-2, a cosmid containing behavioural genes from *R. meliloti*.

Experiments were undertaken to investigate the occurrence of proteins homologous to the MCP's of enteric bacteria in C58C^1. DNA hybridisation to oligonucleotide probes showed DNA that could potentially code for MCP-like proteins exists in both *Agrobacterium and Rhizobium* spp. In addition, an antibody raised against the *E. coli* MCP Tar cross-reacted with a protein of approximately 60kDa. in C58C^1. *In vivo* protein methylation experiments using C58C^1 resulted in the labelling of a 45kDa protein, whose methylation pattern did not change upon addition of chemostimuli. Possible reasons for the difference in size between the labelled protein and that revealed by the antibody are discussed.

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CHAPTER 1 : INTRODUCTION

1.1. The genus *Agrobacterium*.

The genus *Agrobacterium* is a member of the family Rhizobiaceae and is closely related to the genus *Rhizobium* (Kersters & DeLey, 1982). Agrobacteria are part of the soil microflora and are especially found in association with plant roots. Morphologically, the agrobacteria are Gram-negative parallel-sided rods with rounded ends, measuring 0.6-1μm wide and 1.5-3.0μm long. They have 1-6 peritrichous flagella (see fig. 1.1) and are mesophilic, with 28°C being the optimum temperature for their growth. (Lippincott *et al.*, 1981).

An *Agrobacterium* species was first described as a common non-phytopathogenic soil inhabitant (Beijerinck & van Delden, 1902), but a more significant discovery was that *Bacterium tumefaciens* was the agent responsible for crown gall diseases of plants (Smith & Townsend, 1907). The establishment of the genus *Agrobacterium* followed the reclassification of the genus *Phytomonas*, which contained a great number of phytopathogenic bacteria (Conn, 1942).

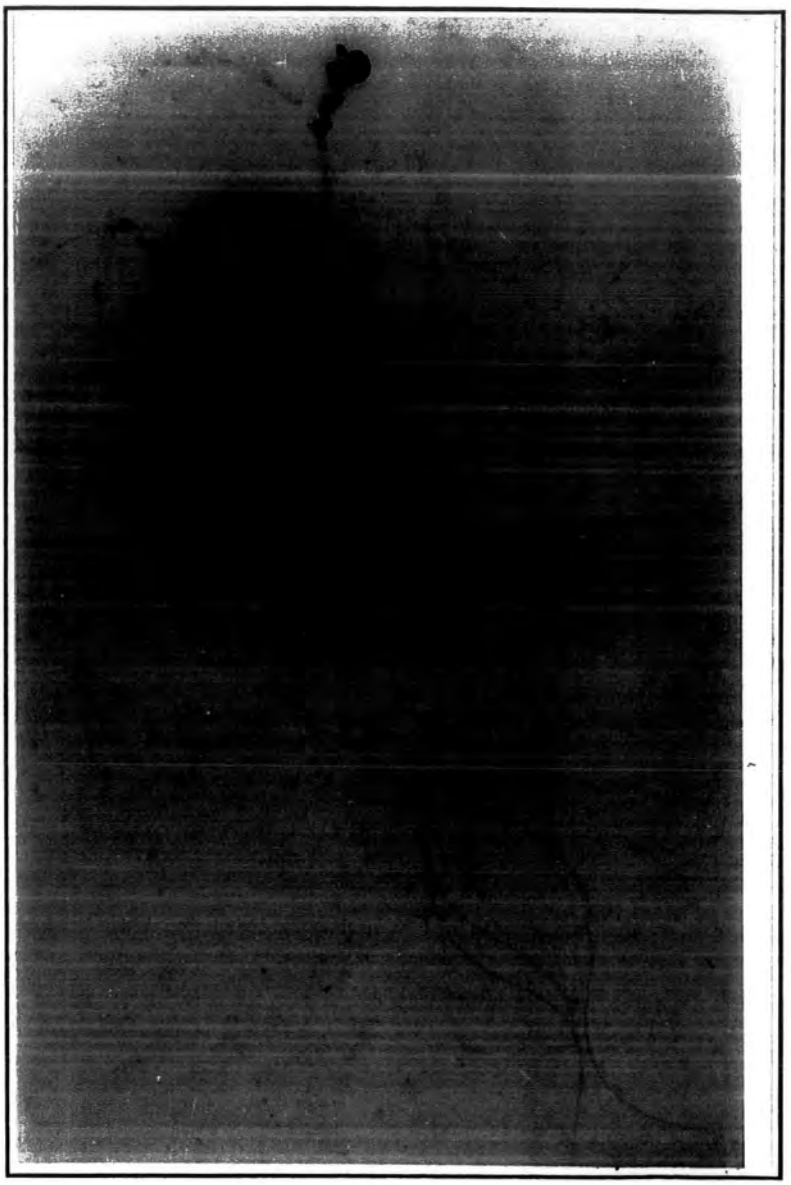
The genus traditionally consists of four species which are characterised by their pathogenicity. *Agrobacterium tumefaciens* is responsible for crown gall tumours on a wide range of dicotyledonous and a few monocotyledonous plants; *A. rhizogenes* causes hairy root disease, and *A. rubi* cane gall on susceptible plants. *A. radiobacter* is a non-phytopathogenic species (Nester & Kosuge,1981).

This system of classification has recently been called into question after the discovery that the pathogenicity of different species is determined by large tumour-inducing (Ti) or root inducing (Ri) plasmids (Zaenen *et al.*, 1974; Watson *et al.*, 1975; Moore *et al.*, 1979; White & Nester 1980). These large plasmids are transferable *in vivo* and *in vitro* (Genetello et al., 1977; Kerr *et al.*, 1977) and recipient cells acquire the pathogenic properties of the donor strain (Van Larebeke *et al.*, 1975; Thomashow *et al.*, 1980). Phytopathogenicity is not therefore a stable characteristic and is unsuitable for species identification.

An alternative method of classification uses a number of biochemical and physiological properties, together with DNA hybridisation studies to classify the agrobacteria (Kerr & Panagopoulos, 1977; Kersters & DeLey, 1982). This system divides the genus into two major and one minor biovars (biotypes), numbered 1, 2 and 3 respectively. The three biovars are genetically and phenotypically distinct and contain both pathogenic and non-pathogenic strains. The chromosome-encoded characteristics used for identification in this system are more valid criteria for classification than phytopathogenicity.

In this work the traditional nomenclature will be used and future sections will mainly discuss crown gall and Ti-plasmids, since all the strains used are derivatives of the biovar 1 strain

Figure 1.1 : Wild type *A. tumefaciens* cell, showing flagella. Cell body is approximately 1.2μm long. Photographed by Dr C.H. Shaw.



Agrobacterium tumefaciens C58 (Van Larebeke *et al.*, 1974) which contains the nopaline Ti plasmid pTiC58.

1.2. Soil ecology and plant transformation.

Agrobacteria occur almost worldwide and are a common component of the soil microflora, with up to 500 cells per gram of soil being reported. They are especially concentrated in the rhizosphere, with the number of bacteria around the roots being roughly one thousand times that found in nearby soil (New & Kerr 1972). Agrobacteria are responsible for crown gall and hairy root diseases, which are both neoplastic overgrowths of plant tissues. Crown gall often impairs and stunts the growth of infected plants and is responsible for significant losses to agriculture and horticulture in areas with temperate to mediterranean climates (De Cleene, 1979; Kerr, 1974; Kennedy & Alcorn, 1980). Infection is dependent on the presence of large Ti or Ri plasmids and in the soil non-pathogenic bacteria,without oncogenic plasmids, greatly outnumber pathogenic strains (Kerr, 1969; Schroth *et al.*, 1971).

Both crown gall and hairy root result from the transformation of plant cells into tumourous cells which undergo uncontrolled proliferation. They are capable of autonomous growth without the tumour-inciting bacteria (White & Braun, 1942) and do not require phytohormones for *in vitro* culture (Braun, 1958). Transformed cells are also characterised by the synthesis and secretion of unusual sugar and amino acid conjugates called opines, which are not present in normal plant cells (Tempe & Goldmann, 1982).

The type of tumour incited on a plant and the type of opine that is synthesized are both dependent on the oncogenic plasmid present in the infecting bacteria (Watson *et al.*, 1975; White & Nester, 1980; Petit *et al.*, 1970). This is because transformation of the plant cells involves the transfer of a small piece of DNA (the transfer or T-DNA) from the Ti or Ri plasmid into the plant genome (Chilton *et al.*, 1977; Chilton *et al.*, 1980; Willmitzer *et al.*, 1980). Integration of the T-DNA into the plant genome and its subsequent expression are responsible for the tumourous phenotype and opine secretion. These will be discussed in later sections together with an account of the Ti plasmid and its functions.

The opines secreted by a crown gall can be catabolized by the infecting *Agrobacterium* strain and used as the sole carbon, nitrogen and energy source. Opine catabolism is also a Ti-plasmid encoded trait. (Bomhoff *et al.*, 1976). Some opines induce the conjugal transfer of self-transmissible Ti-plasmids between *Agrobacterium* strains (Petit *et al.*, 1978; Ellis *et al.*, 1982), which enables non-virulent strains to become tumourigenic and to metabolize opines. *Agrobacterium tumefaciens* infection therefore not only provides an environmental niche within the crown gall, but may give surrounding agrobacteria a competitive advantage over other soil

micro-organisms which cannot utilise opines. The phrase 'genetic colonisation' has been used to describe this mode of parasitism (Schell *et al.*, 1979).

Investigations in the field however have shown that the selective advantage provided by opines to agrobacteria may not be as great as first thought. This is because most *Agrobacterium* species isolated from the soil lack a Ti-plasmid and more importantly, other micro-organisms are capable of metabolizing opines (Rossignol & Dion, 1985; Tremblay *et al.*, 1987; Beauchamp *et al.*, 1990). Opine production probably stimulates growth of the inducing *Agrobacterium* species within a crown gall, but does not play a major role in soil colonization and survival in the rhizosphere.

1.3. The *Agrobacterium* : plant interaction.

Once *Agrobacterium tumefaciens* has become established in the rhizosphere and localized at a plant wound site (events for which chemotaxis is important - see later sections) the plant infection process consists of four main events. They are attachment of the bacteria to the susceptible plant cells; Ti-plasmid virulence (*vir*) gene induction; T-DNA transfer into the plant cell; integration/expression of the T-DNA in the plant chromosomal DNA. Wounding of the plant is a pre-requisite for tumour development (Binns & Thomashow, 1988).

The *Agrobacterium* genes responsible for T-DNA transfer can be divided into two groups. The virulence (*vir*) genes on the Ti-plasmid process and transfer the T-DNA, whilst chromosomal virulence (*chv*) genes are involved with plant cell attachment and *vir* gene induction.

1.3.1 Attachment of the bacteria to plant cells.

The attachment of agrobacteria to the surface of wounded plant cells is an essential stage in tumour development (Lippincott & Lippincott, 1969; Lippincott *et al.*, 1977). It is a two stage process which can be observed with both light and scanning electron microscopy (Matthysse *et al.*, 1981; Pueppke & Hawes, 1985). The first stage is a loose reversible attachment which is poorly understood. No plant cell receptor has yet been isolated although its existence is supported by a number of pieces of work - see Binns & Thomashow 1988 for a discussion of the evidence. The bacterial cell wall component that is involved in binding was thought to be lipopolysaccharide (Whatley *et al.*, 1976) but the characterisation of mutants defective in attachment indicate that carbohydrates have a much more important role to play - see next section.

Following initial attachment, there is tight irreversible binding which results in massive aggregation of bacteria at the cell surface. This is brought about by the synthesis of cellulose

fibrils which anchor the bacteria to the plant and entrap additional bacteria at the wound site (Matthysse *et al.*, 1981). The cellulose fibrils appear to play an important role in firmly anchoring the bacteria and preventing them from being washed off in natural conditions, as cellulose deficient mutants can still attach to plant cells singly and are virulent in sensitive assays. (Matthysse, 1983).

1.3.2 Chromosomal virulence loci.

These genes are characterised by mutants that are avirulent or have severely attenuated virulence accompanied by host range restrictions. So far nine loci have been described, with five studied in some detail. Mutations in *chvD, chvE,* and *Ivr* -211, -223, -225 produce strains that are unable to induce the Ti-plasmid *vir* genes properly, resulting in attenuated virulence (Winans *et al.*, 1988; Huang *et al.*, 1990a; Metts *et al.*, 1991). The function of ChvD and ChvE will be discussed in section. 1.6.3. The other four chromosomal loci *chvA*, *chvB*, *pscA* and *att* are neccessary for the attachment step and mutations in these genes cause avirulence.

chvA and *chvB* (Douglas *et al.*, 1985) map to an 11kb region of the chromosome and define two transcriptional units of 1.5 and 5.0kb respectively. *chvB* defective strains are unable to synthsize a cyclic β-1,2-glucan, that is found both extracellularly and in the periplasmic space of wild-type cells. (Puvanesarajah *et al.*, 1985). *ChvB* encodes a 235kDa inner membrane bound protein that synthesizes β-1,2-glucans from UDP-glucose monomers (Zorreguieta *et al.*, 1988). *ChvA* mutants synthesize the β-1,2-glucans but they are retained in the cytoplasm rather than being exported to the periplasm and extracellular matrix (Cangelosi *et al.*, 1989). In addition, modification of the glucans by anionic substitution that occurs in wild-type cells (Miller *et al.*, 1987) fails to take place (Inon de Iannino & Ugalde, 1989). The *chvA* gene codes for a 75kDa protein that has high homology to bacterial and eukaryotic transport proteins. It is thought that ChvA forms a complex with ChvB in the inner membrane and participates in the transport of glucans into the periplasm, where modifying reactions occur (Cangelosi *et al.*, 1989; Inon de Iannino & Ugalde, 1989).

PscA mutants (Thomashow *et al.*, 1987) cannot synthesize the major acidic extracellular polysaccharide of *A. tumefaciens* - succinoglycan, or β-1,2-glucans. Lack of succinoglycan is probably not the cause of the avirulent phenotype because several mutants that also cannot make it are fully virulent (Cangelosi *et al.*, 1987). The *pscA* locus codes for the enzyme phosphoglucomutase which is required for the production of UDP-glucose, a substrate for β-1,2-glucan and succinoglycan synthesis (Uttaro *et al.*, 1990).

Cyclic β-1,2-glucans are only found in *Agrobacterium* and *Rhizobium* species (Sutherland, 1985) and there are homologous and functionally interchangeable counterparts of the *chvA*, *chvB* and *pscA* genes in *Rhizobium meliloti*. The corresponding chromosomal genes are *ndvA*, *ndvB*

and *exoC* respectively and they are required for effective nodulation of alfalfa (Dylan *et al.*, 1986; Marks 1987).

Mutations in two of the above loci are pleiotropic. In addition to the phenotypes given above, *pscA* mutants cannot produce cellulose fibrils and are non-motile, whilst *chvB* mutant strains lack flagella and as a result are non-motile and resistant to certain bacteriophages. Absence of flagella in these strains is not responsible for impaired attachment, as other non-flagellated mutants are still able to bind plant cells and are virulent (Bradley *et al.*, 1984). The mechanism by which defects in β-1,2-glucan metabolism affect motility and virulence is unknown, but the loss of osmo-adaptation that occurs is not responsible for the pleiotropic effects (Cangelosi *et al.*, 1990).

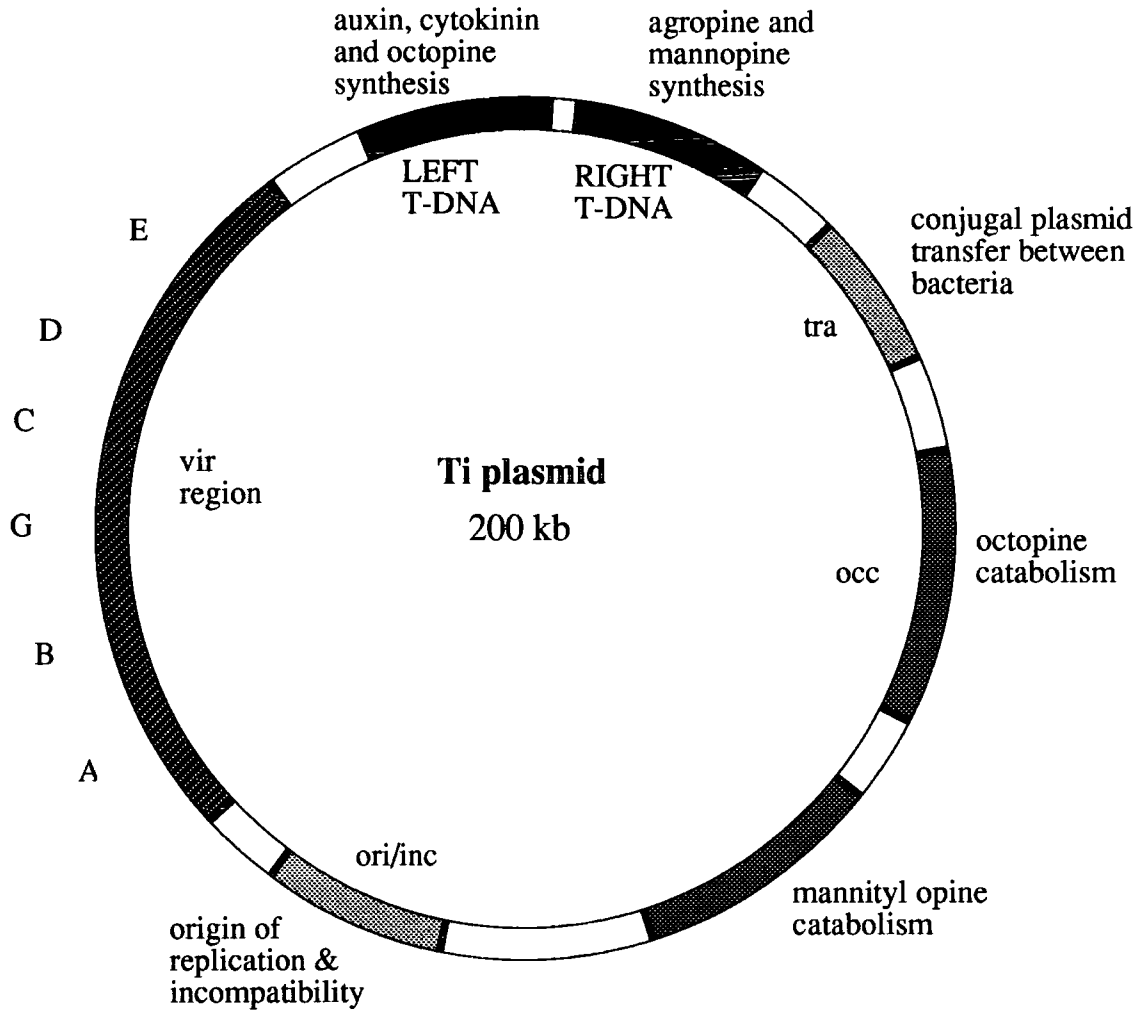
The fourth class of avirulent attachment mutants, termed *att* are all like wild-type strains with regard to motility, flagella, lipopolysaccharide composition and β-1,2-glucan content (Matthysse 1987). They do, however, lack one or two small proteins which presumably play a role in bacterial attachment, possibly in conjunction with β-1,2- glucans.

Chromosomal genes required for phytopathogenicity appear to be functionally conserved in the family Rhizobiaceae. This is demonstrated by the transfer of Ti- or Sym-plasmids into cured bacteria belonging to the 'wrong' genus. *R. leguminosarum* strains are rendered tumourigenic with a Ti-plasmid (Hooykaas *et al.*, 1977) and nodulating *Agrobacterium* strains result from the introduction of Sym-plasmids (Hooykaas *et al.*, 1981; Hirsch *et al.*, 1984). Agrobacteria containing Sym-plasmids need functional *chvA* and *chvB* genes for nodule formation (Van Veen *et al.*, 1987). A *Phyllobacterium* species can also be made to incite crown gall or root nodules with a Ti- or Ri-plasmid (Van Veen *et al.*, 1988). These results suggest that there is a general chromosomally-encoded plant-attachment step among the Rhizobiaceae that occurs before more specific interactions take place.

1.4. The Ti-plasmid.

Apart from the chromosomal virulence genes, all the genetic elements required for crown gall formation are found on Ti-plasmids - see figure 1.2. These are large plasmids, approximately 200kb in size, which have a low copy number (1-3) in the cell. The regions of the Ti-plasmid which are required for pathogenicity are the T-DNA, its flanking border sequences and the virulence (*vir*) genes. The T-DNA becomes integrated in the plant genome and its expression leads to opine production and neoplastic transformation. *Vir* genes are responsible for T-DNA transfer, a process that requires intact border sequences.

Figure 1.2 : A genetic map of an octopine type Ti-plasmid. Adapted from Ream (1989).



Ti-plasmids are divided into four groups which are characterised by the types of opine produced by the tumours they incite. Octopine Ti-plasmids eg. pTiA6 and pTiB6 cause the synthsis of octopine, mannopine and agropine, whilst nopaline and agrocinopine A and B are present when a nopaline plasmid, such as pTiC58 causes transformation. Plasmids which specify neither octopine or nopaline were originally termed null type, but they have subsequently been divided into leucinopine or succinamopine types (Chang *et al.*, 1983; Chilton *et al.*, 1984). Plasmids from the different classes have different restriction patterns, but within each group maps may be similar or even identical. Incompatibility studies have have shown that that the octopine, nopaline and leucinopine Ti-plasmids belong to one group, *inc Rh-1* and the succinamopine plasmids belong to another, *inc Rh-2* (Melchers & Hooykaas, 1987).

DNA required for tumour formation and replication makes up approximately 50% of the Ti-plasmid and a number of other functions have been mapped to the rest of the plasmid. Genes neccessary for the metabolism of several different opines are present, which encode specific uptake systems together with catabolic enzymes (Valdivia *et al.*, 1991). The plasmids also contain genes responsible for opine-stimulated conjugal transfer and, in the case of nopaline and succinamopine plasmids, sensitivity to the bacteriocin agrocin 84 (Murphy & Roberts, 1979). Nopaline plasmids, such as pTiC58, also have a T-DNA-independent locus, *tzs* which encodes an enzyme that produces the phytohormone cytokinin in the bacteria under *vir* - inducing conditions (John & Amasino, 1988).

In addition to Ti or Ri-plasmids, agrobacteria possess cryptic 'Megaplasmids' to which no function has been assigned. (Van Montagu & Schell, 1979). Curing *A.tumefaciens* C58 of the 410kb cryptic plasmid pAtC58 has no effect on the virulence of the bacterium (Rosenberg & Huguet, 1984).

1.5. T-DNA structure and transfer.

T-DNA is the portion of the Ti-plasmid that is found integrated in the nuclear DNA of transformed plant cells. In nopaline plasmids there is one 22kb continuous T-DNA region, which contains within it the nopaline synthase (*nos*) and agrocinopine synthase (*acs*) loci. Octopine plasmids contain two T-DNA regions separated by a central fragment. The 13kb left T-DNA (TL) encodes the genes needed for plant transformation and octopine synthesis, whilst agropine and mannopine synthesis enzymes are produced from the 7.8kb right T-DNA (TR).

T-DNA from both types of plasmid have a highly conserved set of genes which are responsible for phytohormone production and the tumourous phenotype in transformed plant cells (Depicker *et al.*, 1978; Willmitzer *et al.*, 1983). The unorganized and uncontrolled growth of

plant cells is caused by the overproduction of auxin and cytokinin. The genes *iaaM* and *iaaH* code for enzymes that establish a new auxin biosynthetic pathway (Schroeder *et al.*, 1984; Van Onckelen *et al.*, 1986) and the activity of the third oncogene *iptZ* leads to cytokinin production (Buchmann *et al.*, 1985).

The nopaline T-DNA and the TR and TL regions of octopine plasmids are flanked by 25bp direct repeat sequences, called the border repeats (Holsters *et al.*, 1983; Yadav *et al.*, 1982). Any DNA placed within the border repeats is transferred to the plant. Only the right border repeat is essential for T-DNA transfer (Shaw *et al.*, 1984) and it specifes the direction of DNA transfer from the *Agrobacterium* to the plant cell (Wang *et al.*, 1984; Peralta & Ream,1985). In nopaline Ti-plasmids, sequences surrounding the left repeat attenuate polar DNA transfer and those surrounding the right enhance it (Jen & Chilton, 1986; Wang *et al.*, 1987). A 24bp T-DNA transfer enhancer, called overdrive, lies to the right of the right border repeats in octopine Ti-plasmids and is essential for efficient DNA transfer to plant cells (Peralta et *al.*, 1986).

Transfer of T-DNA into the plant cell is brought about by the *vir* genes of the Ti-plasmid and the mechanism is thought to be analagous to bacterial conjugation, at least in the early stages (Howard & Citovsky, 1990; Stachel & Zambryski,1986a). In support of this model, it has been found that the origin of transfer (*oriT*) of the conjugative plasmid pRSF1010 can substitute for T-DNA borders and direct DNA transfer to plant cells (Buchanan-Wollaston *et al.*, 1987). Recent work has shown that the 25bp T-DNA borders contain sequences identical to *nic* regions of *incP* plasmids, which are required for the production of single-stranded DNA conjugation intermediates (Waters *et al.*, 1991). It appears that the Vir proteins, discussed below, function like the products of the *tra* genes of conjugative plasmids and transfer a single stranded T-DNA intermediate into the plant cell. For a comprehensive review of T-DNA structure and function see Zambryski (1988) or Ream (1989).

1.6. The *vir* region.

The virulence region is 35kb in length and lies to the left of the T-DNA on the Ti-plasmid. It encodes over 20 proteins which are required for T-DNA transfer. There is extensive DNA homology in the *vir* loci of different Ti-plasmids and experiments have shown that *vir* genes are interchangeable between the different groups of Ti-plasmids (Hooykaas *et al.*, 1984). Transposon mutagenesis and complementation studies indicate that nopaline and octopine plasmids have 6 and 7 complementaton groups respectively (Rogowsky *et al.*, 1987; Stachel & Nester, 1986).

Nopaline plasmids contain the *virA*, *virB*, *virC*, *virD*, *virE* and *virG* loci and there is an extra group, *virF* in octopine Ti-plasmids (Melchers *et al.*, 1990). Only *virA* and *virG* are monocistronic, with the other complementation groups consisting of operons which code for

several proteins. The organisation of the common six loci in octopine and nopaline plasmids is essentially identical. *virA*, *virB*, *virD* and *virG* are absolutely required for tumour formation, whilst *virC*, *virE* and *virF* are the 'host range loci' that are required for normal tumour development on certain plants only.

Another plant inducible locus, *pinF*, is situated next to *virA* on pTiA6 and is regulated in the same manner as the *vir* genes. Mutants in this region also have attenuated virulence on some plants and it has been suggested that the locus should be called *virH* (Kanemato *et al.*, 1989).

1.6.1. Functions of the *vir* genes.

Five of the virulence loci, *virB*, *virC*, *virD*, *virE* and *virF* encode proteins that are directly involved in tumour formation and they are positively regulated by the action of *virA* and *virG*. These latter two genes are constitutively expressed in minimal medium and they activate transcription of the other *vir* genes and themselves if plant-derived inducing factors are present. (Stachel *et al.*, 1986; Stachel & Zambryski, 1986b). The functions of the regulated genes are as follows.

VirB is the largest locus, being 9.5kb long and containing eleven open reading frames (Kuldau *et al.*, 1990). Nine of these encode proteins that may interact with membranes and it is thought that the *virB* gene products form a membrane-associated transport system and channel that allows T-DNA to move from *Agrobacterium* into the plant cell (Ward *et al.*, 1991). In support of this idea, both VirB10 and VirB11 have been localized to the inner membrane of *Agrobacterium* and the VirB11 amino acid sequence has similarity to a DNA uptake protein in *Bacillus subtilis* (Ward *et al.*, 1990; Christie *et al.*, 1989).

The *virC* complementation group codes for two proteins VirC1 and VirC2 which are involved in host range specificity, since the extent of attenuation of virulence caused by mutations in *virC* depends on the type of plant (Close *et al.*, 1987). The 26kDa protein VirC1 has been shown to enhance T-DNA processing by binding to the overdrive sequence on the Ti-plasmid (Toro *et al.*, 1988; 1989).

The DNA that enters a plant cell on *Agrobacterium* infection is part of a protein/DNA structure called the T-DNA transfer complex. This consists of T-strands (linear single stranded DNA that corresponds to the bottom strand of the T-DNA region) which are coated with a DNA binding protein and have a pilot protein bound at the 5' end. (Howard & Citovsky, 1990). The *virD* and *virE* gene products are required for T-DNA complex formation and some aspects of its transfer.

VirD proteins are involved with T-strand synthesis and are required for transfer and targetting of DNA in the plant. VirD1 and VirD2 participate directly in the formation of T-strands

and have a number of functions. The two proteins catalyze site-specific and strand specific nicking of the T-DNA at the border sequences and VirD2 becomes tightly bound at the 5' end of the nicked T-strand (Howard *et al.*, 1989). This protects the DNA from exonucleolytic degradation (Durrenberger *et al.*, 1989) and probably prevents closure of the nick by ligase. VirD1 is also a topoiosomerase, converting supercoiled DNA into its relaxed form (Ghai & Das, 1989), although the significance of this in T-strand formation is unclear. Once inside the plant cell the bound VirD2 is thought to act as a pilot protein and target the T-DNA to the plant nucleus (Herrera-Estrella *et al.*, 1990). The VirD4 protein, which has homology to the TraG protein of RP4 plasmids, has been localized in the cell membrane of *Agrobacterium* (Okamoto *et al.*,1991) and probably has a role to play in the transfer of the T-complex.

The *virE* locus codes for the second protein component of the T-complex, VirE2. This is a 69kDa non-specific single-stranded DNA binding protein that binds in a co-operative fashion to the T-DNA (Christie *et al.*, 1988; Citovsky et al., 1989). Vir E1 , a 7kDa protein, is also required for full virulence and probably acts to stabilize the VirE2 protein (McBride & Knauf, 1988). Coating of the T-strands is thought to protect the DNA from bacterial and plant cellular nucleases and VirE2 may also interact with the membrane pores created in plant induced *Agrobacterium* cells (Citovsky *et al.*, 1989).

The *virF* locus is poorly characterised as yet, but it has been implicated in the production of opines shortly after the onset of transformation (Melchers *et al.*, 1990). Mutations in both of the 'host range loci' *virE* and *virF* can be complemented by mixed infections with helper *Agrobacterium tumefaciens* strains (Otten *et al.*, 1985) which suggests that both VirE and VirF proteins are exported and function extracellularly during tumour induction.

1.6.2. Regulation of *vir* gene expression.

The virulence genes required for T-DNA processing and transfer are expressed only in the presence of wounded plant cells that are susceptible to infection . This is because of recognition of plant signal molecules and co-ordinate transcriptional induction mediated by the *virA*, *virG* and *chvE* gene products. VirA and VirG are members of a family of two component regulatory systems that respond to a number of environmental stimuli and use phosphorylation for intracellular signalling (Ronson *et al.*, 1987). VirA is the sensor protein of the *vir* induction system and VirG is the regulator that activates transcription of the *vir* genes.

VirA is a transmembrane protein that signals the presence of *vir* inducing molecules to the inside of the cell. It belongs to a family of histidine protein kinases (Stock *et al.*, 1989) and can autophosphorylate a histidine residue in its C-terminal domain (Huang *et al.*, 1990b; Jin *et al.*, 1990b). Phosphorylated VirA brings about *vir* gene induction by transferring the phosphate from its phosphohistidine to an aspartate residue in the N-terminal domain of VirG (Jin *et al.*, 1990a).

VirG is a sequence-specific DNA binding protein that recognizes a 12bp cis-acting sequence called the *vir* box which is found upstream of all genes regulated by VirA and VirG (Pazour & Das, 1990 a & b; Steck *et al.*, 1988). VirG binds to *vir* boxes without being phosphorylated, an interaction that involves the C-terminal domain, but N-terminal phosphorylation is thought to increase binding to the *vir* boxes and activate DNA transcription from the virulence loci (Roitsch *et al.*, 1990).

The *virC* and *virD* loci of the Ti-plasmid are also negatively regulated by a specific repressor, encoded by the chromosomal *ros* gene. This is an autoregulated protein that has pleiotropic effects; *ros* mutants constitutively express VirC and VirD proteins, form colonies with rough surface, are cold-sensitive and do not produce the major extracellular polysaccharide succinylglycan (Close et al., 1987). *virC* and *virD* are still transcriptionally activated by inducing compounds however and virulence is not affected. Analysis of the cloned *ros* gene shows that it is a DNA binding protein that acts as a repressor by binding at the *virC* and *virD* promoters at sites that overlap the *vir* boxes (Cooley *et al.*, 1991).

1.6.3. Vir gene induction

Three classes of environmental stimuli act synergistically to raise the level of phosphorylated VirG and induce expression of the *vir* genes (Winans 1991). Monocyclic aromatic hydrocarbons such as acetosyringone were the first *vir* inducers to be described (Stachel *et al.*, 1985) and a large number of related compounds have subsequently been shown to have inducing activity. These include the naturally occurring lignin precursors sinapyl alcohol and coniferyl alcohol (Spencer & Towers, 1988). The presence of opines potentiates the induction of virulence genes by acetosyringone (Veluthambi *et al.*, 1989).

Specific monosaccharides enhance the induction of the *vir* genes by phenolic compounds and may even be active by themselves (Cangelosi *et al.*, 1990a; Shimoda *et al.*, 1990). This process is mediated by a distinct regulatory pathway that involves *virA* and the *chvE* gene products. ChvE is a periplasmic galactose binding protein (GBP) that is homologous to sugar binding proteins found in *E. coli* (Huang *et al.*, 1990a). ChvE-sugar conjugates probably interact with the periplasmic region of VirA to stimulate *vir* induction in a manner analogous to the interaction of *E. coli* GBP with Trg, a transmembrane signalling protein involved in chemotaxis. Of the eleven monosaccharides that have synergistic *vir* inducing activities, eight are known plant metabolites and seven are monomers of plant cell wall polysaccharides (Ankenbauer and Nester, 1990).

An acidic pH is neccessary for maximal *vir* induction by inducing compounds and causes increased expression of *virG*, a process that requires a functional ChvD protein (Winans *et al.*, 1988). Acidic conditions probably activate the *virG* promoter by inducing the heat shock response

of the bacterium (Winans, 1990), although this does not wholely explain the acid pH optimum for vir induction as a VirG overproducing strain still needs acidic growth media for induction (Chen & Winans, 1991). Phosphate starvation also induces VirG, a process that possibly involves recognition of the *vir G* promoter by the regulator protein of the *Agrobacterium* phosphate regulon, the homolog of *E. coli* PhoB (Winans, 1990).

The positive regulation of *vir* genes is a complex process which requires the interaction of several signalling pathways. It is significant that the environmental stimuli that are required are exactly those found at a plant wound site. Wound sites are acidic and wounding of the plant results in the release of free sugars and oligosaccharides by mechanical means and the activity of cell wall glycosidases. The biochemical pathways that are activated by the oligosaccharides, together with cell wall repair and lignin biosynthesis, result in the release of the phenolic inducers of *vir* genes. The availability of *vir* inducers is one of the main reasons that wounding of plant cells is a pre-requisite for *Agrobacterium* infection. The importance of chemotaxis towards *vir* inducing molecules and the involvement of VirA, VirG and ChvE in that process will be discussed in section 1.8.

1.7. Motility and chemotaxis of rhizosphere bacteria.

Chemotaxis involves the directed movement of motile cells in response to a gradient of attractant or repellent (Adler, 1973). It has been most extensively studied in the enteric bacteria, with *E. coli* providing the model system for the process. Chemotaxis has also been observed in several plant pathogenic and soil-borne bacteria, including *Agrobacterium tumefaciens* (Ashby *et al.*, 1988), *Azospirillum* spp. (Heinrich & Hess, 1985; Zhulin *et al.*, 1988), *Erwinia amylovora* (Raymundo & Ries, 1980), *Pseudomonas* spp (Chet *et al.*, 1973; Scher *et al.*, 1985; Cuppels, 1988) and *Rhizobium meliloti* (Ames *et al.*, 1980). Measurements of the movement of bacteria have shown that *P. putida* can move 1cm in 12 hours towards a soybean seed (Scher *et al.*, 1985) and *Azospirillum brasilense* can travel up to 160cm under field conditions, depending on the plant source (Bashan & Levanony, 1987). All of the above strains are attracted to plant roots, plant exudates, or components of plant exudates in laboratory experiments, but studies on the biological significance of chemotaxis in the soil are limited.

The importance of chemotaxis in the colonisation of plant roots and movement of bacteria in the soil is unclear, with contrasting results from different experiments. Motility and chemotaxis have been shown to be neccessary for efficient spreading of *Rhizobium meliloti* in soil (Soby & Bergman, 1983) and de Weger *et al.*, (1987) found that flagella were required for potato root colonisation by *P. fluorescens*. Alternatively, Howie *et al.*, (1987) showed that non-motile *P. fluorescens* colonized wheat roots as well as wild-type bacteria and Scher *et al.*, (1988)

demonstrated no correlation between motility and root colonization with *Pseudomonas* and *Serratia* spp. The latter study inoculated bacteria directly onto the seeds, which may have removed the need for chemotaxis by the bacteria. Differences in results between these experiments could be the result of differences in soil type, methods of inoculation and estimation of bacterial numbers in the rhizosphere.

Movement of soil bacteria by water may have an equally important role as chemotaxis to play in bacterial colonisation of roots, with cells being carried long distances by percolating water or fluid fronts (Liddell & Parke, 1989; Soby & Bergman, 1983). However, given soil conditions suitable for motility, it seems likely that chemotaxis towards plant exudates is involved in the root colonization process and organisms that could move towards a source of nutrients would presumably have a selective advantage over those that were non-motile.

The importance of chemotaxis in the *Agrobacterium* and *Rhizobium* infection processes has been studied in some detail and it does seem to be important under field conditions (see next section for a discussion of *Agrobacterium* chemotaxis and its importance). In the case of *Rhizobium* spp. it appears that non-chemotactic mutants can nodulate host roots but their nodulation efficiency and competitiveness in comparison with chemotactic strains are reduced (Ames & Bergman, 1981; Caetano-Anolles *et al.*, 1988b).

Rhizobia are attracted to various substances in plant root exudates (Currier & Strobel, 1976; Gaworzewska & Carlile, 1982) and become localized around specific regions of plant roots (Gulash *et al.*, 1984). Attractants are secreted mainly by cells in the zone of elongation and rhizobia therefore move towards the root cells which are most susceptible to infection (Bhuvaneswari *et al.*, 1981). As well as chemotaxis towards some amino acids and sugars, rhizobia are attracted by both aromatic acids and *nod* gene inducing flavones such as luteolin and naringenin (Caetano-Anolles *et al.*, 1988a; Aguilar *et al.*, 1988). Chemoattraction by flavenoid compounds seems to depend on their ability to act as *nod* inducers and requires the presence of functional *nod* genes (Armitage *et al.*, 1988; Caetano-Anolles *et al.*, 1988a). It is thought that motile rhizobia present in the rhizosphere detect luteolin coming from a host root at low concentrations (between 10^{-10} and 10^{-9}M) and move towards the host. When luteolin concentrations reach 10^{-7}M, chemotaxis diminishes and induction of the *nod* genes rapidly takes place (Peters *et al.*, 1986). A similar scenario is envisaged for Ti-plasmid containing *Agrobacterium*, with *vir* inducing compounds acting as chemoattractants at low concentrations and then inducing the genes required for infection of the plant at higher concentrations (Ashby *et al.*, 1987; Shaw *et al.*, 1988a).

The chemotactic interactions with the host plants of both *Agrobacterium* and *Rhizobium* appear to involve two signalling pathways, which can be separated by mutation. An *Agrobacterium* Tn5 insertion mutant has been isolated that is attracted to excised root tips

(wounded cells) but not to isolated root cap cells (Hawes *et al.*, 1988). Similarly *Rhizobium* mutants that respond to root extracts and accumulate at specific sites on roots, but fail to respond to sugars and amino acids have been described (Bergman *et al.*, 1988). The best way to interpret this data is to suggest that a general chemotaxis pathway, which responds to nutrients such as amino acids and sugars, is responsible for maintaining the bacteria in sufficiently large numbers in the rhizosphere for infection to take place. A plasmid encoded pathway, which feeds into the general signalling pathway at some point, is then responsible for movement to susceptible plant cells, whose presence is signalled by specific *vir* or *nod* inducing compounds.

1.8. The importance of chemotaxis in *Agrobacterium*.

During the last decade, significant advances have been made in the understanding of molecular events that lead to T-DNA transfer and crown gall tumour formation. It is only recently however that the importance of chemotaxis during infection has been realised, and a detailed study of *Agrobacterium* chemotaxis undertaken (Ashby *et al.*, 1988; Loake *et al.*, 1988). This has led to the proposal of a mechanism by which *A. tumefaciens* recognises host plants and becomes concentrated around wound sites in sufficiently high numbers to initiate infection. (Shaw *et al.*, 1988b).

A. tumefaciens C58C^1, which is cured of a Ti-plasmid, has a highly sensitive chemotaxis system that responds to a number of sugars which are abundant in plant extracts (Loake *et al.*, 1988). Sucrose, the most common translocated plant sugar produces the most intense and sensitive response with an optimum concentration of 10^{-6}M. Glucose and fructose are detected with equal sensitivity, but the magnitude of the response is smaller. Generally, oligosaccharides are better attractants than their component monosaccharides. The chromosomal virulence gene *chvE*, a galactose binding protein, is required for chemotaxis towards D-galactose, D-glucose, L-arabinose, D-fucose and D-xylose (Cangelosi *et al.*, 1990a). Like homologous proteins from *E. coli*, ChvE is presumably a periplasmic receptor protein that binds sugars and interacts with a transmembrane signalling protein to initiate the chemotactic response. VirA, which binds to ChvE during *vir* induction, is not the signalling molecule involved in chemotaxis, as the cured strain C58C^1 is still responsive to the above sugars (Loake *et al.*, 1988). Other carbohydrate receptors for attractants such as maltose have yet to be identified.

Amino acids are relatively poor attractants for *A.tumefaciens*, with valine, arginine and glutamic acid being the only three so far observed to give a response. This contrasts with *Rhizobium* strains, which show sensitive attraction to a number of amino acids, and some response to all of them. (Götz et al., 1982).

A significant amount of fixed carbon is released into the rhizosphere in plant exudates, and one effect of this is to alter the microbial populations in the vicinity of the root (Hawes, 1990). Accumulation of agrobacteria around roots was recorded a long time ago (Schroth *et al.*, 1971) and it now seems likely that the sensitve chemotactic responses (optimum concentrations are generally 10^3 times lower than those for *E. coli*) evoked by plant sugars are responsible for maintaining agrobacteria in the rhizosphere.

A. tumefaciens also exhibits chemotaxis towards phenolic compounds that are capable of inducing the *vir* genes (Ashby *et al.*, 1988; Parke *et al.*, 1987), although there is controversy about the specific action of these as chemoattractants in the rhizosphere. Ashby *et al.* (1987) demonstrated that acetosyringone, a potent *vir* inducer, elicits a slight, but very sensitive response from *A.tumefaciens* C58C^1 containing a Ti-plasmid. The concentration required for chemotaxis - 10^{-7}M, is 100-fold lower than that giving maximal *vir* gene induction (Stachel *et al.*, 1985). Further work demonstrated a correlation between Ti-plasmid-dependent chemotaxis towards related phenolic compounds and their ability to induce *vir* genes (Ashby *et al.*, 1988). This suggests that Ti-plasmid-containing strains are attracted from the rhizosphere and localized at a wound site by attraction to phenolic compounds, which then induce the virulence genes and initiate the infection process.

virA and *virG* were subsequently shown to be the Ti-plasmid loci that are required for chemotaxis towards acetosyringone (Shaw *et al.*, 1988a) which suggests a multifunctional role for VirA and VirG. The low constitutive level of expression of these two proteins is appparently sufficient for them to be able to initiate movement towards acetosyringone, presumably by interaction with the chromosomally-encoded chemotaxis system. The homology between VirA and CheA (Stock *et al.*, 1989), a protein kinase in the chemotactic signal transduction pathway of *E. coli* , together with similarities between VirG and the *E. coli* chemotaxis proteins CheB and CheY suggests an involvement in *Agrobacterium* chemotaxis, although their exact function during chemotaxis has yet to be elucidated.

Contrasting results were obtained from another group, who reported that acetosyringone is not an attractant for *A. tumefaciens* A348 and chemotaxis towards other *vir* inducers was a chromosomally-encoded phenomenon (Parke *et al.*, 1987). These results may differ from those obtained by Ashby *et al.* (1988) because the strain of *Agrobacterium* used in the experiments - A136 is a less vigorously motile strain than C58C^1 (Loake *et al.*, 1988).

Despite these results and speculation about the exact role of attraction to phenolic compounds, there seems little doubt that chemotaxis is an important part of the infection process in the soil. Positive chemotaxis towards root and shoot extracts of both monocotyledonous and dicotyledonous plants has been shown (Ashby *et al.*, 1988) and Hawes & Smith, (1989) have

demonstrated a requirement for chemotaxis in the pathogenicity of *A. tumefaciens* on pea roots in the soil. Mutants deficient in chemotaxis to root exudates were avirulent on soil grown plants but fully virulent on plants grown in sand showing that chemotaxis is neccessary in some environments only. A mutant deficient in chemotaxis to root exudates, but attracted by wounded cells was also found to be avirulent in soil, suggesting that movement towards wound products is of secondary importance to attraction by naturally released carbohydrates (Hawes & Smith 1989). Attraction of agrobacteria by wounded cells is only likely to be important on a micrometre scale (Shaw *et al.*, 1989) and chemotaxis is probably required for pathogenicity in order to maintain high numbers of bacteria in the rhizosphere.

1.9. Motility and chemotaxis in *Agrobacterium tumefaciens.*

A. tumefaciens is a motile bacterium and exhibits swarming on semi-solid agar plates (swarm plates) - see figure 1.3. Chemotactic bacterial strains inoculated onto the centre of such a plate begin to metabolize nutrient compounds that are present and set up concentration gradients of attractants in the process. Motile bacteria are attracted up such gradients and form large circles as they move through the soft agar towards the edge of the plate. Separate rings are often seen, due to attraction towards different sources of carbon as nutrients are depleted.

Motile *A. tumefaciens* can readily be isolated by the use of such swarm plates, which provides a rapid method for the enrichment of motile populations. After passage through two or three swarm plates, cells are picked from the edge of the swarm for use in chemotaxis assays or for microscopic examination.

Under microscopic observation C58C^1 proves to be an active swimmer, whilst other genotypes such as LBA4301 and A136 are relatively poorly motile (Loake *et al.*, 1988). Subsequent investigations into *Agrobacterium* chemotaxis (Loake, 1989) concentrated on C58C^1 for this reason and almost all the bacteria used in this work are derivatives of this strain.

Motility of C58C^1 was characterised by long straight or curved runs, rarely interrupted by the sudden changes of direction and tumbling motions that are observed in *E. coli* - see section 1.11. Average speeds of 60μms^{-1} were recorded on run lengths of 200μm, whilst longer runs of up to 500μm produced mean speeds of 50μms^{-1}. The direction of rotation of tethered cells, attached by their flagella to glass coverslips or slides, suggest that the flagella rotate unidirectionally in a clockwise direction. Overall, the behaviour of *A. tumefaciens* is distinct from *E. coli* and strongly resembles that of *Rhizobium meliloti* (Götz & Schmitt, 1987).

A. tumefaciens possesses an unusual flagellation pattern, with a polar tuft of 2 flagella together with 2-4 lateral flagella, see figure 1.1. Flagellar filaments generally show a typical

Figure 1.3 : Swarming colonies of wild type *A. tumefaciens*. The cells were grown in L-B media swarm plates, containing 0.2% agar.

sinusoidal curvature in electron micrographs. Preliminary results suggest that *A. tumefaciens* flagella have a helical structure and may be of the complex type found on *Rhizobium meliloti* (Krupski *et al.*, 1985).

Studies on possible chemoattractants that are abundant in plant extracts demonstrate that the chromosomally encoded chemotaxis system in C58C1 is responsible for attraction by a few amino acids and a number of saccharides of varying sizes (Loake *et al.*, 1988). The different carbohydrates evoke responses with optimum concentrations that range from 10^{-6} to 10^{-4}M and there is a trend for the least sensitive responses to have the lowest magnitude. The order of response magnitudes is sucrose >> maltose > lactulose > glucose > galactose/fructose > stachyose/arabinose/raffinose. Xylose, cellobiose and lactose are sugars that produce no detectable chemotactic response. Attraction by oligosaccharides may be due to breakdown in the periplasm and synergistic effects of the component saccharides. In support of this idea is the fact that 10^{-6}M fructose and glucose together elicit a response of almost the same magnitude as that with 10^{-6}M sucrose, whereas separately their responses are much smaller (Loake, 1989). It has been proposed that failure to metabolize the carbohydrates in the periplasm or the impermeability of the outer membrane is responsible for some compounds being non-attractants.

The high sensitvity of the chemotactic response to sugars is not apparent with amino acids that are attractants, as the concentration needed for a response, 10^{-3}M, is higher than that required for any sugar and compares to values obtained from *E. coli* .

1.10. Analysis of chemotaxis genes in *A. tumefaciens.*

Analysis of motility and chemotaxis in a number of bacteria has been strongly influenced by a model for the process that has developed for the enteric bacteria. The general use of *E. coli* as a model bacterial system and the extremely powerful genetic techniques available for use on it mean that it is the only bacterium for which the complex events underlying chemotaxis have been worked out - see sections 1.11 - 1.13. The unavailabilty of a generalized transducing phage and relatively low transformation efficiencies obtained with *A. tumefaciens* meant that the genetic dissection of chemotaxis (Loake, 1989) has been done in a different way from the methods used in *E. coli* .

Bacteria with mutations in the chemotaxis pathway can be obtained by isolation of spontaneous mutants or by mutagenesis with chemicals or transposons. Enrichment for spontaneous mutants can be achieved by continually picking bacteria from the centre of a swarm plate and re-inoculation on further plates. After several rounds of swarming, analysis of the motility of single colonies allows chemotaxis mutants to be identified. This was the method used

to isolate behavioural mutants of *R. meliloti* (Ames *et al.*, 1980). Chemical mutagenesis of *A. tumefaciens* with EMS, followed by screening for wild-type motility was carried out to assess the variety and ease of isolation of chemotaxis mutants (Loake, 1989). Six behavioural mutants were isolated on the basis of swarm morphology but due to potential problems with isolation of complementing DNA they were not studied further.

In order to facilitate easier subsequent isolation of DNA that codes for chemotaxis genes, transposon mutagenesis with Tn5 was carried out followed by selection for non chemotactic mutants. Tn5 is a 5.8kb transposon encoding kanamycin resistance that has a number of properties that make useful for genetic analysis (Simon *et al.*, 1989). It moves with high frequency in a wide range of Gram-negative bacteria, generally shows little target specificity, exhibits a low probability of genome rearrangements during insertion and has high stability once established. DNA flanking the inserted Tn5 and corresponding to mutated genes is easily isolated using standard cloning procedures.

1.10.1. Construction of Tn5 insertion chemotaxis mutants

A number of plasmids have been constructed which can be used to deliver the insertion mutagen Tn5 (Beringer *et al.*, 1978; Simon *et al.*, 1983). Investigation of the mutagenic efficiency of several of these 'suicide vehicles' (Loake, 1989) demonstrated that pJB4JI gave the highest transfer frequency and this was the vector used in mutant construction. 1830/pJB4JI was mated directly with rifampicin resistant *A.tumefaciens* C58C^1 and transconjugants (occurring at a frequency of 10^{-6}) were screened for behavioural mutation by plating in L-swarm agar containing Rif and Km using the mini-swarm assay method (Parkinson, 1976). Colonies with mutation in motility and chemotaxis genes could be isolated because of a visible phenotype of altered swarm morphology.

1.10.2. Classes of behavioural mutants.

Chemotaxis mutants of *E. coli* can be divided into groups depending on their motility and the compounds that are required for a chemotactic response (Parkinson, 1981). *fla* mutants lack flagella and are hence non-motile, forming tight dense colonies on swarm plates. *mot* mutants have morphologically normal flagella but they fail to rotate due to defects in the flagellar motor or energy transduction mechanism and the bacteria are non-motile. *mot* mutants again produce small dense colonies on swarm plates and microscopic examination is required to distinguish them from *fla* mutants. Non-chemotactic (*che*) mutants are fully motile but form smaller swarms than wild-type bacteria because they fail to respond to any or a specific number of attractants. Generally non-chemotactic mutants do not respond normally to any compounds because the central intracellular signalling pathway is defective. Specifically non-chemotactic mutants do not respond

to either a limited subset of stimuli (transducer defective) or a single stimulus (primary receptor mutation).

22 *A. tumefaciens* behavoural mutant candidates were originally isolated, at a frequency of $1.8x10^{-2}$ per Km resistant transconjugant and further characterised (Shaw *et al.*, 1991). All three classes of behavioural mutants are found in *A. tumefaciens* - see figure 1.4. Two of the mutants were discarded because of slow growth in liquid culture and the remaining mutants analysed by flagella staining, electron microscopy.and motility studies with a phase contrast microsope.

Fourteen of the behavioural mutants are non-motile, with seven *mot* mutants and seven *fla* mutants which have no visible flagella (see table 1.1). One of the remaining mutations produces paired cells which are joined extracellularly at the poles and four are normally attracted to L-broth in chemotaxis assays, despite intermediate swarm size. The nature of the lesion in these small swarm mutants is unknown. Only one mutant, designated *che2*, appears to be a genuine non-chemotactic mutant as it tumbles continuously under microscopic examination. This is likely to be a generally non-chemotactic mutant as the swarm plates used during its isolation contained a large number of attractants.

1.10.3. Biology of the behavioural mutants.

Virulence of the 20 mutants on sunflower seedlings, after introduction of the Ti-plasmid pGV2201, was unaffected by the Tn5 insertion at the behavioural loci (Shaw *et al.*, 1991). The virulence assay did, however, circumvent the need for chemotaxis and motility in the infection process, as the bacteria were inoculated directly onto the seedlings. The root colonising ability of one of the mutants was assessed using the method of De Weger (1987) with potato seedlings. *mot-1* appears to be incapable of colonizing newly grown roots (Loake, 1989), which supports the idea of Hawes and Smith (1989) that chemotaxis is required for sufficient rhizosphere colonisation in the soil.

1.10.4. Strategies for cloning *A.tumefaciens* behavioural genes.

In order to isolate DNA that codes for chemotaxis and motility in *A. tumefaciens*, a representative genomic library of $C58C^1$ genomic DNA was constructed. (Loake, 1989). BamH1 partially digested $C58C^1$ DNA was size fractionated on a sucrose gradient and 23kb fragments ligated into the cosmid vector pLAFR-3 (Staskowicz *et al.*, 1987) using protocols contained in that paper. The ligation mixture was used to transfect *E. coli* HB101 after *in vitro* packaging and 1200 recombinant clones (white colonies on Xgal/tetracycline LA plates) selected. These were grown in separate wells of microtitre dishes before addition of glycerol and storage at -80°C. A culture containing a mixture of all the isolates was also made and stored. Using this library, two

Figure 1.4 Behavioural mutants of *A. tumefaciens* in a 0.2% agar swarm plate. Non-motile strains on left, wild-type in middle and intermediate swarmers on the right

Table1.1 : Classes of behavioural mutants.

CLASS	SUBCLASS	FEATURES	MUTANT	GENE LOCUS
Non-motile	possess flagella	normal flagella*	5	*mot5*
			14	*mot14*
		abnormal flagella*	1	*mot1*
			4	*mot4*
			6	*mot6*
			9	*mot9*
		no polar tuft	12	*mot12*
	no visible flagella		3	*fla3*
			7	*fla7*
			8	*fla8*
			10	*fla10*
			11	*fla11*
			15	*fla15*
			19	*fla19*
Motile	non-chemotactic	tumbles incessantly	2	*che2*
	tandem paired cells	poor motility	17	*tpc-17*
	small swarms		18	*ssw18*
			20	*ssw20*
			21	*ssw21*
			22	*ssw22*

*as judged from electron micrographs

approaches can be used to isolate DNA that complements the Tn5 insertion mutants.

Direct complementation of the mutants after introduction of cosmid DNA from the combined library is a possibility, with screening of transformed cells on Rif/Km/Tc swarm plates. Transcription start and stop signals would have to be in the right place on the vector and insert for this to work, and large numbers of separate transformed lines screened.

The second method utilises the 'molecular tagging' of each behavioral gene by Tn5 that results from the insertion inactivation nature of transposon mutagenesis. The drug resistance encoded by Tn5 can serve as a positive selectable marker for the recovery of flanking DNA sequences that correspond to the mutated gene. Figure 1.5 outlines the procedure for isolating Tn5 flanking sequences. These can be used as probes for screening the genomic library to recover cosmids to use in complementation tests.

Using the latter method a cosmid clone, pDUB1900 has been isolated that complements seven of the mutants : *mot 1*, *mot 4*, *mot 9*, *mot 14*, *fla 3*, *fla 11* and *fla 15*. DNA flanking the Tn5 in *fla 3*, which was used to isolate pDUB1900 is also homologous to cloned *R. meliloti* behavioural genes (Loake, 1989).

Chemotaxis in *E. coli* - a model system.

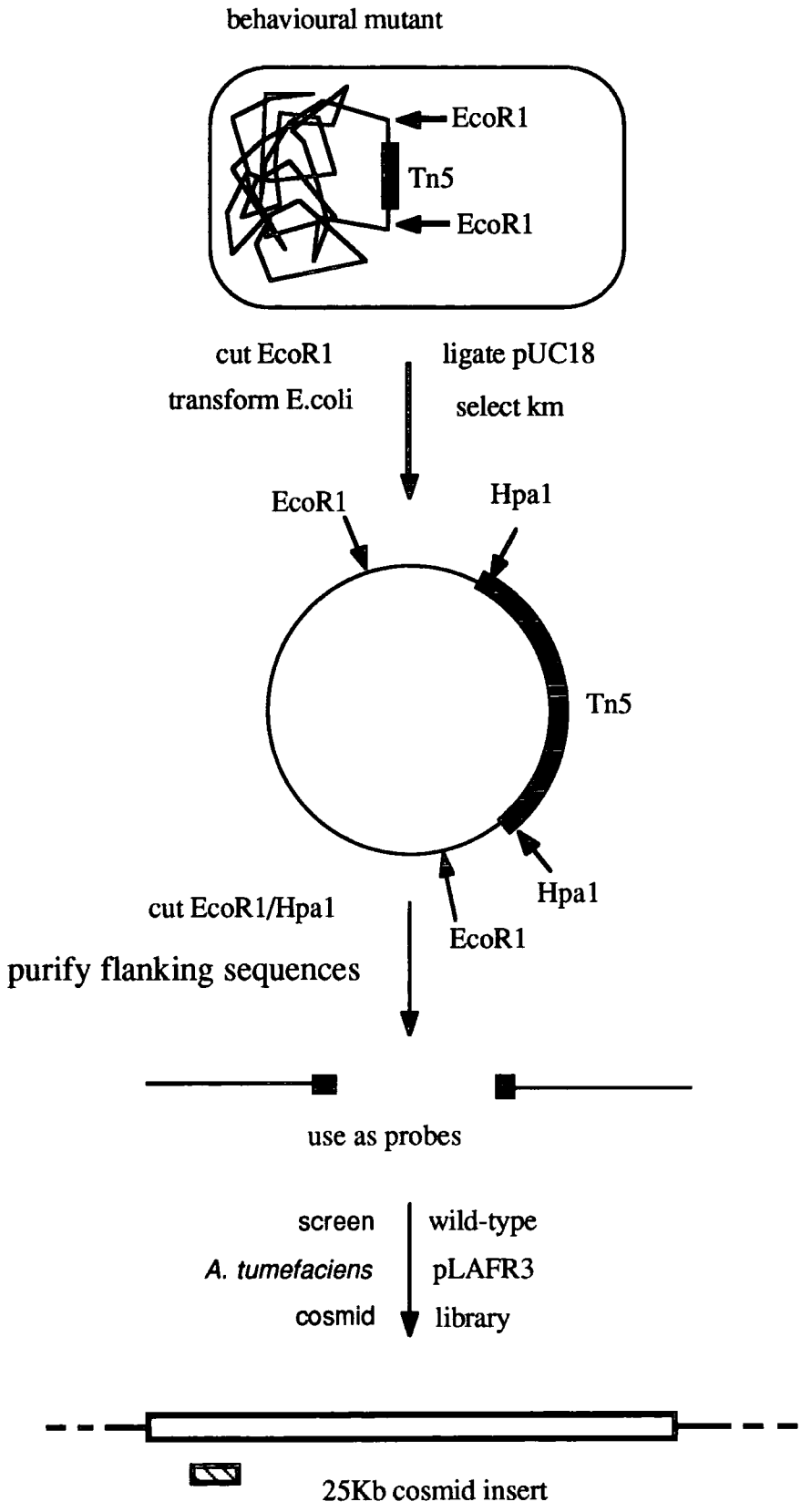
1.11. Motility and behaviour of *E. coli*.

E. coli possesses a number of peritrichous flagella and is a motile bacterium, capable of movement in a liquid medium. The flagella are helical filaments that rotate to propel the cell forward (Silverman & Simon, 1974a), with the transmembrane proton potential being used as the energy source for rotation (Larsen *et al.*, 1974a; Manson *et al.*, 1977). Recently one of the proteins of the flagellar motor, MotA, has been shown to be a protein that allows protons to cross membranes (Blair & Berg, 1990).

The flagellar motor can move both ways and it continuously switches back and forth between clockwise (CW) and counterclockwise (CCW) rotation (Silverman & Simon, 1974a). This continuous switching accounts for the behaviour of the bacterium in a uniform medium and changes in the probability of either CW or CCW rotation are responsible for chemotaxis (Larsen *et al.*, 1974b).

In the absence of any gradients of attractants or repellents *E. coli* cells move in a series of smooth, quite straight runs lasting 1-2 seconds which are followed by a tumble lasting 0.1-0.2

Figure 1.5 : Isolation of Tn5 flanking sequences.



seconds which re-orientates the cell randomly (Berg & Brown, 1972). Runs are caused by CCW rotation of the flagella, which individually form a left handed structure before aggregating together in a large bundle to drive the bacterium forward. CW rotation of a flagellum results in a right handed helical conformation being adopted and on switching, a wave of conformational change spreads out from the cell. CW rotation of some or all of the flagella causes the flagellar bundle to fall apart and individual flagella can be seen in transition between left and right helices. The tumble that occurs on CW rotation is a reaction to a number of rotating flagella that are in transition from one form to another (Macnab & Ornston, 1977). Recently the motor has also been shown to pause (Lapidus *et al.*, 1988). Pausing is thought to be a result of incomplete switching events and may be necessary to prevent all of the flagella filaments acquiring the less stable right hand helical conformation and being able to aggregate into a bundle that can propel the bacterium (Eisenbach, 1990; Eisenbach *et al.*, 1990).

The continuous switching and pausing of the flagella motor results in a three dimensional random walk in the absence of chemical stimuli. In a gradient of attractants or repellents, suppression of tumbling (ie. CW rotation of flagella) if the cell is moving in a favourable direction results in a biased random walk that produces net progress (Berg & Tedesco, 1975).

The most effective attractants for *E. coli* are amino acids, sugars, carboxylic acids and oligopeptides (Adler, 1975), with serine and and aspartate evoking the strongest response. Repellents include organic molecules such as phenol, benzoate, indole and ethylene glycol and inorganic ions such as Co^{2+}, Ni^{2+} and S^{2-}.

E. coli do not spatially sense chemical concentrations along their length but instead use a temporal sensing mechanism which includes a simple memory (Macnab & Koshland, 1972; Segall *et al.*, 1986). Chemotaxis therefore involves the temporal sensing of chemical stimuli and the ability to use the information gained to control the probability of clockwise rotation of flagella. The way this is achieved has been most thoroughly investigated in methylation dependent chemotaxis mediated by the methyl accepting chemotaxis proteins (MCP's) - see section 1.13. For more detailed reviews of chemotaxis in *E. coli* see Stewart & Dahlquist, 1987; Macnab, 1987b; Hazelbauer, 1988; Stock *et al.*, 1989 and Stock *et al.*, 1990.

1.12. Genes involved in chemotaxis.

Chemotaxis and motility in *E. coli* requires over 60 genes, the majority of which are highly clustered on the chromosome - see figure 1.6. Some 40 genes, designated *fli*, *flg* and *flh* (Iino *et al.*, 1988), are involved in the synthesis of the flagellar filaments and their associated membrane structures (Macnab, 1990). The *che* genes code for proteins in the central intracellular signalling pathway of chemotaxis and *mot* gene products are part of the motor complex of the flagella. *tar*

and *tap* are MCP genes in region II and the other two MCP genes, together with sequences encoding primary receptor proteins are isolated around the chromosome. The four major regions of genes are divided up into at least 14 operons which code for between 1 and 9 proteins. Expression of the operons is controlled by secondary sigma factors for RNA polymerase (Arnosti *et al.*,1989; Helmann & Chamberlin, 1987) and takes place in an ordered manner, with proteins from some operons being required for transcription of others. Activation of the first operon *flhD* is controlled by cAMP levels, via the CAP regulatory protein (Silverman & Simon, 1974b). For reviews of flagellar gene regulation and details of flagellum structure and assembly see Macnab,1987a and Macnab, 1990.

1.13. Mechanism of chemotaxis.

Increasing concentrations of attractants and decreasing concentrations of repellents suppress clockwise rotation of the flagella and hence tumbling frequencies. There are at least three systems for chemoreception that must link together in the central signalling pathway of chemotaxis.

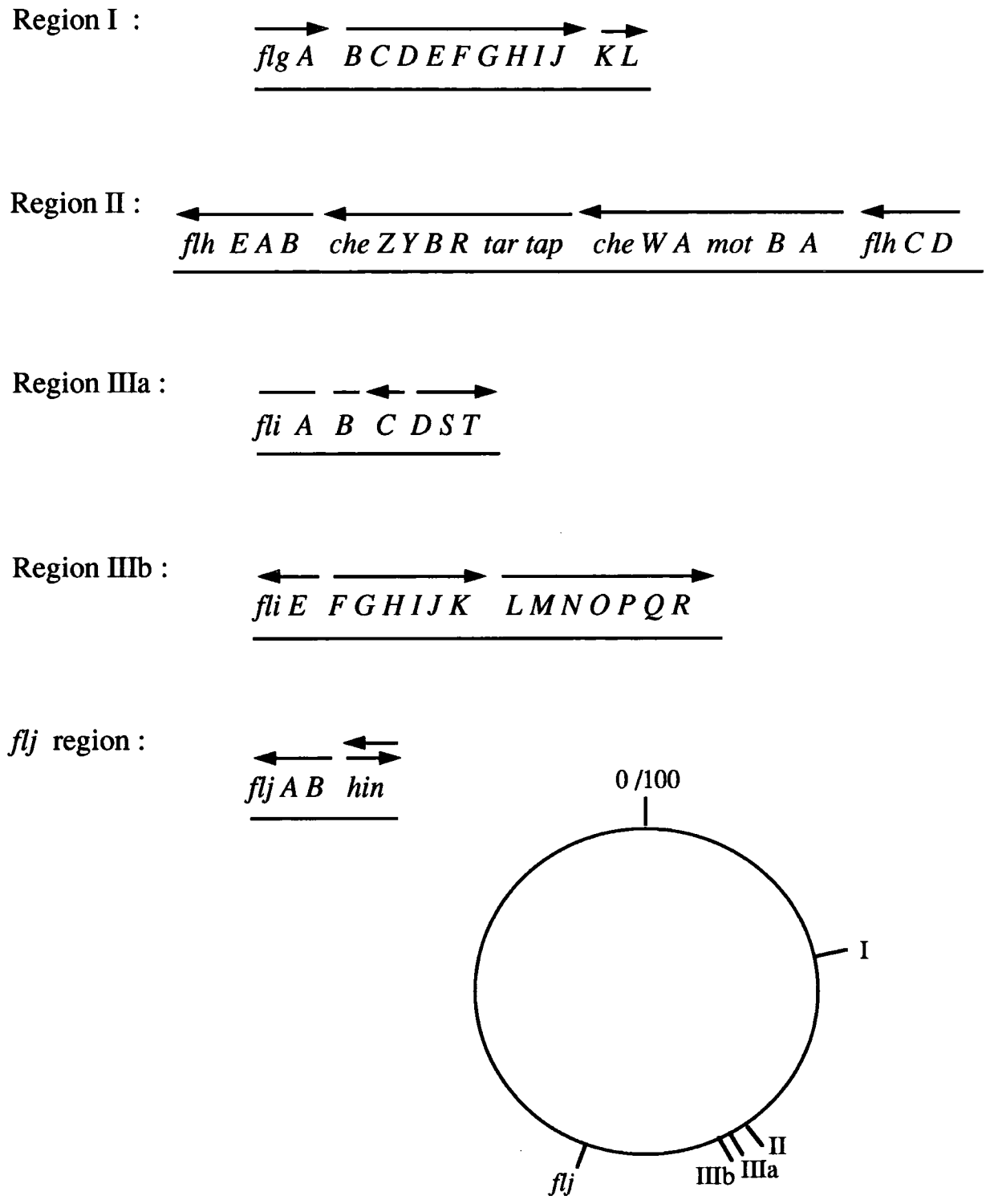
Oxygen acts as a chemoattractant by increasing the flow of electrons through the respiratory chain and increasing the proton transmembrane potential. The putative 'proton-meter' protein, that interacts with the components of other chemotaxis systems has yet to be identified. (Shioi *et al.*, 1988).

Carbohydrates transported by the phosphoenolpyruvate dependent phosphotransferase system - PTS (for review see Postma & Lengeler, 1985) act as attractants, but the response is not mediated by MCP's and is methylation independent. Uptake and phosphorylation, but not metabolism of the carbohydrate is neccessary for chemotaxis, and the mechanism by which the histidine protein (HPr) of the PTS affects flagellar rotation is currently being investigated (Taylor & Lengeler, 1990; Grubl *et al.*, 1990). Responses to PTS attractants are much weaker than many of the methylation dependent responses to amino acids, ions, some repellents, and sugars such as ribose, galactose and maltose. The chemotactic responses evoked by these compounds utilise two sorts of covalent modification of proteins - methylation and phosphorylation to produce co-ordinated net movement in response to a number of chemical stimuli.

1.13.1. Methyl accepting chemotaxis proteins (MCP's).

The chemotactic response evoked by an attractant consists of a rapid excitation phase, during which motor direction is reversed, followed by an adaptive phase. This phase allows the bacteria to resume a random walk even in the presence of the stimulus. MCP's are involved in initiation of the excitatory phase via the cytoplasmic signalling pathway (see section 1.13.3) and are responsible for adaptation, which is brought about by their methylation (see section 1.13.2).

Figure 1.6 : Chemotaxis genes in *E. coli* and their position on the chromosome.



(Left ends of maps point anticlockwise on chromosomal map.
Arrows show direction of transcription of operons or genes)

MCP's are integral membrane proteins, each approximately 60kDa, that function as transducing proteins signalling the presence of an attractant or repellent from the outside to the inside of the cell. The sequences of the four MCP's in *E. coli* Tar, Tap, Tsr and Trg (Boyd *et al.*, 1983; Krikos *et al.*, 1983; Bollinger *et al.*, 1984) show that the proteins are closely related and have the same structural domains, summarised in figure 1.7. Two hydrophobic transmembrane sequences located near the N-terminus surround a hydrophilic ligand-binding domain that extends into the periplasm. A large hydrophilic C-terminal domain is found in the cytoplasm and is responsible for intracellular signalling and adaptation after methylation. MCP's exist as dimers in the bacterial membrane (Milligan & Koshland, 1988).

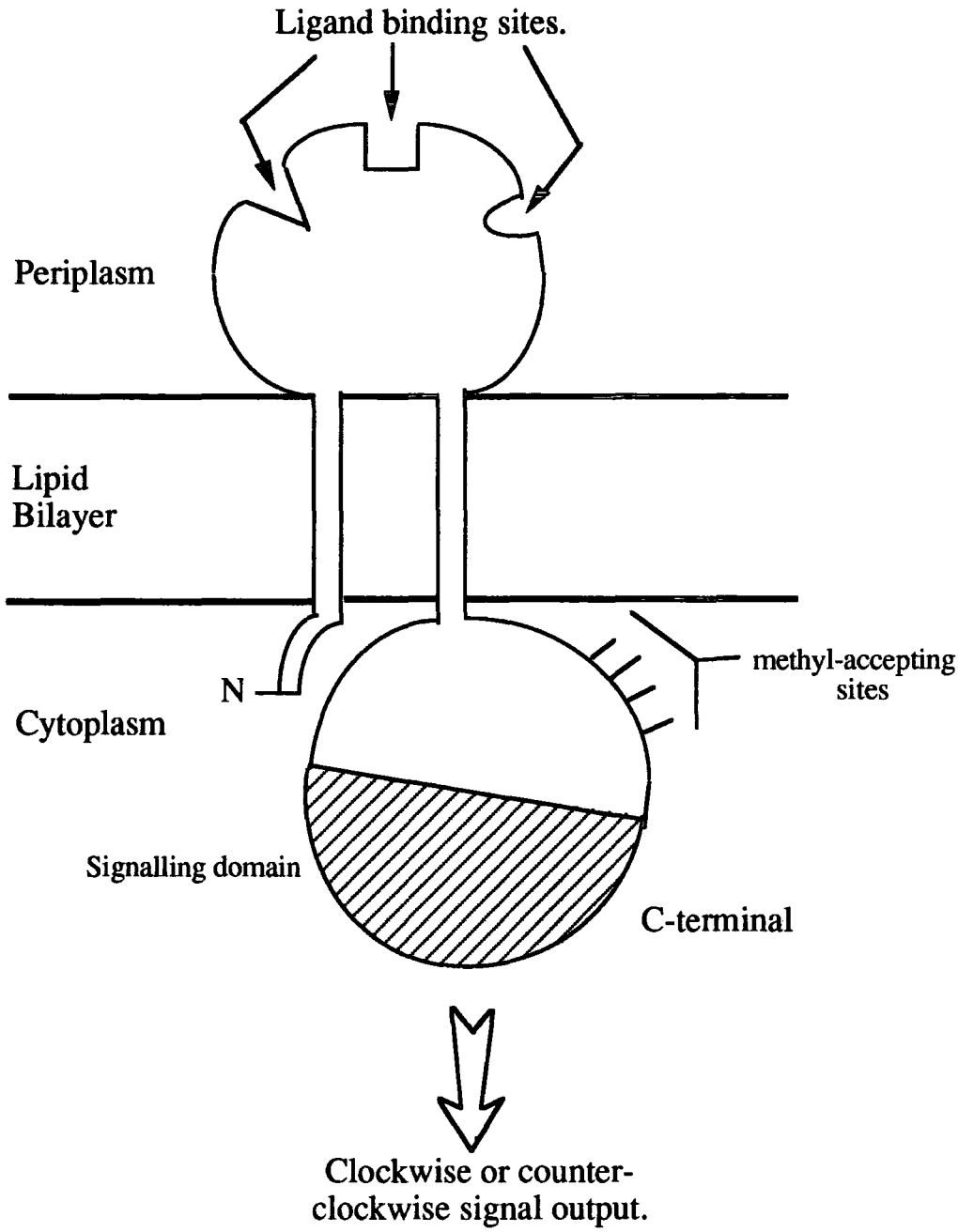
MCP's bind some compounds directly to initiate a response and three of them also interact with periplasmic binding proteins that are the primary receptors for some compounds. (Hazelbauer *et al.*, 1990). Tar binds aspartate directly and mediates maltose taxis by interaction with the maltose binding protein MalE. Trg is responsible for for taxis to galactose and ribose after they have been bound by their respective periplasmic binding proteins and Tsr is required for movement towards serine. Tap mediates the response to dipeptides, which involves interaction with a dipeptide binding protein that has been recently characterised (Abouhamad *et al.*, 1991). Each of the transducers is also responsible for movement away from one or more repellents (Yamamoto *et al.*, 1990). Binding sites for attractants, repellents and periplasmic binding proteins are distinct and a single protein sends an additive signal if more than one compound is bound. Thus, maltose is able to suppress tumbling even if all the aspartate binding sites on Tar are saturated (Mowbray & Koshland, 1987) and the repellent response to phenol, mediated by Trg is abolished with addition of ribose (Yamamoto *et al.*, 1990).

In the unstimulated state, the ligand-binding sites are unoccupied and the methylation sites are continuously methylated and demethylated to give a steady state level of one methyl group per protein (Hazelbauer *et al.*, 1990). On binding of an attractant, the excitatory signal is a conformational change that decreases interaction between the MCP cytoplasmic domain and the intracellular signalling system and suppresses tumbling. Signal transduction and changes in the demethylation rate result in increased levels of methylation of the MCP's, which cancels out the excitatory signal and allows the protein to cause tumbling again, which restores the random walk of the cell. Repellents reverse the conformational changes caused by attractants and bias the flagellar motors in a clockwise direction.

1.13.2. Adaptation.

Reversible methylation of the MCP's takes place during adaptation and is involved in the memory component of chemotaxis that allows temporal sensing of compounds - for reviews see Hazelbauer *et al.*, 1990 and Stock, 1990. MCP's can be reversibly methylated at four, five or six

Figure 1.7 : Diagramatic structure of methyl accepting chemotaxis proteins Redrawn from Taylor and Lengeler (1990).



glutamate residues in the cytoplasmic domain, two of which are the product of glutamine deamidation (Rice & Dahlquist, 1991). The methyl donor is S-adenosylmethionine and γ-glutamyl esters are formed (Van der Werf & Koshland, 1977). CheR is the methyltransferase that methylates the transducers (Springer & Koshland, 1977) and CheB is the methylesterase (Stock & Koshland, 1978) which also deamidates some glutamines to form some of the glutamate residues that take part in adaptation. CheR appears to work at a constant rate, whereas the activity of CheB is regulated by phosphorylation (see next section).

After the excitatory signal, increased levels of methylation return the MCP to a null signalling state, even though attractants are still bound to the periplasmic domains. In this state the level of methylation and demethylation are again equal. The time taken for adaptation and return to a random walk instead of straight runs varies from seconds to minutes, depending on the size of the chemotactic stimulus. Attractants increase the methylation levels of transducer proteins, whereas repellent adaptation is accomplished by loss of methyl groups (Goy *et al.*, 1977).

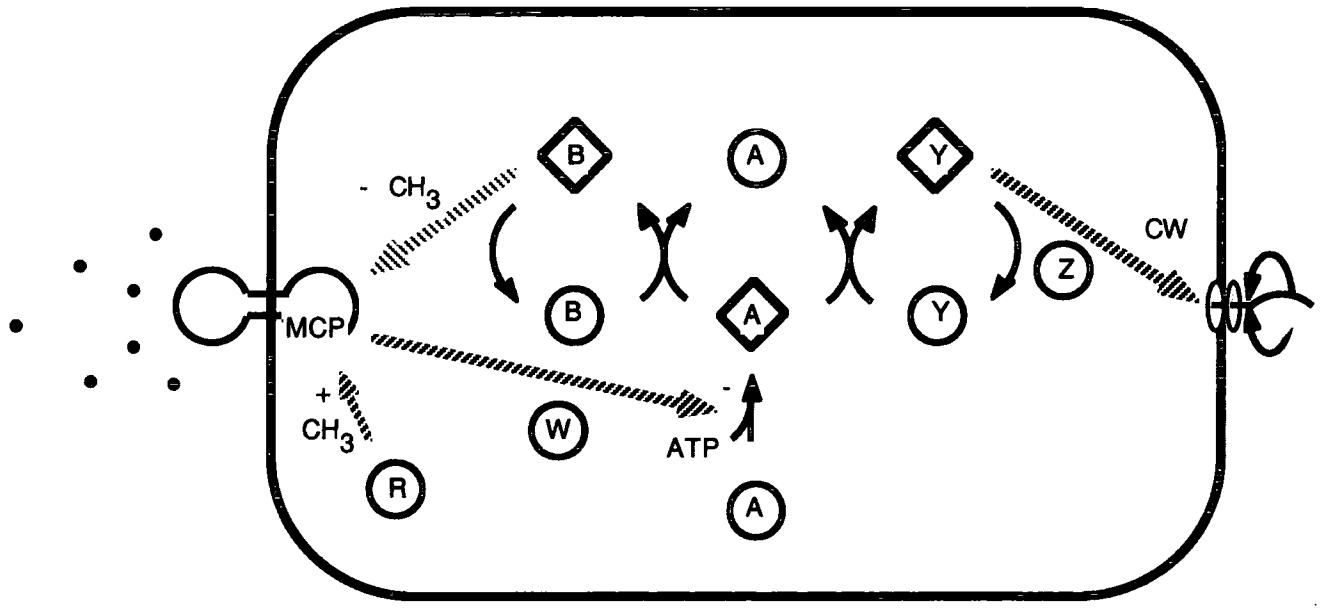
It is the time difference between fast MCP conformational changes and relatively slow methylation level changes on binding of compounds that allows a record of the past environment to be kept by the bacterium. The state of methylation of the the transducers corresponds to the chemical stimuli a few seconds previously, whilst the conformation of the protein reflects the occupancy of the ligand binding sites at that moment. The balancing of information from ligand occupancy and covalent modification achieved by the protein during signal output to the flagella allows the comparison between current and past concentrations of compounds. Thus if a bacterium with transducers in the adapted null state moves in a long run down an attractant gradient, the still methylated but no longer ligand bound MCP's bias the flagella towards CW rotation and induce tumbling. Only if no more attractant molecules bind do the proteins become demethylated and return to the unstimulated state.

1.13.3. The intracellular signalling pathway.

The cytoplasmic proteins that link the MCP's and flagella during chemotaxis are CheA, CheW, CheY, Che Z, CheR and CheB. Intra- and intermolecular phosphorylation reactions, summarised in figure 1.8, are used during intracellular signalling (Parkinson, 1988; Oosawa *et al.*, 1988) which modifies the activity of CheB and CheY to bring about behavioural responses and adaptation.

The CheB methylesterase is activated by phosphorylation (Lupas & Stock, 1989) and phospho-CheY promotes clockwise rotation of the flagella, presumably by interacting with the FliG, FliM and FliN motor switch complex (Smith *et al.*,1988; Kuo & Koshland, 1987). Both

Figure 1.8 : Chemotaxis in E. coli, the intracellular signalling pathway



Phosphorylated species are represented by diamonds, non-phosphorylated by circles. MCP represents a methyl-accepting chemotaxis protein which is methylated by CheR and demethylated by CheB. CW refers to the clockwise rotation of the bacterial flagellum favoured by phosphorylated CheY.

CheR and CheZ, the phosphatase that hyrolyses phospho-CheY and inactivates it (Stock & Stock, 1987; Kuo & Koshland,1987) appear at the moment to work at a constant rate and no covalent modifications of them has been reported.

CheA is the protein that phosphorylates CheY and CheB (Hess *et al.*, 1988b; Sanders *et al.*, 1989) after transferring a phosphate from ATP onto one of its histidine residues (Hess *et al.*, 1988a). CheA and CheY are members of a family of two-component regulatory systems in bacteria (including VirA and VirG) that use phosphorylation for signalling (Bourret *et al.*, 1989; Stock *et al.*, 1990). Transducer proteins bias flagellar rotation directions by altering the rate at which CheA autophosphorylates and produces phospho-CheY. This process requires the presence of CheW (Borkovich *et al.*, 1989; Borkovich & Simon, 1990; Liu & Parkinson, 1989; Ninfa *et al.*, 1991). Genetic evidence suggests that CheW directly binds MCP's during the excitatory signal (Liu & Parkinson, 1991) and CheW also has been shown to form a complex with CheA which increases the rate of CheA autophosphorylation and affinity for CheY (Gegner & Dahlquist, 1991; McNally & Matsumura, 1991).

Binding of attractants by MCP's decreases the rate at which CheA autophosphorylates and there is bias towards straight runs due to decreased levels of phospho-CheY. Increased levels of methylation of the transducers, resulting from less phospho-CheB, reverses this effect of the conformational change that occurs on excitation allowing the tumble frequency to return to previous levels.

1.14. Evidence for a methionine-dependent MCP system in *A.tumefaciens.*

It has been sugggested that most motile bacteria use an MCP system like that found in enteric bacteria (Nowlin *et al.*, 1985). Antibody studies show proteins homologous to *E. coli* MCP's are present in *Bacillus subtilis* and *Spirochaeta aurantia,* which means they are present in 3 of the major lines of eubacterial descent. *Rhodobacter sphaeroides* is the only bacterial species so far described that has no MCP like proteins according to a number of biochemical, behavioural and genetic studies (Sockett *et al.*, 1987).

The occurrence of MCP's in the sensitive chemotactic responses of *A. tumefaciens* was investigated using biochemical methods (Loake, 1989). A methionine auxotrophic mutant of *A. tumefaciens* - C58C^{1} *met81* was fully motile under microscopic examination but failed to give a chemotactic response to 10^{-6}M sucrose when starved of methionine. Chemotaxis toward 10^{-6}M sucrose was restored by the addition of methionine above a threshold level of 10^{-5}M. The abolition of chemotaxis caused by the methionine starvation was not due to loss of protein synthesis, as chloramphenicol did not prevent chemotaxis in the presence of added methionine.

Methionine analogues could also restore chemotaxis to the auxotrophic mutant, their ability to do so being correlated to their abilty to act as substrates for the S-adenosylmethionine (SAM) synthesis. The requirement for SAM implies that methylation is involved in chemotaxis of *A.tumefaciens* towards sucrose.

1.15. Aims of this work.

1. To isolate and characterise DNA flanking Tn5 insertions in a number of the 20 behavioural mutants created by Loake, (1989).

2. To recover and map further cosmids containing behavioural genes from an *A. tumefaciens* genomic library and assess the clustering of the mutants.

3. To further investigate the occurrence of MCP-like proteins in *A. tumefaciens* using biochemical, DNA hybridisation and immunological studies.

CHAPTER 2 : MATERIALS AND METHODS

2.1. Materials.

All chemicals and biological reagents,with the exception of those listed below, were from Sigma chemical company Ltd, Poole, UK.

Restriction endonucleases, 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) and T4 DNA ligase were from Northumbria Biologicals Ltd, Cramlington, Northumberland, UK.

Agarose and LMP agarose were from Bethesda Research Labs (UK) Ltd, BRL, Cambridge.

Yeast extract and agar were from Oxoid Ltd, Basingstoke, Hampshire, England

BBL trypticase peptone was from Becton Dickinson and co, Cockeysville, MD, USA.

Bacto agar was from Difco labs, Detroit, Michigan, USA.

Tris (hydroxy methyl) aminomethane was from BDH Ltd, Poole, England.

Caesium chloride was from Boeringer corporation (London) Ltd, Lewes, East Sussex, England.

Sodium chloride was from A+J Beveridge Ltd, Derwenthaugh Industrial Estate, Swalwell, Newcastle Upon Tyne, England.

Ficoll 400 was from Pharmacia Fine Chemicals, Uppsala, Sweden.

Nitrocellulose filters were from Schleicher and Schuell, Postfach 4, D-3354, Dassel, Germany.

3MM paper was from Whatman Ltd, Maidstone, Kent, UK.

Minisart filters were from Sartorius GmbH, Postfach 3243, D-3400, Gottingen, Germany.

Polaroid 667 film was from Polaroid (UK) Ltd, St Albans, Hertfordshire, England

Radiochemicals were from Amersham International plc., Amersham, Bucks, UK.

2.2 Growth media and conditions.

All bacteria with the exception of *Rhizobium* spp. were grown in complex (LB broth) medium containing $10gl^{-1}$ tryptone, $5gl^{-1}$ yeast extract and $5gl^{-1}$ NaCl. Rhizobia were grown in *Rhizobium* initiation medium, containing $5gl^{-1}$ tryptone, $3gl^{-1}$ yeast extract and $1.3gl^{-1}$ $CaCl_2.2H_2O$. Liquid cultures were incubated on an orbital shaker at 200rpm at a temperature of 37°C for *E.coli* and 28°C for *Agrobacterium* and *Rhizobium* strains. Agar was added at a concentration of 1% for solid plates, and 0.16% for swarm plates

Antibiotics were added as required to LB media after autoclaving to final concentrations of ampicillin - $50\mu gml^{-1}$, kanamycin - $50\mu gml^{-1}$, rifampicin - $100\mu gml^{-1}$ and tetracycline - $15\mu gml^{-1}$. When selecting for the presence of inserts in the multiple cloning sites of pUC18 or 19 by inactivation of the β-galactosidase gene, $50\mu gml^{-1}$ ampicillin and $40\mu gml^{-1}$ X-gal (stock $20mgml^{-1}$ in DMF) were incorporated into the the media.

2.3. Bacterial strains and plasmids.

Bacterial strain	Remarks	Source/reference
E. coli strains		
DH5α	*F⁻ recA1 endA1 supE44 thi-1 λ⁻ hsdR17(r_k^- m_k^+) gyrA96 relA1 Δ(lacZYA-argF)U169 φ80lacΔM15*	Lab stock
HB101	*F⁻ hsdS20(r_b^- m_b^-) recA13 proA2 leu6 thi-1 rpsL20 ara14 galK2 lacY1 xyl5 mtl1 supE44*	Lab stock
ED8767	*recA met* Helper strain for triparental matings	Lab stock
A. tumefaciens strains		
C58C1	Rif^R	Loake (1989)
GM19023	cured of cryptic plasmid	Rosenberg & Huguet (1984)
C58C1 *che-2*	Tn5 insertion mutant forming small swarms	Loake (1989)
C58C1 *mot-6*	non-motlile Tn5 insertion mutant	Loake (1989)

C58C^1 *fla-7*	Tn5 insertion mutant with no flagella	Loake (1989)
C58C^1 *fla-8*	Tn5 insertion mutant with no flagella	Loake (1989)
C58C^1 *fla-11*	Tn5 insertion mutant with no flagella	Loake (1989)

Plasmids	Remarks	Reference
Plasmid vectors		
pBR322	Amp^R Tc^R	Soberon *et al.*, 1980
pUC18/pUC19	Amp^R	Yanisch-Perron *et al.*, 1985
Helper plasmid for triparental matings		
pRK2013	ColE replicon containing transfer function of RK2. Km^R	Figurski & Helinski, 1979
Recombinant plasmids containing cloned behavioural genes		
pAK108	pACYC184 containing *tar-tap* region of *E. coli* . Tc^R	Boyd *et al.*, 1981
pRZ2/pRZ4	pLAFR-1 broad host range vector containing cloned behavioral genes of *R. meliloti*. Tc^R	Ziegler *et al.*,1986
Recombinant plasmids containing C58C^1 chromosomal DNA that overlaps Tn5 insertion sites		
pDUB1900	C58C^1 library cosmid containing *Bam* HI chromosomal fragment in pLAFR-3. Tc^R	Loake 1989
pDUB1901	"	This study
pDUB1902	"	"
pDUB1903	"	"
pDUB1905	"	"
pAB1	pBR322 containing 12kb *Bam* HI fragment from pDUB1905. Amp^R	"

Recombinant pUC plasmids containing Tn5 plus flanking sequences from mutants.

pDUB1802	Amp^R Km^R	This study
pDUB1806	Amp^R Km^R	"
pDUB1807	Amp^R Km^R	"
pDUB1808	Amp^R Km^R	"
pDUB1811	Amp^R Km^R	"

2.4. Preparation of motile bacteria.

Motile strains of bacteria were periodically enriched for motile cells by the use of L-broth swarm agar plates. A loopful of bacteria was inoculated into the centre of the plate, which was subsequently incubated for 2 days at the optimum temperature for the strain. Motile cells moved towards the outside of the plate and cells from the outside of the swarm were picked and re-inoculated on another swarm plate. This process was carried out at least three times before the bacteria were streaked for single colonies on a master plate with antibiotic selection. Single colonies were picked from this for use in procedures such as *in vitro* MCP labelling (See section 2.14.1.).

2.5. Complementation analysis of *A.tumefaciens* motility mutants.

2.5.1. The mini-swarm assay technique.

Mutant bacteria containing *A. tumefaciens* library cosmids were screened for restoration of motility using this method. The concentration of transconjugant bacteria in cell suspensions arising from tri-parental mating experiments was determined by plating dilution series on selective media. Approximately 200 cells were mixed into 10ml of antibiotic-containing L-swarm agar in a capped test tube held at 42°C. The resulting suspension was poured into a petri dish and the plates left to dry for 30 minutes. They were then sealed with Nesco film to prevent drying and incubated for 48 hours at 28°C before analysis of colony morphologies.

2.5.2. Motility analysis of strains containing pDUB1905.

Mutant bacteria into which pDUB1905 had been transferred by conjugation were confirmed as *Agrobacterium* strains using the 3-keto lactose plate test (see section 2.7) and the presence of the plasmid checked with DNA minipreps. The strains were then put into swarm plates together with corresponding plasmid-less mutants using a sterile metal dissection needle to try and ensure approximately equal numbers of cells were inoculated. The needle was flamed, cooled and dipped into an overnight culture of the bacteria before being stabbed to the bottom of a swarm agar plate. The plates were then wrapped in Nesco film and incubated at 28°C for 48 hours.

2.6. Conjugation of plasmids into *Agrobacterium*.

Plasmids were transferred into *Agrobacterium* strains by triparental mating using a method based on that of Ditta *et al.*, (1980), with pRK2013 as a helper plasmid. Cultures of the *E. coli* plasmid donor and helper strains, together with the recipient *Agrobacterium* were grown to mid log phase and 100µl of each culture mixed together in an eppendorf tube. 300µl of the mixture was carefully pipetted onto a 0.22µm nitrocellulose filter disc placed on the surface of an L-agar plate, which was then incubated overnight at 28°C in an upright position.. The disc was transferred to a bottle containing 10ml of sterile 10mM $MgSO_4$ and the bacteria released with vigorous vortexing. Dilutions of the resulting cell suspension were made and plated on selective plates to determine the concentration of transconjugants.

2.7. The 3-keto-lactose test for *Agrobacterium*.

All the *Agrobacterium* strains used in this work are derivatives of C58C[1] which belongs to the biovar 1 group and they can be differentiated from bacteria belonging to other genera by the use of the 3-keto-lactose test. The method used was that of Bernaerts and De Ley, (1963). Presumptive *Agrobacterium* colonies were streaked onto plates containing 2% $CaCO_3$, 2% glucose, 1% yeast extract and 1% agar which were incubated overnight at 28°C. A loopful of the bacteria was then transferred to a plate containing 1% lactose, 0.1% yeast extract and 1% agar to make a thick area of cells <0.5cm in diameter. After incubation at 28°C for 2 days, the plates were flooded with Benedicts reagents (173g sodium citrate, 100g sodium carbonate and 17.3g copper sulphate per litre) and left at least 1 hour at room temperature. Yellow rings could be seen around *Agrobacterium* colonies - see figure 2.1.

Figure 2.1 : The 3-keto-lactose plate test for *Agrobacterium*.

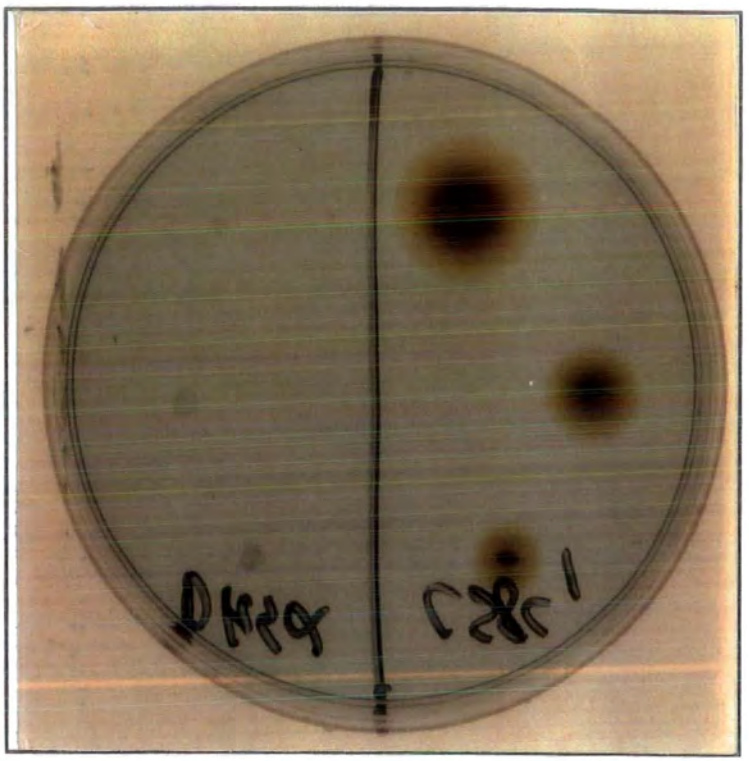


Figure 2.1 : The 3-keto-lactose plate test for biotype 1 strains of *Agrobacterium.*

The figure shows the result of a test using the method of Bernaerts and De Ley (1961). The *E. coli* strain DH5α is on the left of the plate and *Agrobacterium tumefaciens* $C58C^1$ on the right. The plate was flooded with Benedict's reagent for 2 hours, with the yellow rings first visible after 15 minutes.

2.8. Transformation of bacteria.

2.8.1. Calcium chloride transfomation method.

This was based on the method of Mandel and Higa (1970) and was used when a large number of transformations were to be carried out or when competent cells were to be stored.

100ml of L-broth was inoculated with 1ml of an overnight culture of *E. coli* and incubated, with shaking at 37°C until the absorbance at 550nm was 0.4 - 0.5 (2-4 hours). The culture was chilled on ice for 10 minutes before the cells were pelleted in chilled centrifuge tubes at 4000g for 10 minutes at 4°C. After removal of the supernatant, the pellet was gently resuspended in 50ml of ice-cold 100mM $CaCl_2$, 10mM Tris.HCl pH8.0 and the suspension stored on ice for 15 minutes. The cells were then re-centrifuged as before and finally resuspended in 6ml of the same solution. 0.2ml aliquots were dispensed into eppendorf tubes which were stored on ice a further 1-2 hours Cells were used straight away for transformation or stored for future use. For storage of the competent cells, 20% glycerol was added and the tubes rapidly frozen in liquid nitrogen before transfer to a -80°C freezer. Frozen cells were thawed on ice before transformation.

Transformation of the competent cells was carried out as follows. Pure DNA in TE buffer or a ligation mixture was added to the cells and mixed gently by inversion before incubation on ice for 30-60 minutes. The cells were heat-shocked for 2 minutes at 42°C and placed back on ice for 2 minutes before the addition of 1ml of pre-warmed L-broth. The tube was then incubated at 37°C in a heating block for 1 hour before appropriate aliquots, usually 1/10th and 9/10ths of the tube, were spread on selective agar plates.

2.8.2. Rubidium chloride transformation method.

This method was used for a small number of transformations or when higher transformation efficiencies were required. The method has previously been described by Kushner (1978).

100µl of an overnight culture was used to inoculate 5ml of L-broth, which was incubated with shaking until the cell density was approximately $2x10^8$ cells per ml ($O.D._{680}$ = 0.4 - 0.5). 1.5ml aliquots were dispensed into eppendorf tubes and the cells pelleted with a 30 second spin in a microfuge. The supernatant was discarded and the pellet washed in 500µl of solution A (10mM MOPS pH7.0, 10mM RbCl). The cell suspension was immediately re-centrifuged for 15 seconds and the cell pellet resuspended in 500µl of solution B (100mM MOPS pH6.5, 10mM RbCl, 50mM $CaCl_2$) before storage on ice for 90 minutes. After a further 10 second centrifugation the

bacteria were finally resuspended in 150µl of solution B and 3µl of DMSO added, together with 1-250ng of DNA in TE. Following incubation on ice for 1 hour, the cells were heat-shocked for 30 seconds at 55°C and placed back on ice for 1 minute before the addition of 1ml of pre-warmed L-broth. The cells were then incubated at 37°C for 1 hour before spreading of aliquots on pre-warmed antibiotic containing plates.

2.8.3. Transformation of *Agrobacterium.*

The method of introducing plasmids directly into *Agrobacterium* strains was a slightly modified version of that reported by Ebert *et al.* (1987).

100ml of L-broth was inoculated with 4ml of an overnight culture of *A.tumefaciens* and incubated with vigorous shaking at 28°C until $O.D._{600}$ equalled 0.5 - 1.0. The cells were harvested in a bench top centrifuge (Wifug) at 3500rpm for 10 minutes and then resuspended in 2ml of L-broth. 100µl aliquots in eppendorf tubes were held on ice for 30 minutes before the addition of DNA (up to 1µg) which was followed by two rapid cycles of freeze-thawing using liquid nitrogen and a 37°C heating block. After the final heat-shock, 1ml of L-broth was added and the tubes incubated for 2 hours at 28°C with gentle shaking. The cells were finally collected with a 1 minute microcentrifuge spin and resuspended in 100µl L-broth before plating on selective media.

2.9. Isolation of DNA.

2.9.1. Plasmid minipreps.

Small amounts of plasmid DNA were prepared according to Sambrook *et al.* (1989).

A single colony was grown overnight in 5ml of L-broth with appropriate antibiotic selection and 1.5ml transferred to a sterile eppendorf tube. The culture was centrifuged for 1 minute in a microfuge (MSE microcentaur, approximately 10000g) and the supernatant discarded. The pellet of cells was resuspended in 100µl of ice-cold solution 1 (1% glucose, 10mM EDTA pH8.0, 25mM Tris.HCl pH8.0) and allowed to stand at room temperature for 5 minutes. 200µl of solution 2 (0.2M NaOH 1% SDS) was then added and the contents of the tube mixed by gentle inversion before being placed on ice for 5 minutes. 150µl of ice-cold solution 3 (11.5ml of glacial acetic acid and 28.5ml of distilled water added to 60ml of 5M of potassium acetate pH4.8 with no correction) was then added and the tube vortexed briefly. After a further 5 minutes on ice, the tube was centrifuged for 5 minutes in a microcentrifuge to remove bacterial debris. The

supernatant was transferred to a fresh tube and extracted with an equal volume of TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1) before another 2 minute spin in the microcentrifuge. The aqueous phase was transferred to a clean tube and 2 volumes of ethanol added to precipitate the plasmid DNA. After 5 minutes at room temperature, the DNA was collected by centrifugation for 5 minutes and washed in 70% ethanol, before being dried under vacuum. The pellet was resuspended in TE (10mM Tris.HCl pH8.0, 1mM EDTA pH8.0) with RNase A added to a concentration of 20μgml^{-1}.

2.9.2. Large scale plasmid preparation.

5ml of L-broth containing the appropriate antibiotic was inoculated with a single colony of the plasmid containing strain and grown overnight. The whole culture was added to 500ml of L-broth plus antibiotic which was then incubated overnight with shaking. The culture was transferred to 250ml centrifuge bottles and the bacteria harvested by centrifugation at 4000rpm in an MSE High Speed18 centrifuge. After removal of the supernatant, the cells were washed once in 20ml STE (TE buffer plus 0.1M NaCl) before re-centrifugation at 4000rpm and removal of the supernatant again. The bacteria were resuspended in 10ml of solution 1 and the cell suspension put into two 50ml Oakridge centrifuge tubes.before storage at room temperature for 5 minutes. 10ml of solution 2 was added to each tube which were then incubated on ice for 20 minutes after mixing by gentle inversion. 7.5ml of ice-cold 3M sodium acetate pH4.8 (solution 3) was then added to each tube, mixed well and the mixture stored on ice a further 10 minutes. Bacterial debris was removed by centrifugation at 20000rpm for 20 minutes in the MSE 18 and the supernatant removed to sterile 50ml polyethylene tubes. The cell-lysate was extracted once with phenol:chloroform and the aqueous phase transferred to 30ml Corex tubes. 0.6 volumes of isopropanol was then added and the DNA precipitated at room temperature for 15 minutes. The DNA was pelleted by centrifugation at 8000rpm, washed in 70% ethanol, dried and resuspended in 5ml of TE.

2.9.3. Bacterial chromosomal DNA preparation.

A 5 ml culture of the strain was grown up overnight with appropriate selection and 1.5ml aliquots placed in eppendorf tubes. The cells were harvested with a 2 minute spin in a microfuge and the supernatant removed. The bacterial pellet was resuspended in 380µl of protease buffer (50mM Tris.HCl pH8.0, 2mM EDTA pH8.0, 0.08% SDS) and 20µl of 20$mgml^{-1}$ Protease XI added before incubation for 1 hour at 37°C. The resulting clear viscous liquid was pipetted up and down a pasteur pipette in order to shear the chromosomal DNA. The solution was then extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), the aqueous layer bring transferred to a fresh tube each time. A single chloroform extraction was done finally and DNA

precipitated by addition of 1/10th volume 3M sodium acetate pH4.8 and 2 volumes of ethanol. After storage at -70°C for 20 minutes (two hours if DNA not immediately apparent) the DNA was pelleted by centrifugation for 10 minutes in a microfuge. The pellet was washed with 70% ethanol, dried and resuspended in 300µl of TE, with DNA from several tubes being combined before density gradient centrifugation.

2.9.4. Caesium chloride/ethidium bromide density gradient centrifugation.

DNA from large scale plasmid and chromosomal DNA preparations was purified using the following method.

20.6g of caesium chloride and 0.3ml of 10mgml^{-1} ethidium bromide solution were placed in a clean measuring cylinder and the DNA sample, together with TE buffer, added to give a final volume of 27ml. The solution was transferred to a medium Beckman Quickseal centrifuge tube using a needle and syringe and the tube filled with a top-up solution (0.76g CsCl and 11µl 10mgml^{-1} ethidium bromide per ml). The tube was balanced, heat-sealed and centrifuged at 50000rpm at 15°C in a Sorvall 65 OTB ultracentrifuge with a Beckman 70Ti rotor. Following centrifugation for at least 16 hours, the tubes were observed under UV light in order to visualize DNA. Two bands were usually visible, the upper one consisting of chromosomal DNA together with open circular plasmids and the lower one supercoiled plasmid DNA. The top of the tube was punctured and the relevant band collected with a syringe and needle. The DNA solution was transferred to a sterile plastic tube and exhaustively extracted with sodium chloride saturated isopropanol (isopropanol stored over 5M NaCl) to remove the ethidium bromide. DNA was precipitated without dialysis by addition of 600µl 0.45M sodium acetate and 540µl isopropanol to 300µl aliquots of the DNA solution.

2.10. DNA manipulations.

2.10.1. Spectrophotometric quantitation of DNA.

The DNA sample was diluted 1:50 or 1:100 with TE to give final volume of 1ml. This was put into a quartz cuvette and the absorbance of the solution at 260 and 280nm measured with a UV spectrophotometer (LKB) using TE as a blank. A pure DNA sample has an $A_{260/280}$ ratio of 1.8. An A_{260} value of 1.0 is equivalent to a concentration of 50µgml^{-1} of double stranded DNA or 20µgml^{-1} of single stranded oligonucleotides.

2.10.2. RNase digestion of DNA solutions.

Contaminating RNA was removed from plasmid miniprep DNA samples by the addition of RNase A and incubation at 37°C for an appropriate length of time. Usually the plasmid DNA was resuspended in TE buffer with 50μgml^{-1} of RNase present and removal of the RNA occurred during later restriction enzyme digestion. For some large scale plasmid and chromosomal DNA preparations, RNA was digested with 50μgml^{-1} RNase for 1 hour at 37°C and the enzyme removed by phenol:chloroform extraction before subsequent precipitation and collection of the DNA. RNase stock solution was made as follows. Pancreatic RNase A was dissolved at a concentration of 10mgml^{-1} in 15mM NaCl, 10mMTris.Cl pH7.5 and the solution boiled for 15 minutes to inactivate any DNase activity. After slow cooling to room temperature, aliquots were stored at -20°C.

2.10.3. Restriction enzyme digests.

Plasmid DNA was generally digested in a volume of 10-30μl with 5 units of the required restriction enzyme and 0.1 volumes of the appropriate 10x concentrated enzyme buffer. The volume was made up with sterile distille water and the reaction incubated at the recommended temperature (generally 37°C) for 1-2 hours. Chromosomal digests were made up in the same way except the volume was usually 100μl and 20 units of enzyme were added, with the digestion taking place for 16 hours. Digestions which were to be analysed by gel electrophoresis were terminated by the addition of 0.1 volumes of stop dye, made as follows:-

1ml	10%SDS
2ml	250mM EDTA pH8.0
0.2ml	1M Tris.HCl pH8.0
5ml	glycerol
1.8ml	distilled water
10mg	bromophenol blue

If DNA was being used in subcloning procedures, the digestion was stopped by phenol/chloroform extraction - see section 2.11.4. Restriction enzyme digests generally had a requirement for one of the following buffers.

	BUFFER (x10)		
	Low (µl)	Medium (µl)	High (µl)
5M NaCl	0	100	200
1M Tris.Cl pH7.4	100	100	500
1M $MgSO_4$	100	100	100
1M DTT	10	10	10
distilled water	790	690	190

2.10.4. Partial restriction digests.

In order to aid in the mapping of some plasmid inserts, partial restriction digests were done before gel electrophoresis. The digestion was set up as in section 2.11.2, with sufficient DNA so that nine samples could be taken for electrophoresis and the correct number of units of the enzyme added so that digestion was complete after 1 hour. At time points 1, 3, 5, 10, 15, 20, 30, 45 and 60 minutes after the addition of the enzyme, aliquots of the digestion were taken and added to an equal volume of 100mM EDTA pH8.0. Stop dye was added to all the samples and agarose gel electrophoresis carried out with DNA size markers to which 50mM EDTA had been added.

2.10.5. Phenol:chloroform extraction of DNA.

Protein was removed from DNA samples by the addition of an equal volume of redistilled Tris saturated phenol in equilibration with 0.1M Tris.HCl pH8.0. After vortexing for approximately 30 seconds, the phases were separated by centrifugation for 2 minutes in a microcentrifuge. The upper aqueous layer was carefully removed to another tube and re-extracted with an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1).saturated with the same buffer.

2.10.6. Ethanol precipitation of DNA.

DNA was precipitated by the addition of 1/10th volume 3M sodium acetate pH4.8 and 2 volumes of ethanol followed by vortexing. Chromosomal DNA precipitated after 15 minutes at

room temperature, whilst plasmid and DNA fragment precipitations were carried out by storage at -80°C for at least 30 minutes. DNA was recovered by centrifugation for 10 minutes at 10000g in a microcentrifuge. The pellet was usually washed with 70% ethanol and dried under vacuum before resuspension in TE or sterile distilled water.

2.10.7. Phosphatase treatment of DNA.

In order to prevent re-circularisation of single enzyme digested vector DNA during routine subcloning calf intestinal phosphatase was used to remove the 5' phosphates from the linear DNA.

1µl of calf intestinal phosphatase was added to the restriction enzyme digest 30 minutes before the end of the reaction. On completion, 0.1 volumes of 0.1M nitriloacetic acid was added to the digest and the mixture heated to 70°C for 15 minutes in order to inactivate the phosphatase. The solution was then phenol:chloroform extracted and the DNA ethanol precipitated before resuspension in sterile water or TE, ready for use in a ligation reaction.

2.10.8. Ligation of DNA.

DNA fragments with compatible cohesive or blunt termini generated by digestion with restriction enzymes were ligated together by the action of T4 DNA ligase. The fragments to be ligated were mixed together in the approximate ratio of 3:1 insert to vector moles of termini, together with 0.1 volumes of 10x concentrated ligation buffer (0.66M Tris.HCl pH7.5, 50mM $MgCl_2$, 50mM DTT, 10mM ATP) and 1-2 units of T4 DNA ligase. 100-300ng of DNA per 10µl of ligation mixture was the usual concentration of DNA. For blunt ended ligations, the amount of enzyme was incresed to 5 units. The ligation rection was incubated overnight at 4°C or at room temperature for 2-3 hours in the case of cohesive termini and overnight at 15°C for blunt ended ligation.

2.10.9. Agarose gel electrophoresis.

Gel electrophoresis was carried out with large 18x15cm maxigels (volume 200ml), or 10x8cm minigels (volume 70ml) run in a Pharmacia gel apparatus GNA-100 electrophoresis tank. The agarose concentration of a gel depended on the size of DNA to be separated - see Sambrook *et al.* (1989), with 0.7% routinely being used. The correct amount of agarose was added to TAE buffer (10x concentrated - 242g Tris, 100ml 0.5M EDTA pH8.0, 57.1ml glacial acetic acid per

litre) and dissolved by heating the mixture in a microwave. 0.2μgml^{-1} ethidium bromide was added and the agarose solution cooled before being poured into a gel mould and allowed to set. After the gel was placed in TAE buffer containing 0.2μgml^{-1} ethidium bromide and the DNA samples loaded, electrophoresis was carried out at 5-10 Vcm^{-1} for the desired length of time. DNA was visualized with UV light on a transilluminator (UVP Inc.) and a photographic record of the gel made if neccessary using Polaroid 667 film in a polaroid RP4 Land camera with a red filter attached.

2.10.10. DNA fragment isolation.

Two different methods were used for the isolation of DNA fragments from agarose gels for use in subcloning procedures or for hybridization probes.

Method 1 employed low melting point agarose gels. After running of the gel, the DNA was visualized with long wave UV light and the appropriate band cut out with a sterile scalpel blade. The gel slice was transferred to an eppendorf tube which was incubated for 10 minutes at 65°C in order to melt the agarose. 2 volumes of 50mM Tris.HCl pH8.0, 0.5mM EDTA pH8.0 were then added and the tube placed at 37°C for a few minutes after mixing. Phenol extraction of the resulting solution was carried out twice and the final aqueous layer extracted once with chloroform:isoamyl alcohol (24:1) before DNA precipitation with 0.1 volumes 3M sodium acetate pH4.8 and 2 volumes of ethanol. DNA was recovered with a 10 minute spin in a microfuge, washed with 70% ethanol, dried and resuspended in TE with an aliquot being used to check recovery.

Method 2 was a modified freeze-squeeze protocol. The bottom of a 0.5ml eppendorf tube was punctured with a hot needle and the hole plugged with a roll of sterile siliconised glass wool. The appropriate gel slice was isolated as for method 1 and put on top of the glass wool before incubation of the tube for at least 30 minutes at -80°C. The small tube was then placed in a 1.5ml eppendorf tube with the top removed and centrifuged for 10 minutes in a microfuge. The DNA solution left in the bottom of the large tube was extracted once with chloroform before DNA precipitation and recovery as above.

2.11. DNA hybridisation procedures.

2.11.1. Transfer of DNA to membranes

DNA was transferred to nitrocellulose or nylon filters for hybridisation studies using a modification of the method of Southern (1975).

The DNA containing gel was photographed with a ruler down the side and then soaked for 45 minutes in denaturation buffer (1.5M NaCl, 0.5M NaOH) with occasional shaking. (Depurination and fragmentation of the DNA by soaking in 1% HCl was not usually done as the DNA was of a sufficiently small size). The gel was rinsed twice in distilled water and then soaked in neutralisation buffer (1.5M NaCl, 0.5M Tris.HCl pH7.0) for a further 45 minutes. For single sided blots the gel was placed, wells uppermost, on a long piece of 3MM paper that was soaked in 10xSSC (20x - 3.0M NaCl, 0.3M Na.citrate pH7.0) and positioned over a glass plate with its ends dipped in a reservoir of 10xSSC. A nylon or nitrocellulose membrane cut to the same size as the gel was then put on top of the gel and any air bubbles removed. Nylon was placed on the gel straight away, but nitrocellulose had to be pre-wetted with distilled water and equilibrated with 10xSSC before use. The transfer membrane was covered with 3 pieces of 3MM paper soaked in 10xSSC followed by 2 layers of disposable nappies and these were covered with a glass plate. A weight of at least 1kg was placed on top. Care was taken that everything placed on top of the filter was cut to the same size as the gel and nothing touched the bottom layer of 3MM paper. For double-sided blots the gel was sandwiched between two piles consisting of glass plate, 2 layers of disposable nappies, 3 pieces of 3MM and transfer membrane, treated as above. In this case liquid retained in the gel transferred the DNA out onto both membranes. DNA transfer with both methods was allowed to proceed for at least 16 hours.

The apparatus was then dismantled and the filter(s) removed after the position of the wells had been marked. DNA was fixed onto the transfer membrane(s) by baking for one hour at 80°C in a vacuum oven in the case of nitrocellulose and exposure to UV light from a transilluminator for 2 minutes for nylon filters.

2.11.2. Hybridisation of radio-labelled probes to Southern blots.

The baked nitrocellulose or UV cross-linked nylon filter was soaked in 6xSSC for 2 minutes and then put iside a plastic bag heat-sealed along three sides. 200µl of pre-hybridisation fluid (6xSSC, 5x Denhardts solution [50x Denhardts is 1% ficoll, 1% polyvinylpyrrolidine, 1% BSA fraction V], 0.5% SDS, 0.1% tetrasodium pyrophosphate and 100µgml^{-1} denatured salmon sperm DNA) were added per cm^2 of the filter, any air bubbles removed, and the bag heat-sealed. The bag was placed in a plastic sandwich box and incubated, with gentle shaking for 2-4 hours at a temperature of 42°C or 65°C. After pre-hybridisation was complete the pre-hybridisation solution was removed and replaced with 50µl/cm^2 hybridisation solution (as above plus 0.01M EDTA) containing the boiled DNA probe. The bag was put into the plastic box after re-sealing and hybridisation carried out for 16 hours with shaking at a temperature of 42 to 65°C depending on the desired stringency. The hybridisation fluid was discarded or transferred to a glass container if required for further use and the filter placed in the plastic sandwich box.

2.11.3. Washing of probed Southern blots.

All washing steps were carried out without allowing the filters to dry out. For high stringency washing, nitrocellulose filters were washed twice for 10 minutes in 2xSSC, 0.1%SDS at room temperature followed by two washes of 1 hour at 65°C in 0.1xSSC, 0.5% SDS. In the case of nylon filters, the first two washes were the same but they were followed by a 30 minute wash in 2xSSC, 0.1% SDS at 65°C and a 15 minute wash at 65°C in 0.1xSSC, 0.1% SDS. Lower stringency washes were done with 2xSSC at temperatures varying from 42 to 65°C. If higher stringency washing after exposure was not required, filters were then air-dried and wrapped in cling film before exposure to X-ray film. If further washing was required the filters were wrapped wet. Removal of radioactive probes from nylon filters for re-probing of the same DNA with another probe was done by washing with 0.4M NaOH at 45°C for 30 minutes followed by a wash with 0.2M Tris.Cl pH7.5, 0.1xSSC, 0.1% SDS for 30 minutes at 45°C.

2.11.4. Detection of hybridising probes.

Radioactive hybridised probes were detected by exposing Fuji RX-100 X-ray film to the filters for varying lengths of time. Film sheets were pre-flashed once in order to sensitize the film and exposure was carried out in film cassettes with intensifying screens at -80°C. Exposed films were developed with Ilford phenisol developer for 2 minutes and fixed with Kodok Unifix fixer for a further 2 minutes. Radioactive ink spotted onto 3MM paper in the film cassette allowed the position of the wells in the original agarose gel to be marked on the film and the size of any hybridizing fragments could be worked out with reference to the gel photograph.

2.11.5. *In situ* hybridisation to bacterial colonies.

Colony hybridisation was carried out as described in Sambrook *et al.*, (1989).

A gridded nitrocellulose filter was placed on a selective agar plate and allowed to wet from beneath. Individual colonies to be screened were picked with sterile cocktail sticks and patched onto the squares of the grid and also onto a master plate.The plates were incubated overnight at 37°C and both the filter and master plate marked in three positions in order to identify positive clones. Using sterile forceps the filter was placed on 3MM paper soaked in 10% SDS for 3 minutes in order to lyse the cells and the filter was then transferred to 3MM paper saturated with denaturation solution (1.5M NaCl, 0.5M NaOH) for 5 minutes. This was followed by 5 minutes

on 3MM soaked in neutralising solution (1.5M NaCl, 0.5M Tris.Cl pH7.0) before the filter was air dried at room temperature for 30-60 minutes and baked for 1 hour at 80°C in a vacuum oven.

To prepare the filter for hybridisation it was floated on the surface of a tray of 6xSSC until thoroughly wetted from beneath and then submerged for 5 minutes. A pre-washing step followed in order to remove loose bacterial debris, with the filter being incubated at 65°C for 1-2 hours in 100ml of washing solution (1M NaCl, 1mM EDTA, pH8.0, 0.1% SDS, 50mM Tris.Cl pH8.0). Pre-hybridisation, hybridisation and detection of positive clones were carried out as described for Southern blot hybridisations except 1xSSC was used for the final washing step, with 0.2xSSC being used only if the background was very high.

2.11.6. Labelling of DNA fragments.

DNA fragments for use in colony hybridisation or Southern blot analysis were labelled with [α-^{32}P] dCTP by the random primer labelling method using a kit from Amersham.

The DNA to be labelled (30-50ng) in a total volume of 28µl was boiled for 5 minutes and then held on ice for 2 minutes. 10µl of labelling buffer, 5µl of random hexanucleotide primers and 5µl ^{32}P-dCTP (equivalent to 50µCi) were then added. After the addition of 2µl of Klenow enzyme the labelling reaction was incubated for 2-3 hours at 37°C or left overnight at room temperature. The labelled DNA was then either boiled for 5 minutes and used immediately or stored for future use at -20°C.

2.11.7. Labelling of oligonucleotide probes.

The oligonucleotides used in this study were synthesized without a 5' phosphate group and therefore could be labelled with [γ-^{32}P] dATP by the action T4 polynucleotide kinase (PNK).

DNA to be labelled was resuspended in TE and then precipitated again to ensure there were no ammonium ions present. The DNA was resuspended in TE finally and the concentration determined spectrophotometrically. 1µl of the DNA solution, diluted to contain 25-50ng of the oligonucleotide, was placed in an eppendorf containing 35µl of milli-Q water and 10µl of 10x kinase buffer (0.5M Tris.Cl pH7.6, 0.1M $MgCl_2$, 50mM DTT, 1mM spermidine.HCl, 1mM EDTA pH8.0). 1µl of T4 PNK and 5µl of [γ-^{32}P] dATP were then added. After incubation at 37°C for 30 minutes, the enzyme was inactivated by heating to 70°C for 5 minutes and the unincorporated nucleotides removed as follows. 10µl of a stop-dye mix containing 60mM EDTA, 100mgml^{-1} blue dextran and 1mgml^{-1} xylene cyanol were mixed with the labelling reaction and

the resulting blue solution loaded onto a 7cm Biogel P-60 or Sephadex G-25 column made in a glass pasteur pipette. The DNA washed through with TE buffer. The probe came off with the first dye front (blue dextran) and was collected in a volume of approximately 0.5ml whilst unincorporated nucleotides eluted with the xylene cyanol. Labelling efficiency (dpm.μg^{-1} DNA) could be worked out after counting 5µl of the probe solution in a scintillation counter.

2.12. Western blotting and antibody binding protocols.

2.12.1. Bacterial protein extracts.

Protein extracts were made from bacteria by ultrasonic disruption of cells as follows.

Bacterial cells from 100ml of stationary phase culture were harvested by centrifugation at 4000g for 5 minutes at 4°C and then washed in 20ml of 0.1M sodium phosphate buffer pH7.5. After a further centrifugation step, the pellet was resuspended in 4ml of the same phosphate buffer and the cells disrupted with ultrasonic oscillations for 30 seconds at 0°C using an MSE 100W ultrasonic disintegrator set at 7µm peak to peak amplitude. Remaining intact cells and cell wall material were removed by centrifugation at 20000g for 20 minutes at 4°C. The concentration of protein in the remaining solution was estimated as described below before separation by polyacrylamide gel electrophoresis.

2.12.2. Protein concentration estimation.

The concentration of protein in the cell free extracts was estimated using the method of Bradford (1976).

0.25 volumes of a commercial Bradford assay dye reagent were added to 100µl samples of a dilution series of the protein sample in a microtitre dish. The colour reaction was allowed to develop for 15 minutes at room temperature before measurement of the absorbance at 595nm. Crystalline rabbit IgG was used as the standard protein for the construction of the calibration curve required to calculate the protein concentration.

2.12.3. SDS polyacrylamide gel electrophoresis.

For antibody binding studies the proteins were separated using the polyacrylamide gel buffer system of Schägger and von Jagow (1987), with gels being cast and run in a Bio-rad Protean II vertical gel unit.

The 10% resolving gel was made by mixing 8.10ml of acrylamide stock solution (48% acrylamide, 1.5% N,N'-methylene-bis-acrylamide) with 13.33ml gel buffer (3M Tris, 0.3% SDS, pH to 8.45 with HCl), 5.33ml glycerol and 13.23ml distilled water. The mixture was de-gassed and 130µl of fresh 10% ammonium persulphate (APS) solution and 30µl of N,N,N',N'-tetramethylethylethylenediamine (TEMED) added before the gel was poured. Distilled water was layered on top of the gel before it set to ensure a level surface. The 4% stacking gel consisted of 1ml of the stock acrylamide solution mixed with 3.1ml gel buffer, 8.4ml distilled water, 50µl 10% APS and 25µl TEMED. The cathode buffer was 0.1M Tricine, 0.1M Tris, 0.1% SDS (pH8.25 without addition) and the anode buffer 0.2M Tris.Cl pH8.9. Protein samples were made up to a volume of 25µl with distilled water and mixed with an equal volume of 2x sample buffer (4% SDS, 20% glycerol, 120mM Tris.Cl pH6.8, 0.005% bromophenol blue with 10% β-mercaptoethanol added immediately before use). The sample tubes were boiled for 5 minutes and spun for a few seconds in a microfuge before being applied on top of the gel. Both pre-stained and unstained markers (Sigma cat. no. SDS 7 and SDS 7B) were used as molecular weight size standards. The gel was stacked at 30v and run at 75v overnight.

2.12.4. Western blotting.

Proteins were transferred to a nitrocellulose membrane for immunoblot screening using liquid transfer apparatus. After separation of proteins, the gel was placed in 200mls of transfer buffer (25mM Tris, 192mM glycine, 20% methanol) for 30 minutes, together with a nitrocellulose membrane cut to the same size as the gel. A piece of 3MM paper soaked in transfer buffer was placed on top of a fibre pad in the gel holder cassette and the gel was placed on top of this. The nitrocellulose membrane was placed on top of the gel, followed by another piece of soaked 3MM paper, with all air bubbles being smoothed out. The cassette was closed and totally immersed in transfer buffer in the transfer tank, with the membrane towards the anode. Transfer was at 0.8mA per cm^2 for 90 minutes. To ensure transfer of the proteins onto the membrane and allow marking of the position of protein standards, the nitrcellulose was stained with Ponceau S (0.2% in 1% acetic acid) for 5 minutes, with shaking at room temperature. The membrane was destained with Tris-buffered saline (10mM Tris, 150mM NaCl, pH to 7.4 with HCl) before immuno-screening.

2.12.5. Immunoblot screening.

Non-specific antibody binding sites on the nitrcellulose filter were blocked at room temperature with shaking using a 3% low fat milk powder or 1% gelatin solution in Tris-buffered saline (TBS) for thirty minutes. The primary antibody was then added in 50µlcm^{-2} fresh blocking solution and the filter incubated overnight at room temperature with shaking. After removal and storage of the antibody solution, the blot was washed twice in TBS containing 0.5% Triton X-100 for 20 minutes and rinsed for a further 20 minutes in TBS. The secondary antibody was added in fresh blocking solution for 2 hours at room temperature with shaking and the blot washed as for the primary antibody before the colour reaction was carried out. The colour reaction for the horseradish peroxidase conjugated secondary antibody was done by immersing the filter in 45ml TBS mixed with 25µl H_2O_2, 12.5mg diaminobenzidine and 5ml 0.3% $CoCl_2$ for 15 minutes in a dark container. The reaction was stopped by vigorous washing of the filter in distilled water.

2.12.6. Pre-adsorbance of the primary antibody.

The rabbit serum used as the primary antibody source was pre-adsorbed with an acetone powder from the *E. coli* strain HCB437 (Wolfe *et al*, 1987) as described in Harlow and Lane (1988).

Cells from 100ml of stationary phase *E. coli* HCB437 liquid culture were harvested and washed in 0.5 volumes of 0.9% NaCl. After re-centrifugation, the pellet was resuspended in 4ml 0.9% NaCl and the cell suspension placed on ice for 5 minutes. 16ml of acetone held at -20°C were then added and the mixture vortexed before incubation on ice for 30 minutes. The precipitate was collected by centrifugation at 10000g for 10minutes at 4°C and the pellet resuspended in 16ml fresh -20°C acetone. After a further incubation on ice for 10 minutes and re-centrifugation at 10000g, the precipitate was spread out on a clean piece of filter paper to air dry before use. The powder was added to a final concentration of 1% in the serum, which was then stored at 4°C for 30 minutes. After centrifugation at 10000g for 10 minutes, the supernatant was used for immunoblot screening.

2.13. *In vivo* methylation protocols.

2.13.1. Radiolabelling of proteins.

The method used was a modified version of that described by Springer *et al*, (1977).

50ml of L-broth was inoculated with 20µl of a stationary phase culture of swarmed *A. tumefaciens* and incubated with shaking for 18 hours at 28°C. The cell density was measured with a Coulter Counter and the number of cells adjusted to $1x10^9ml^{-1}$ by addition of L-broth before harvesting by centrifugation at 10000g. The bacteria were washed twice in chemotaxis medium (0.1mM EDTA pH7.0, 10mM KH_2PO_4 pH7.0) containing $40\mu gml^{-1}$ L-methionine and once in chemotaxis medium alone before resuspension of the cells in chemotaxis medium at a density of $1x10^9$ cells per ml. 0.5% glycerol was added to the bacterial suspension and 1ml aliquots transferred to Eppendorf tubes in a waterbath at 28°C. $200\mu gml^{-1}$ puromycin was added to the tubes which were then incubated at 28°C for a further 20minutes. 15µCi of L-[3H-methyl]-methionine (specific activity 85Ci per mmol) were added, giving a final label concentration of 0.18µm. After further incubation for an appropriate length of time and the addition of possible attractants or repellents, the cells were lysed and proteins precipitated by the addition of 4% formalin and 10% TCA or 1% SDS and 15% TCA. The tubes were held on ice for 20 minutes and the precipitate collected by centrifugation for 20 minutes in a microfuge. The supernatant was discarded and the pellet was held on ice a further 20 minutes after addition of 0.5ml ice-cold acetone to remove TCA. After re-centrifugation, the pellet was resuspended in 100µl 1x SDS PAGE sample buffer (see section 2.13.3) and boiled for 5 minutes before separation of proteins by polyacrylamide gel electrophoresis.

2.13.2. SDS-PAGE.

The labelled proteins were generally separated using the gel electrophoresis method of Laemmli (1970).

The 12% resolving gels consisted of 16ml of acrylamide stock solution (37.5:1 acylamide : bis-acrylamide), 5ml 0.8% SDS, 5ml 3M Tris.Cl pH8.8 and 12ml distilled water, with 2ml 0.28% ammonium persulphate and 80µl TEMED added after de-gassing. The 5% stacking gels were made with 2.4ml of the acrylamide solution, 1.875ml 0.8% SDS, 1.875ml 1M Tris.Cl pH6.8 and 8.1ml distilled water, with 0.75ml 0.28% ammonium persulphate and 60µl TEMED added. The gels were stacked at 75v and run at 50v for the appropriate length of time. After separation, proteins were stained overnight with Coomassie brilliant blue R-250 (0.1% solution in 10% isopropanol, 10% acetic acid) and destained in 10% acetic acid, 10% isopropanol. Standard molecular weight markers were the same as those described in section 2.13.3.

2.13.3. Fluorography.

Sodium salicylate was used as the fluorographic agent in the labelling experiments. After destaining, the gel was placed in a 1M solution of sodium salicylate in 20% v/v isopropanol and 4% v/v glycerol for 1 hour at room temperature. A wet piece of 3MM paper larger than the gel was placed on the slab gel drier and covered with a piece of dry 3MM paper cut to the same size. A piece of cellophane soaked with distilled water was placed on top of this followed by the gel, another piece of soaked cellophane and a further piece of dry 3MM paper. Any air bubbles were removed at each stage and the gel was then dried under vacuum for 2 hours at 60°C The dried gel sandwiched between cellophane sheets was removed from the 3MM paper, taped to the inside of an X-ray cassette and covered in cling film.The gel was exposed to light-sensitized Fuji RX 100 film (flashed side next to gel) at room temperature for an appropriate length of time.

2.13.4. Methyl-ester diffusion assay.

The nature of the protein-methyl linkage was investigated using the diffusion assay method described in Chelsky *et al*, (1984).

After exposure to X-ray film, the relevant gel lane was sliced into 2mm strips (width approximately 1cm) and these were placed in 0.5ml eppendorf tubes from which the tops had been removed. The eppendorf tubes were then individually placed in 6ml scintillation vials which contained 2ml of Ecoscint A. 100µl 1M NaOH was added to each Eppendorf tube in turn with the caps immediately being replaced on the vials after the addition. All of the vials were incubated a 37°C for 12 hours and the amount of volatile radioactive methanol released quantified by scintillation counting for 5 minutes using the ^{3}H counting programme.

CHAPTER 3

Cloning and mapping of Tn5 insertion loci in C58C1 motility mutants.

Two different methods were used to try and isolate C58C^1 library cosmids containing the sequences mutated by Tn5 insertion in the C58C^1 motility mutants. The first was mutant complementation, with transfer of the library cosmids into mutant strains followed by analysis of colony morphology on swarm plates. Cloning of the DNA flanking Tn5 in the mutants and use of the sequences isolated as probes in colony hybridisation to the library was the other method used.

3.1. Complementation of mutants with C58C^1 cosmid library.

Conjugation or transformation of the C58C^1 library into a motility mutant, followed by analysis of the swarm morphology of the resulting cells on LB-swarm plates, provided a potentially rapid method for the isolation of chromosomal DNA which contained the insertion site for Tn5 in the mutant strain. Transcription of the corresponding complementing gene from either the *lac* promotor in pLAFR-3 or a promotor in the insert, together with translation in the correct reading frame were required for complementation to work.

Initially transformation of the C58C^1 motility mutants, using the modified method of Ebert *et al.* (1987) was used to try and introduce the library cosmids. 1ml of an overnight culture of the combined library was used to inoculate 1 litre of L-broth containing tetracycline and a large scale plasmid preparation carried out after incubation at 37°C for 16 hours. 300µg of cosmid DNA was recovered and 1µg samples used to transform *fla-7* and *fla-8* cells. The transformation efficiency achieved using either of these two strains was very low, with only 10-100 Rif/Km/Tc resistant transformants per µg of DNA being recovered. Introduction of library cosmids using transformation was not efficient enough to enable sufficient numbers of cosmid-containing bacteria to be screened for restoration of motility. Conjugation of the library into mutants was therefore attempted.

The library was transferred into both *fla-7* and *fla-8* mutants by tri-parental mating, using ED8767/pRK2013 as the helper strain. Trial mating experiments with *fla-7* as the recipient strain demonstrated that liquid matings, in which the recipient, helper and combined library donor strains were mixed and the cell suspension left overnight at 28°C were unsuccessful as no Rif/Km/Tc resistant cells were produced. Solid matrix matings using a 0.22µm nitrocellulose filter as described in materials and methods were successful, with a 1:1:1 ratio of the three strains giving the greatest number of transconjugants.

Transfer of the library into both *fla-7* and *fla-8* was performed twice. The swarm morphology of approximately 6000 transconjugants from each mating was studied using the mini-swarm assay technique with plates containing Rif/Km/Tc. No swarming cells were seen in either library-containing mutant, with all colonies appearing as tight dense spots on the swarm

plates. The number of recombinant pLAFR-3 clones required to constitute a library with a probability of 99% is 820 (Loake 1989). Analysis of the motility of 12000 colonies of the two library-containing mutants should therefore have allowed isolation of a complementing cosmid if the method was a viable one. Isolation of cosmids by complementation may have been successful for other mutants, but further experiments with other mutants were not carried out and cosmids were isolated from the library using cloned Tn5 flanking sequences as probes.

3.2. Isolation of DNA flanking Tn5 insertion loci.

The kanamycin resistance gene in Tn5 was used as a selectable marker for the isolation of DNA flanking the transposon. Tn5 does not contain an *Eco* RI site and cloning of chromosomal *Eco* RI fragments that coded for kanamycin resistance from the mutant strains enabled the Tn5 flanking sequences to be isolated from a number of motility mutants.

Southern blot analysis of chromosomal DNA from ten mutants (number 2 to 11 in the original mutant classification - See table 1.1 p24) was used to ensure Tn5 was present in the genome and to assess the number of transposon copies present. Chromosomal DNA from each mutant was isolated, purified by density gradient centrifugation, digested to completion with *Eco* RI and separated on a 0.8% gel. After transfer to a nitrocellulose filter, the resulting blot was hybridised to the 1.1kb *Bgl* II fragment from pAG50 (Colbère-Garapin *et al.*, 1981) at 65°C, with washing carried out at 65°C in 0.1xSSC (figure 3.1). The probe fragment from pAG50 codes for the kanamycin resistance protein APH (3')-II in Tn5. The Southern blot hybridisation showed that there was only a single copy of this gene in all the mutants studied and since the size of the hybridising bands was greater than 5.8kb, Tn5 could be cloned from each strain together with flanking sequences defined by *Eco* RI. Based on the sizes of fragments hybridising to the Tn5 probe, the insertions in *che-2*, *mot-6*, *fla-7* and *fla-8* appeared to be in the same *Eco* RI fragment. With the exception of *fla-7*, this was subsequently shown to be the case. Similarly *mot-4*, *mot-9* and *fla-10* apparently contained Tn5 insertions in the same *Eco* RI fragment and this was also confirmed by subsequent experiments.

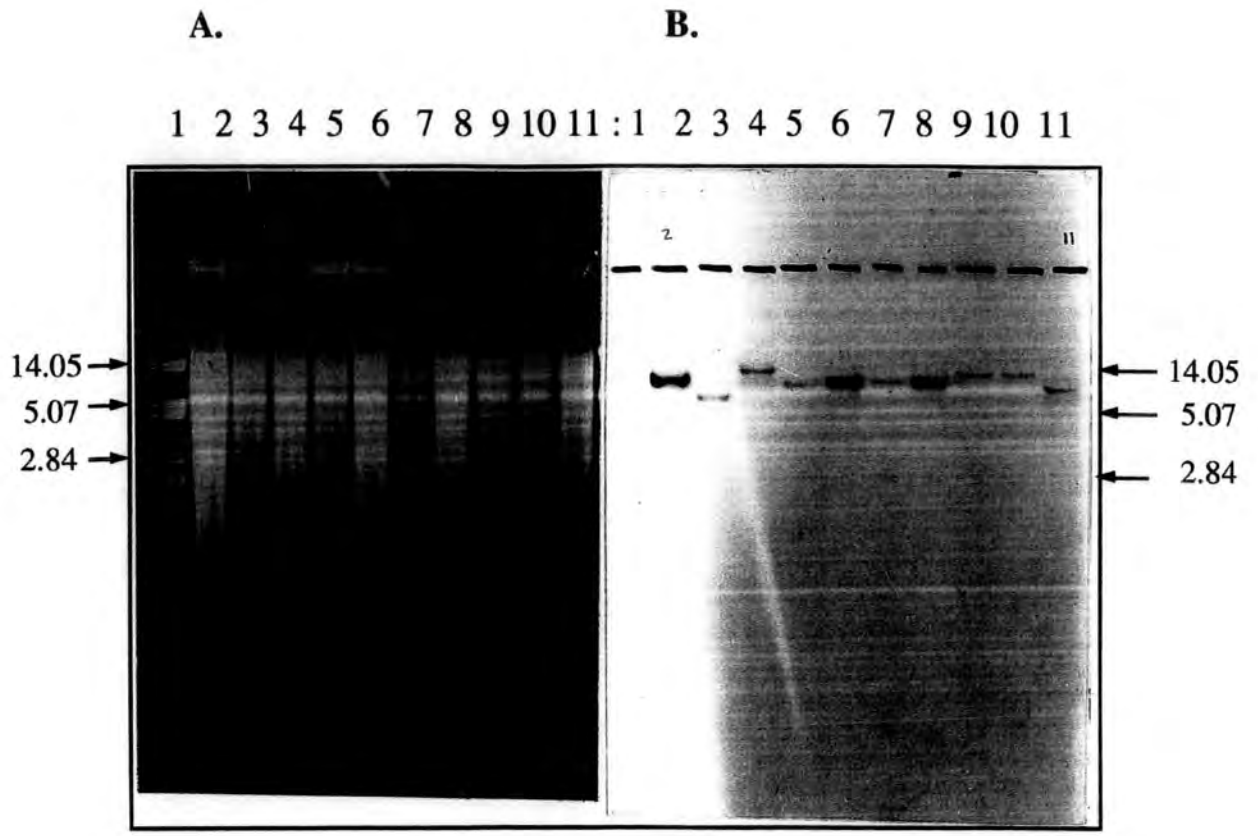
Chromosomal *Eco* RI fragments containing Tn5 were isolated from motility mutants as follows. Genomic DNA from each mutant was isolated, purified on caesium chloride density gradients and 12µg digested to completion with *Eco* RI. This was added to 4µg of *Eco* RI cut pUC18 or 19 to a give a total volume of 500µl and the mixture ligated overnight at 4°C. The insert - vector mass ratio of 3:1 decreased the probability of vector religation. The total number of recombinant pUC clones was titred after transformation of rubidium chloride competent DH5α cells with 5µl of the ligation mixture. The cells were plated on X-gal/Amp plates and the number of white colonies counted.

Figure 3.1 : Southern blot analysis of mutant chromosomal DNA with Tn5 probe.

Panel A shows a photograph of agarose gel electrophoresis of mutant chromosomal DNA's cut with *Eco* RI and panel B the resulting blot after transfer of the DNA to nitrocellulose and probing with radiolabelled *Bgl* II 1.1kb fragment from pAG50. The film was exposed for 16 hours at -80°C. The lanes contained DNA from the following $C58C^1$ motility mutants.

Lane 1	*Pst* I cut λ-DNA
Lane 2	*che-2*
Lane 3	*fla-3*
Lane 4	*mot-4*
Lane 5	*mot-5*
Lane 6	*mot- 6*
Lane 7	*fla-7*
Lane 8	*fla-8*
Lane 9	*mot-9*
Lane 10	*fla-10*
Lane 11	*fla-11*

Figure 3.1. : Southern blot analysis of mutant chromosomal DNA with Tn5 probe from pAG50.



The number of recombinant clones that had to be screened for growth on kanamycin plates to achieve a 99% probability of cloning the Tn5 kanamycin resistance gene was 4830. This estimate was calculated using the formula described by Clarke and Carbon (1976), assuming that the hexanucleotide cutter *Eco* RI digested every 4kb on average and the *Agrobacterium* genome was 4.2Mb in size. Sufficient numbers of tubes containing rubidium chloride competent DH5α were transformed with 5μl aliquots of the ligation mixture to produce 8000 recombinant clones. Transformants were plated on ampicillin and kanamycin plates, which were incubated for 24 hours at 37°C before inspection for any colonies.

Eco RI fragments containing Tn5 were isolated from *che-2, mot-6, fla-7, fla-8* and *fla-11* using the above procedure. One kanamycin resistant colony was found using *che-2* and *fla-7* DNA, two with *mot-6* and *fla-8* and four using total DNA from *fla-11*. The Tn5-containing plasmids in the kanamycin resistant colonies were named pDUB18••, with the last two digits corresponding to the original classification number of the mutant from which the pUC insert came.

The plasmids in the resistant colonies were isolated and digested with *Eco* RI and *Hpa* I. Tn5 contains a *Hpa* I restriction site 184bp from each of its ends and there is no site for the enzyme in pUC18 or 19, so the double digests produced pUC18 or 19, a 5.4kb Tn5 fragment and the Tn5 flanking sequences (figure 3.2). One of the flanking sequences in pDUB1807 was 2.7kb in size and ran as a doublet with the pUC18 in gel electrophoresis of *Eco* RI/*Hpa* I digests - figure 3.2 C. To isolate this DNA fragment a triple digest including *Sca* I, which cuts pUC18 once to produce 0.95 and 1.7kb fragments had to be carried out - figure 3.2 D.

Almost all of the kanamycin resistance clones from mutants that gave rise to more than one colony (*mot-6, fla-8* and *fla-11*) were identical within each mutant class and produced the same restriction fragments on digestion. The exception was one of the plasmids from the *fla-11* ligation, which had an extra 2.8kb *Eco* RI fragment present.- figure 3.2 A, lane 5. The fragments obtained on *Hpa* I digestion of the two plasmids isolated from *fla-8* were not identical (figure 3.2 B, lanes 3 and 6) even though *Eco* RI/*Hpa* I digestion of the two clones produced apparently identical fragments (figure 3.2 lanes 4 and 7). Investigation into potential differences between the two plasmids was not undertaken and all subsequent experiments using pDUB1808 DNA were carried out using the plasmid digested in lanes 1-3 of figure 3.2B.

Table 3.1 details the sizes of the Tn5 flanking sequences observed after *Eco* RI/*Hpa* I digestion of the plasmids obtained from five mutants and used as hybridisation probes in library cosmid screening and mapping. Both the *mot-6* and *fla-8* Tn5 flanking sequences contained an extra *Hpa* I site - figure 3.2 B, and the size of restriction fragments created after *Eco* RI /*Hpa* I double digestion were apparently identical after gel electrophoresis.

Figure 3.2 : Isolation of Tn5 flanking sequences.

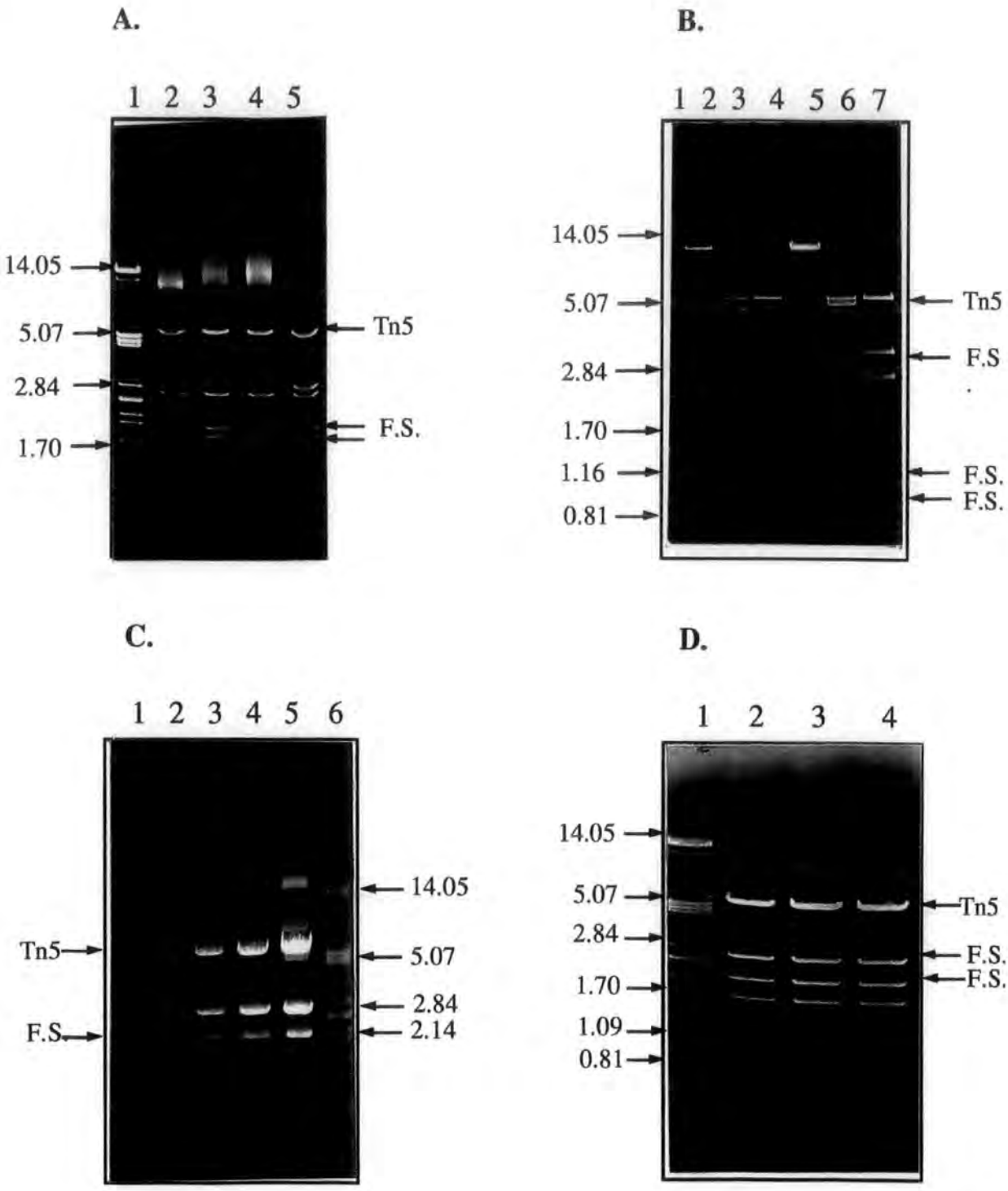


Figure 3.2 : Isolation of sequences flanking Tn5 insertion loci.

Photographs show agarose gel electrophoresis of digested plasmids containing cloned Tn5 from *fla-7*, *fla-8* and *fla-11*. The central region of Tn5 and the isolated flanking sequences are marked F.S. in each case. The gels contained DNA from the following sources.

Panel A : Lane 1, *Pst* I cut λ-DNA. Lanes 2 to 5, four separate pDUB1811 (*fla-11*) clones digested with *Eco* RI and *Hpa* I.

Panel B : Lane 1, *Pst* I cut λ-DNA. Lanes 2 to 7, two separate pDUB1808 (*fla-8*) clones digested with *Eco* RI (2 and 5), *Hpa* I (3 and 6) and *Eco* RI/*Hpa* I (4 and 7).

Panel C : Increasing concentrations of *Eco* RI/*Hpa* I digested pDUB1807 (from *fla-7*), lanes 1 to 5. *Pst* I digested λ-DNA, lane 6.

Panel D : Lane 1, *Pst* I cut λ-DNA. Lanes 2 to 5, pDUB1807 (from *fla-7*) digested with *Eco*RI/*Hpa* I/*Sca* I - with both flanking sequences visible.

Table 3.1 Sizes of behavioural mutant Tn5 flanking sequences.

Mutant	Plasmid	Vector	Size of flanking sequence (kb)
che-2	pDUB1802	pUC 18	1.4, 2.0
mot-6	pDUB1806	pUC 19	0.9, 1.2, 3.3
fla-7	pDUB1807	pUC 18	2.1, 2.7
fla-8	pDUB1808	pUC 19	0.9, 1.2, 3.3
fla-11	pDUB1811	pUC 18	1.8, 1.9

3.3 Isolation of cosmids containing Tn5 insertion loci.

Flanking sequences from the plasmids described above were used as hybridisation probes for isolation of library cosmids that contained the Tn5 insertion loci that led to altered motility. The flanking sequences were purified from agarose gels and 50ng labelled with 50μCi of [α^{32}P-dCTP] using the random primer method before colony or Southern blot hybridisation. Probes were used to investigate possible homologies to the inserts of cosmids already isolated, by Southern blot analysis of *Bam* HI digests, before being used in colony hybridisation experiments with the whole library.

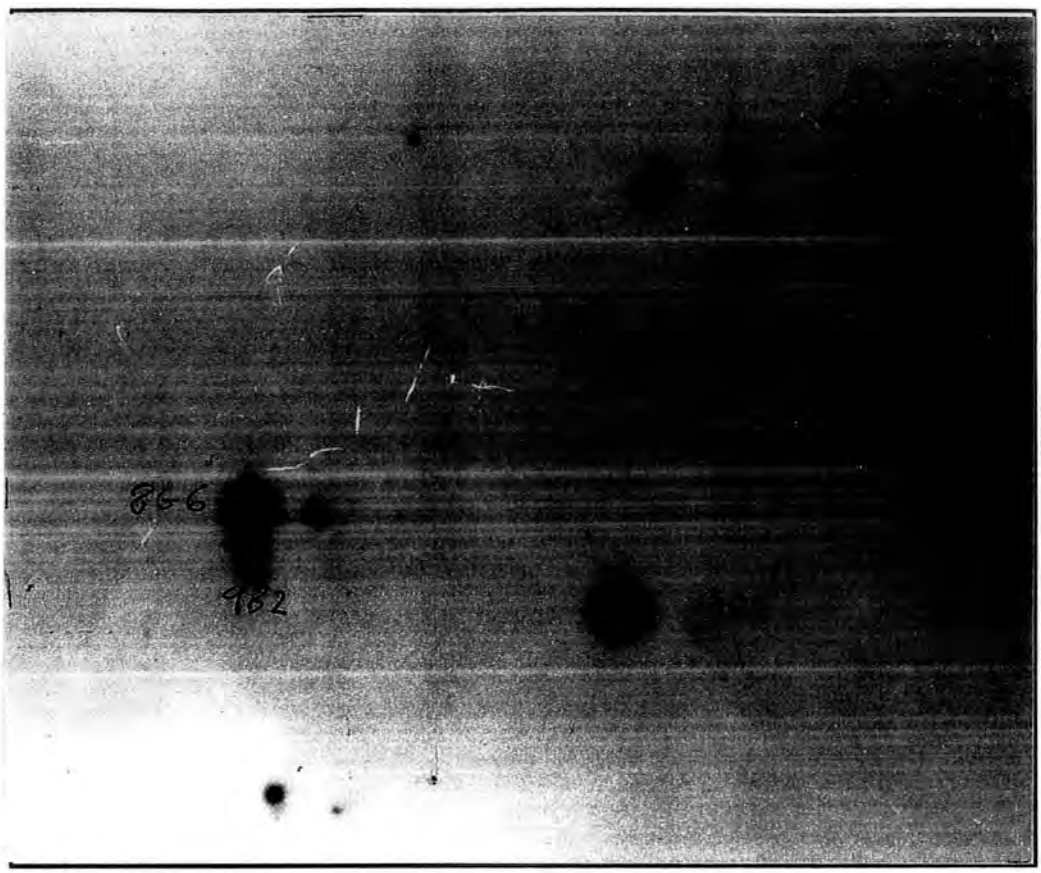
For colony hybridisation to the library cosmids, 960 individual clones were inoculated onto a 22x22cm nitrocellulose filter placed on a tetracycline containing L-agar plate. The filter had previously been marked with a grid and 1μl aliquots from the wells in the microtitre dishes in which the library was stored were spotted onto the marked squares in order. The plate was incubated overnight at 37°C in an inverted position before lysis of the cells and hybridisation to labelled flanking sequences as described in materials and methods. Any positive colonies detected after hybridisation and cells from neighbouring wells in the microtitre dishes were picked and retested by colony hybridisation. Gradient purified pLAFR-3 cosmids from positive colonies were then digested with *Bam* H1 and Southern blot analysis carried out at high stringency (washing at 65°C in 0.1xSSC) to ensure sequences homologous to the Tn5 flanking sequence probes were present in the cosmid inserts isolated.

Library cosmids that contained the wild-type Tn5 insertion sites were named pDUB19••, where the last two digits corresponded to the order in which they were discovered. The cosmid isolated with the *fla-3* flanking sequence (Loake, 1989) had already been labelled pDUB1900.

Figure 3.3 : Primary library screen with 2.1kb Tn5 flanking sequence from pDUB1807.

The photograph shows the colony hybridisation result after the library was plated onto nitrocellulose, lysed and probed with the 2.1kb fragment produced by *Eco* RI and *Hpa* I digestion of pDUB1807. Numbers beside the colonies refer to well numbers of the microtitre dishes in which the library was stored. The film was exposed to the filter for 64 hours at -80°C.

Figure 3.3 : Primary library screening with pDUB1807 flanking sequence.



Four positive clones were detected with the 2.1kb Tn5 flanking sequence from pDUB1807 - figure 3.3. The colony labelled $9B_2$ in the figure proved to be a false positive on secondary screening, but the other three cosmids contained a common 14kb *Bam* H1fragment that hybridised to both the 2.1 and 2.7kb Tn5 flanking sequences in pDUB1807. Cosmids from the three positive library clones in figure 3.3 were designated pDUB1901 ($6H_2$), pDUB1902 ($8G_6$) and pDUB1903 ($9E_5$).

The 3.3kb *Hpa* I fragment from pDUB1806 was used to isolate the cosmid pDUB1905 from the library, with hybridisation occurring to a 12kb *Bam* HI fragment present in the cosmid insert. Use of the 3.3 and 1.2 kb flanking sequences from pDUB1808 together as a mixed probe gave identical results in Southern blot analysis of restriction digests of pDUB1905 as the 3.3kb probe used for its isolation. Flanking sequences from both pDUB1806 and 1808 hybridised to a 12kb *Bam* HI fragment and a 5kb *Eco* RI fragment in the insert. The 5.0kb *Eco* RI band is the expected size if 400bp, which correspond approximately to the ends of Tn5, are subtracted from the total size of the flanking sequences in pDUB1806.

Southern blot analysis of *Bam* HI digests of the cosmids pDUB1900, 1901, 1903 and 1905 demonstrated that the 1.4kb *che-2* Tn5 flanking sequence also hybridised to the 12kb *Bam* HI fragment in pDUB1905. Probing of identical Southern blots of digested pDUB1905 (made by double-sided transfer of fragments from a single gel on to nylon as described in Materials and Methods) with both flanking sequences from pDUB1802 showed that they are homologous to a 5kb *Eco* RI fragment (figure 3.4).

The size of the C58C^1 *Eco* RI fragment that contained the *che-2* Tn5 insertion site was 3.0kb. This raised the possibility that the hybridisation occurring to pDUB1905 was due to the presence of a second homologous sequence to the probe in the C58C^1 genome. An example of this occurring in motility genes is the *flaA* and *flaB* flagellin genes of *Rhizobium meliloti,* whose sequences are 87% identical (Pleier and Schmitt, 1989). To investigate this possibility, the library was screened with the 1.4kb Tn5 flanking sequence from *che-2*, but this only detected pDUB1905 again.

Use of the 1.8 and 1.9kb flanking sequences from pDUB 1811 as separate probes on a double-sided Southern blot of *Bam* HI digests of cosmids pDUB1900-1903 (figure 3.5) showed that the Tn5 insertion in *fla-11* had occurred in the C58C^1 DNA present in pDUB1900. The gel for this Southern blot contained *Eco* RI digested pDUB1811, to act as a positive control for transfer of DNA to both filters and labelling of the probe. The hybridisation to pDUB1900 observed with *fla-11* Tn5 flanking sequences was expected, because it had been shown that this cosmid complemented the *fla-11* mutation and restored motility (Loake, 1989).

Figure 3.4 : Southern blot analysis of pDUB1905 with Tn5 flanking sequences from mutant *che-2*.

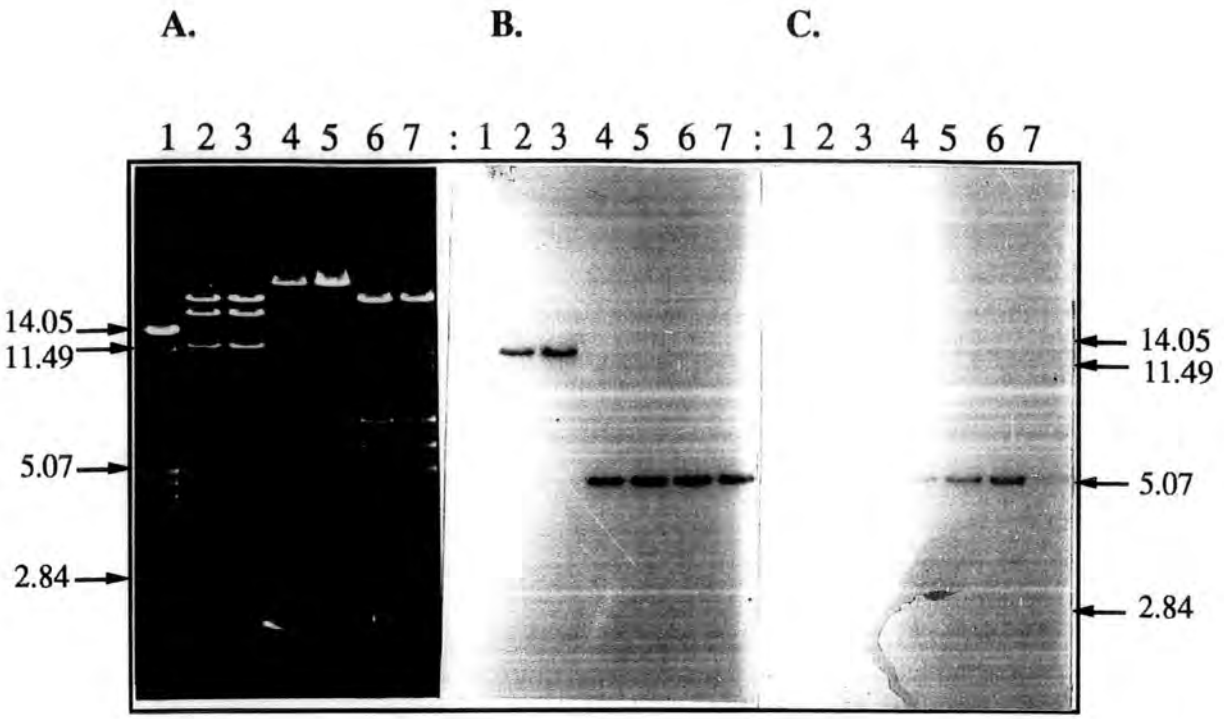


Figure 3.4 : Southern blot analysis of restriction fragments of pDUB1905 with Tn5 flanking sequences from *che-2*.

Panel A shows a photograph of agarose gel electrophoresis of digested pDUB1905 and panels B and C show the resulting blots after double-sided transfer of the DNA to nylon and probing with radiolabelled 1.4kb (panel B) and 2.0kb (panel C) fragments from *Eco* RI/*Hpa* I digested pDUB1802. The films were exposed to the filters for 16 hours (panel B) and 8 hours (panel C) at room temperature. The gel contained DNA from the following restriction digests.

Lane 1	λ-DNA	*Pst* I
Lanes 2 and 3	pDUB1905	*Bam* HI
Lanes 4 and 5	pDUB1905	*Eco* RI
Lanes 6 and 7	pDUB1905	*Bam* HI/*Eco* RI

Figure 3.5 : Southern blot analysis of cosmids pDUB1900-1903 with Tn5 flanking sequences from *fla-11*.

Panel A shows a photograph of the agarose gel electrophoresis of digested C58C[1] library cosmid clones and panels B and C the resulting Southern blots after transfer of the DNA to nitrocellulose and hybridisation with radiolabelled pDUB1811 Tn5 flanking sequences of 1.8 and 1.9 kb respectively. The filters were exposed to X-ray film for 24 hours at -80°C. The gel contained the following DNA samples.

Lane 1	λ-DNA	*Pst* I
Lane 2	pDUB1900	*Bam* HI
Lane 3	pDUB1901	*Bam* HI
Lane 4	pDUB1902	*Bam* HI
Lane 5	pDUB1903	*Bam* HI
Lane 7	pDUB1811	*Eco* RI

Figure 3.5 : Southern blot analysis of C58C1 library cosmids with flanking sequences from *fla-11*.

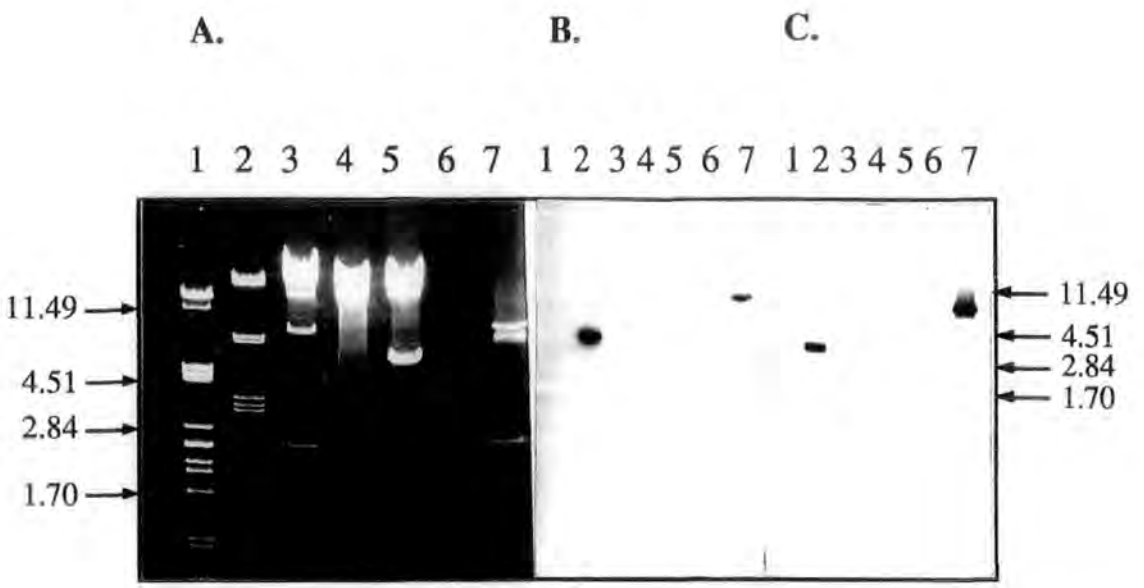


Table 3.2 summarises the cosmid clones isolated from the library and figure 3.6 the order of *Bam* H1 fragments revealed in subsequent mapping experiments.

Table 3.2 Cosmids isolated from library.

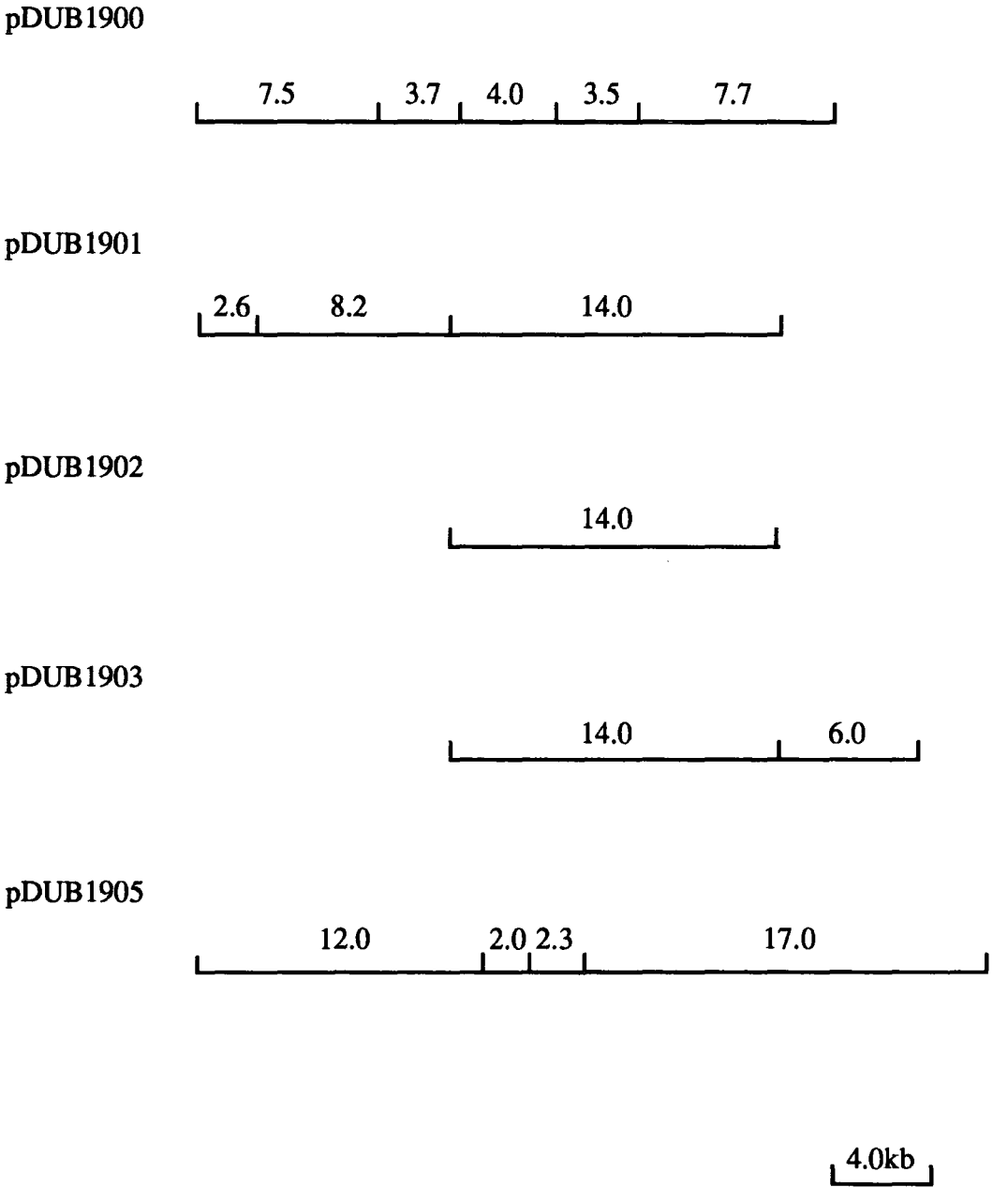
Cosmid	Mutant used for isolation	Size of *Bam* H1 fragments in pLAFR-3 insert
pDUB1900	*fla-3*	3.5
		3.7
		4.0
		7.5
		7.7
pDUB1901	*fla-7*	2.6
		8.2
		14.0
pDUB1902	*fla-7*	14.0
pDUB1903	*fla-7*	6.0
		14.0
pDUB1905	*fla-8*	2.0
		2.3
		12.0
		17.0

3.4 Complementation of mutants.

Loake (1989) showed that a cosmid isolated with flanking sequences from a single mutant (fla-3) could complement the mutations and restore motility to several other mutants, including *fla-11*. This provided some evidence that genes required for chemotaxis and motility are clustered together on the *Agrobacterium* chromosome. In order to discover if pDUB1905 complemented any of the mutants, which might have pointed to the occurrence of Tn5 insertion loci other than those of *che-2*, *mot-6* and *fla-8* being present in the insert of the cosmid, pDUB1905 was transferred to all twenty mutants.

Triparental matings of HB101/pDUB1905 x ED8767/pRK2013 x mutant strains were carried out using nitrocellulose filters, as described in the materials and methods, using a 1:1:1

Figure 3.6 : *Bam* HI maps of cosmids isolated from C58C1 library using flanking sequences of motility mutant Tn5 insertion sites .



volume ratio of the three strains. Transconjugants were selected on Rif/Km/Tc solid agar plates for 24 hours at 28°C. *Bam* H1 digestion of plasmid minipreps from selected colonies confirmed the presence of pDUB1905 in the mutants and cells were inoculated into swarm agar plates from the miniprep cultures, together with the corresponding motility mutant. Swarming transconjugant cells could be easily identified by comparison of colony morphology with the relevant mutant strain after incubation at 28°C for 48 hours. The 3-keto-lactose plate test was carried out with any motile cells, to ensure they were *Agrobacterium* biotype 1 strains, rather than contaminating Tc resistant *E. coli*.

Transfer of the cosmid to the mutants showed that it complemented only *che-2*, *mot-6* and *fla-8*, restoring wild-type swarm morphology (figure 3.7). This result suggests that none of the other mutant Tn5 insertion loci were present in the genomic insert in pDUB1905, although lack of transcription or translation may have prevented complementation of any other mutants.

DNA hybridisation experiments localized the the Tn5 insertion sites of all but one of the non-motile behavioural mutants to specific library cosmids (Shaw *et al.*, 1991). Only the *fla-7* mutation mapped to the cosmids pDUB1901-1903, which meant it was extremely unlikely that these plasmids would complement any of the mutants apart from *mot-5*, whose insertion site has not been localized. Conjugation of all three of these plasmids into *mot-5* was tried on a number of occasions but no tetracycline resistant colonies could be obtained.

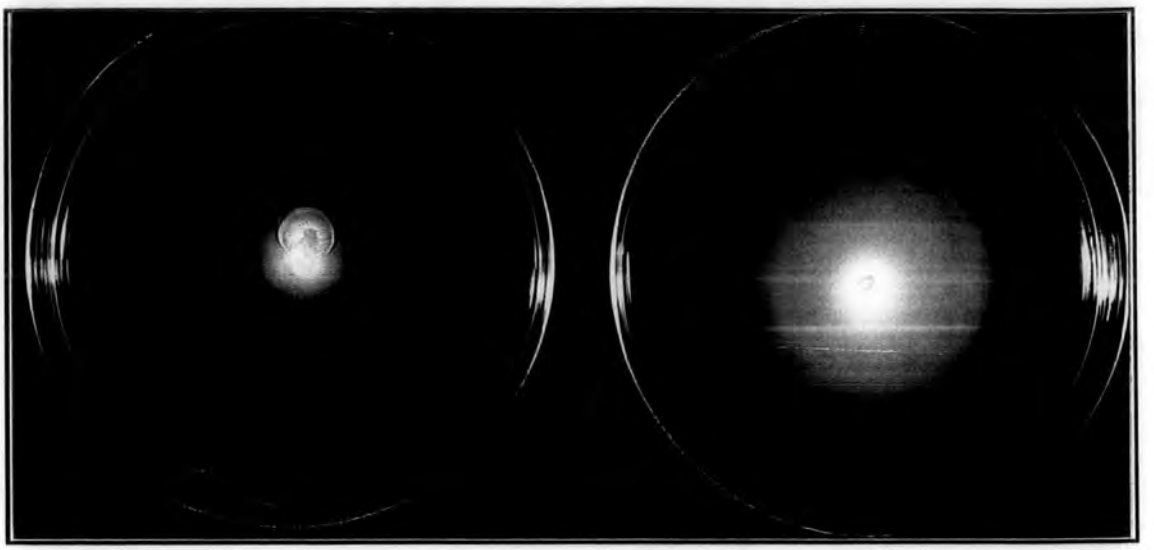
3.5 Mapping of pDUB1905.

Hybridisation of the 1.4 and 2.0kb *Eco* RI/*Hpa* I fragments of pDUB1802 to a 5kb *Eco* RI fragment in pDUB1905 (figure 3.4) suggested that a deletion had occurred on insertion of Tn5 into the *che-2* genome. This would have meant that the Tn5 flanking sequences in pDUB1802 were not adjacent in the *Agrobacterium* genome and hybridisation data obtained for mapping using these fragments would be difficult to interpret. To investigate the possibility that a deletion had occurred in *che-2*, both of the flanking sequences were labelled by random priming and used as probes against a double-sided blot of *Eco* RI digested chromosomal DNA from *che-2* and C58C^1 (figure 3.8). The probes were hybridised at 65°C and washed in 0.1xSSC at 65°C.

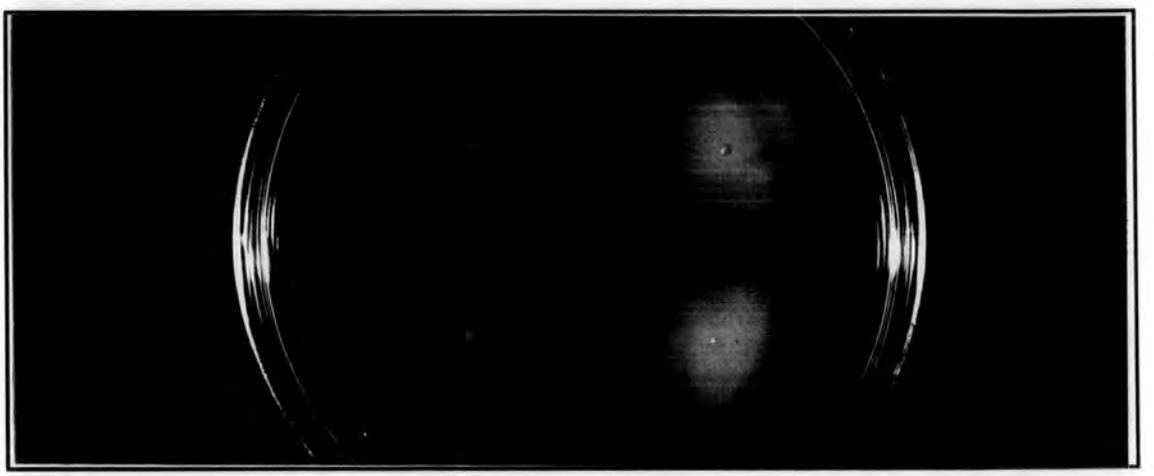
Both flanking sequences hybridised to a 5.0kb fragment in C58C^1 DNA, as expected from the data obtained from pDUB1905. Two fragments of 10.8 and 8.8kb were observed in the *che-2* lane, with the upper band of 10.8kb binding both probes to a much greater extent. These two bands were not therefore the result of a partial digestion, as the 8.8kb fragment would have been the more strongly hybridising species. The 10.8kb band corresponds to a 5kb *Eco* RI fragment

Figure 3.7 : Complementation of mutants by pDUB1905.

A.



B.



C.

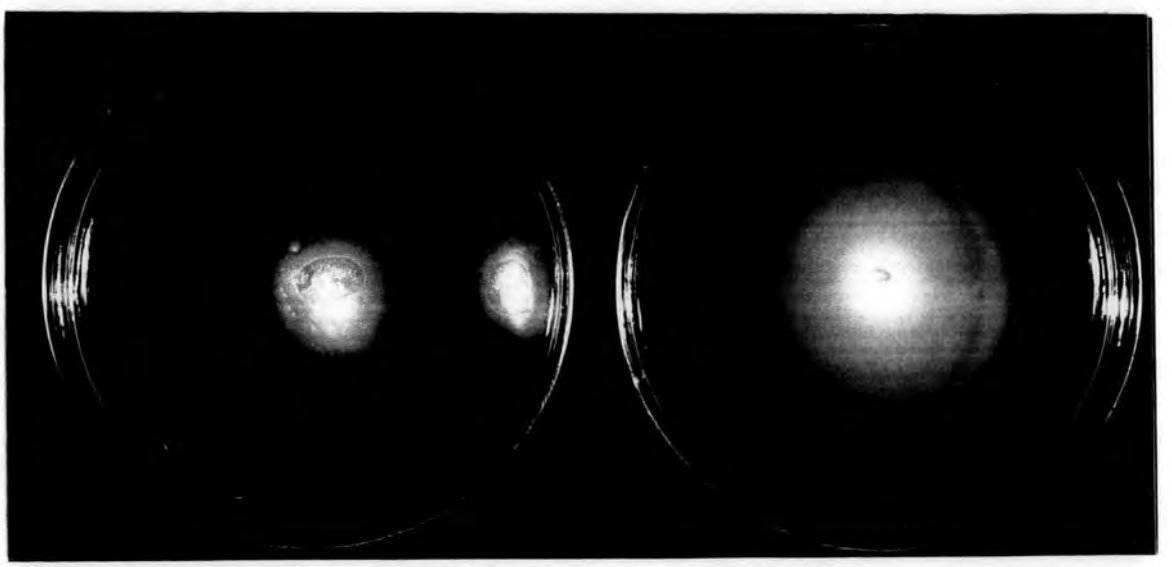


Figure 3.7 : Complementation of mutants by pDUB1905.

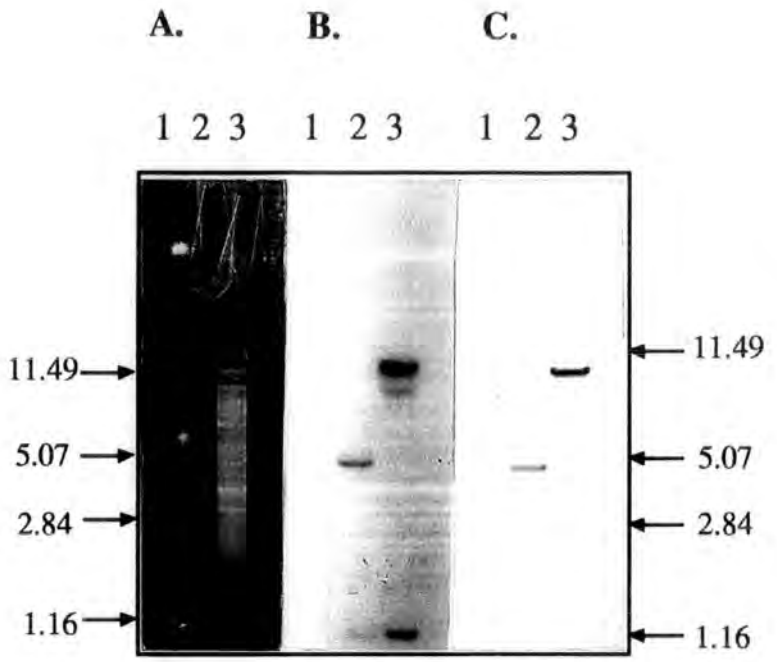
Panels show photographs of 0.2% agar swarm plates with mutants on the left and corresponding complemented strains on the right . Strains are *mot-6* - panel A, *fla-8* - panel B, and *che-2* - panel C.

Figure 3.8 : Southern blot analysis of C58C[1] and *che-2* chromosomal DNA with Tn5 flanking sequences from pDUB1802.

Panel A shows a photograph of the agarose gel electrophoresis of chromosomal DNA and panels B and C the resulting Southern blots after double-sided transfer of the DNA to nylon and hybridisation to radiolabelled DNA fragments of 1.4 (panel B) and 2.0 (panel C) kb arising from *Eco* RI/*Hpa* I digestion of pDUB1802. The filters were exposed to X-ray film for 96 hours at -80°C in both cases. The gel contained DNA from the following sources.

Lane 1	*Pst* I cut λ-DNA	
Lane 2	C58C[1]	*Eco* RI
Lane 3	*che-2*	*Eco* RI

Figure 3.8 : Hybridisation of pDUB1802 Tn5 flanking sequences to C58C1 and *che-2* chromosomal DNA.



containing Tn5, which showed that a deletion had not occurred and the *che-2* flanking sequences were continuous in C58C[1] wild-type DNA.

The sizes of the flanking sequences observed in pDUB1802 were probably the result of *Eco* RI star activity during the chromosomal digest used for the construction of the plasmid and this activity was apparent again in the digest for figure 3.8, producing the weakly hybridising 8.8kb fragment observed. The 1.2kb fragment that bound the 1.4kb probe relatively weakly presumbly contained a homologous, but not identical sequence to the probe which meant that it was not detected during colony hybridisation to the library using the 1.4kb *che-2* flanking sequence.

Tn5 flanking sequences from *che-2* and *mot-6* were used in Southern blot analysis of single and double-digested pDUB1905, to construct a map for the chromosomal insert of this cosmid. pDUB1905 was digested with *Bam* HI, *Eco* RI and *Hind* III in single and double digests and the fragments separated on a 0.7% gel. After transfer of the DNA to nylon filters in a double-sided blot, the DNA was hybridised to radiolabelled Tn5 flanking sequences from pDUB1802 at 65°C. The filters were washed in 0.1xSSC at 65°C (figure 3.9a). The fragments that hybridised to the probe in panel B were the following sizes. Lane 2 - 12.0kb, lane 3 - 5.0kb, lane 4 - 8.0 and 3.6kb, lane 5 - 5.0kb, lane 6 - 2.4 and 2.6kb, lane 7 - 8.8 and 3.6kb.

The *che-2* Tn5 flanking sequences were then removed from the filter and the blots hybridised to the 1.2kb *Eco* RI/*Hpa* I and 3.3kb *Hpa* I fragments from pDUB1806. The 3.3kb probe hybridised to the same fragments as those seen in panel B and the 1.2kb fragment produced a similar hybridisation pattern, except the 3.6kb *Hind* III and 2.4kb *Eco* RI/*Hind* III fragments were not present. Analysis of the Southern blot hybridisation data and the sizes of restriction fragments in figure 3.9a enabled a *Bam* HI and *Hind* III map of the chromosomal insert of pDUB1905 to be constructed - figure 3.9b. The 5.0kb *Eco* RI chromosomal fragment, that contained the Tn5 insertions in *che-2, mot-6* and *fla-8* , mapped to the position indicated.

The mapping of *Eco* RI sites in the chromosomal insert, apart from those shown in figure 3.9b, was complicated by the occurrence of 13 restriction sites for this enzyme. Total digestion of pDUB1905 with *Eco* RI produced 14 restriction fragments, including a number that were too small to order by double enzyme restriction analysis (figure 3.10 panel A). In order to be able to map some of the *Eco* RI sites, and to aid in construction of a more detailed map of the region containing the Tn5 insertion loci, the 12kb *Bam* HI fragment of pDUB1905 was cloned into pBR322. This vector was chosen because of its widely spaced restriction sites, which made analysis of double digests easier.

After ligation of the 12kb fragment into alkaline phosphatase-treated *Bam* HI cut pBR322, recombinants were selected by the inactivation of the *tet* gene which occurs on cloning into the

Figure 3.9 a : Mapping of pDUB1905 by hybridisation of Tn5 flanking sequences from pDUB1802.

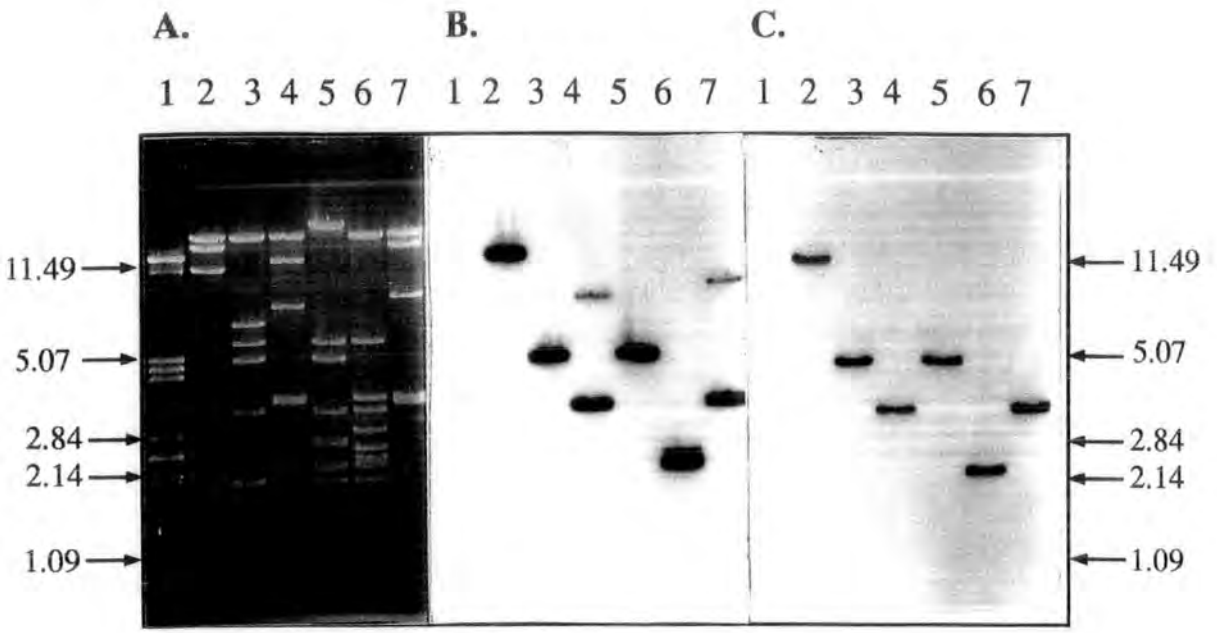


Figure 3.9 b : Map of chromosomal insert in pDUB1905

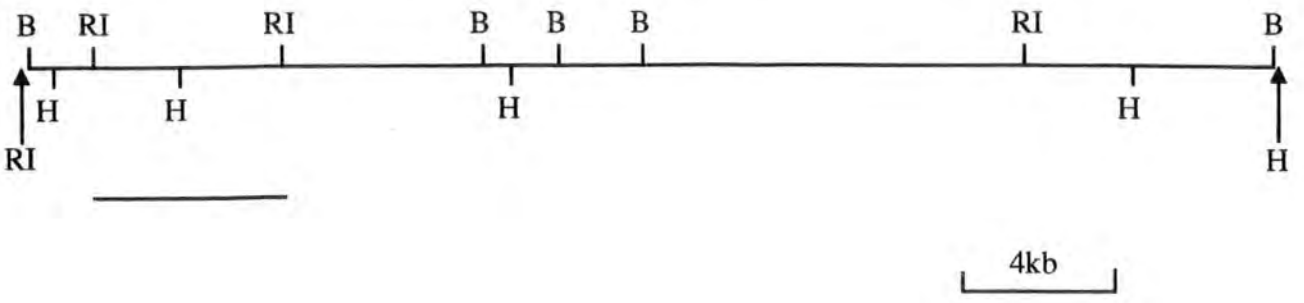


Figure 3.9 a : Southern blot analysis of digested pDUB1905 withTn5 flanking sequences from pDUB1802.

Panel A shows the agarose gel electrophoresis of restriction fragments of pDUB1905 and panels B and C the resulting Southern blots after double-sided transfer of the DNA to nylon and hybridisation to the *Eco* RI/*Hpa* I fragments of 1.4kb (panel B) and 2.0kb (panel C) from pDUB1802. The filters were exposed to X-ray film for 24 and 60 hours respectively at -80°C. The gel contained DNA from the following restriction digests.

Lane1	λ-DNA	*Pst* I
Lane 2	pDUB1905	*Bam* HI
Lane 3	"	*Bam* HI/*Eco* RI
Lane 4	"	*Bam* HI/*Hind* III
Lane 5	"	*Eco* RI
Lane 6	"	*Eco* RI/*Hind* III
Lane 7	"	*Hind* III

Figure 3.9 b : Map of chromosomal insert in pDUB1905

Restriction sites are designated as B - *Bam* HI, RI - *Eco* RI and H - *Hind* III. The sites marked under the vertical arrows occur in the polylinker of pLAFR-3. The line underneath the map illustrates the 5.0kb *Eco* RI fragment to which Tn5 flanking sequences frm *che-2* and *mot-6* hybridise. Only the *Eco* RI sites that define this fragment are shown.

Figure 3.10 : Analysis of small restriction fragments surrounding the *che-2* Tn5 insertion site.

The panels show photographs of 1.4% agarose gels containing the following DNA samples. The size markers are *Pst* I cut λ-DNA in lane 1 of each panel and *Hind* III digested λ-DNA in lane 5 of panel B.

Panel A : pDUB1905 digested with *Bam* HI - Lane 2, *Bam* HI/*Eco* RI - lane 3, *Eco* RI - lane 4 and *Eco* RI/*Hpa* I - lane 5.

Panel B : pAB1 (pBR322 containing the 12kb *Bam* HI fragment of pDUB1905) digested with *Sph* I/*Hind* III - lane 2, *Sph* I - lane 3 and *Hind* III - lane 4.

Panel C : pUC18 containing the 1.4kb *Eco* RI/*Hpa* I Tn5 flanking sequence from *che-2* digested with *Eco* RI/*Hind* III - lane 3, *Sal* I - lane 4, *Sal* I/*Hind* III - lane 5 and *Hind* III - lane 6. Lane contained *Hind* III digested pUC18.

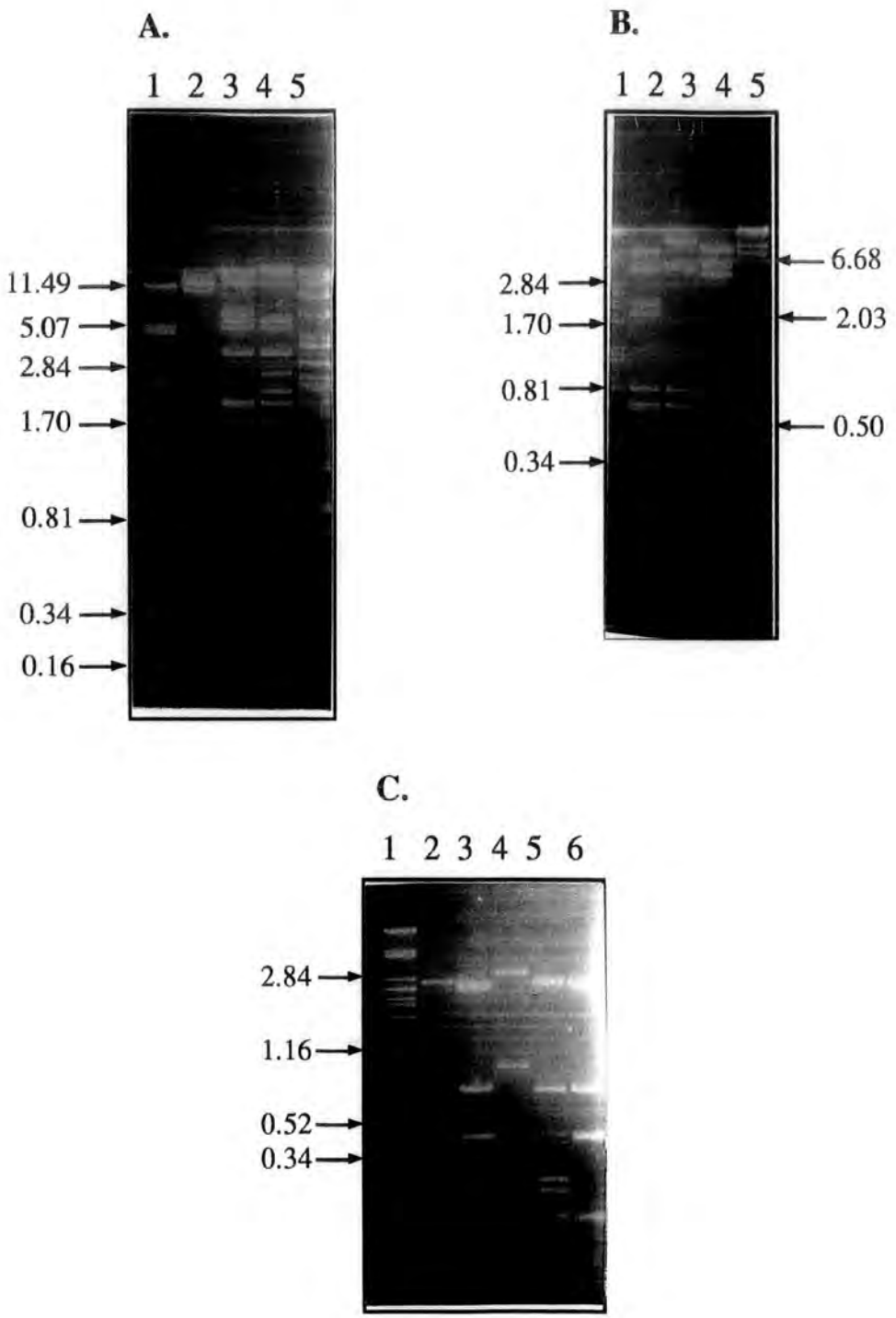
Figure 3.11 a : Partial digestion of pAB1 for mapping of restriction sites.

Panels A and B show photographs of agarose gels of partial digests of the plasmid pAB1. In panel A *Eco* RI was the enzyme used and in panel B *Sal* I. Lane 1 contained *Pst* I digested λ-DNA in both cases and lanes 2 to 10, DNA samples from time points 1, 3, 5, 10, 15, 20, 30, 45 and 60 minutes respectively after the onset of digestion.

Figure 3.11b : *Eco* RI map for part of pDUB1905 obtained after partial digestions.

Restriction sites are designated as B - *Bam* HI, RI - *Eco* RI and H - *Hind* III.

Figure 3.10 : Analysis of small restriction fragments surrounding the *che-2* insertion.



Bam HI site of pBR322. Ampicillin resistant transformants were replica-plated onto ampicillin and tetracycline containing L-B plates and plasmids contained in several tetracycline sensitive colonies further analysed. One plasmid obtained during this procedure, designated pAB1 was used for mapping of restriction sites in the 12kb insert by three different methods. These were partial enzyme digestion, single and double enzyme restriction digests and Southern blot analysis of digests with a Tn5 flanking sequence from *mot-6*.

Restriction fragments present in partial digests, but not in complete digests enabled restriction fragments for a single enzyme to be located adjacent to each other in the plasmid. Partial digestions of the plasmid pAB1 were obtained by taking aliquots at timed intervals from a digest set up to produce complete digestion after 1 hour and adding them to 100mM EDTA. Figure 3.11a shows the results for *Eco* RI and *Sal* I partial digestion of the plasmid. Figure 3.11b is a map of part of pDUB1905 including the *Eco* RI sites located after partial digests.

Southern blot analysis of double digests of pAB1 was carried out with the 3.3kb Tn5 flanking sequence from pDUB1808 to provide data on the order of some of the restriction fragments in the pBR322 insert. Double digests including *Hind* III were carried out as a site for this enzyme was contained in the 3.3kb probe and it had already been mapped in pAB1. Restriction fragments were separated on a 0.7% gel and transferred to a nylon filter before hybridisation at 65°C to the radiolabelled probe. The blot was washed at high stringency in 0.1xSSC at 65°C (figure 3.12a).

Fragments that hybridised to the probe were adjacent in the plasmid and analysis of the Southern blot, together with single and double enzyme restriction data, provided the information for the construction of a detailed map of the region containing Tn5 in three motility mutants (figure 3.12b)

The site of insertion of Tn5 in the C58C^1 motility mutants could be determined by comparison of restriction maps from the wild type chromosomal DNA present in the library cosmids with those of the sequences flanking Tn5 in the pDUB18•• plasmids. The 5.0kb *Eco* RI fragment in pDUB1905 that overlaps the three Tn5 insertion loci is cut once by *Hpa* I to give fragments of 1.2 and 3.8kb (figure 3.10a). pLAFR-3 contains one *Hpa* I restriction site 2.6kb from the *Hind* III of the vector and digestion of pDUB1905 produced two fragments of 25 and 30kb. These results meant there was a single *Hpa* I site in the insert of pDUB1905, in the position shown in figure 3.13a.

Eco RI/*Hpa* I restriction fragments of both pDUB1808 and 1806 contained a 1.2 kb fragment and the presence of a 4.8kb fragment on *Hpa* I digestion of these plasmids meant that the 3.3kb fragment was adjacent to Tn5. The Tn5 insertions that caused the *mot-6* and *fla-8* can therefore be localized to a region 700bp from one end of the 5.0kb *Eco* RI fragment as shown in

Figure 3.12 a : Southern blot analysis of pAB1 fragment with 3.3kb Tn5 flanking sequence from pDUB1808.

Panel A shows the agarose gel electrophoresis of digested pAB1 and panel B the Southern blot resulting from transfer of the DNA to nylon and hybridisation to radiolabelled 3.3kb Tn5 flanking sequence arising from *Eco* RI/*Hpa* I digestion of pDUB1808. The filter was exposed to X-ray film for 9 hours at -80°C. Apart from lane 1, the DNA samples were pAB1 cut with the following enzymes.

Lane 1	*Pst* I digested λ-DNA
Lane 2	*Bam* HI/*Hind* III
Lane 3	*Eco* RI/*Hind* III
Lane 4	*Pst* I/*Hind* III
Lane 5	*Pvu* II/*Hind* III
Lane 6	*Sal* I/*Hind* III
Lane 7	*Sph* I/*Hind* III

Figure 3.12 b : Detailed map of 12kb *Bam* HI fragment of pDUB1905

The figure shows a map for the 12kb *Bam* HI fragment in pAB1 and pDUB1905 to which Tn5 flanking sequences from *che-2*, *mot-6* and *fla-8* hybridise. B denotes the *Bam* H1 sites defining the fragment. The position of the C58C[1] DNA in the 3.3kb probe used for figure 3.12a is shown.

Figure 3.13 : Mapping of Tn5 insertion sites in pDUB1905.

Figure 3.13a. is a map of the 12kb *Bam* HI fragment in pDUB1905 for the enzymes *Eco* RI and *Hind* III and a more detailed map for the 5kb *Eco* RI fragment which contains the Tn5 insertions in the motility mutants *che-2*, *mot-6* and *fla-8*. The proposed site for *Eco* RI star activity that caused the shortened insert in pDUB1802 is marked E*. Restriction sites are marked B -*Bam* HI, RI - *Eco* RI, H - *Hind* III, Hp - *Hpa* I, S - *Sal* I and Sp - *Sph* I throughout the figure.

Figure 3.13b shows the maps of the Tn5 flanking sequences of *che-2* and the *Eco* RI pUC insert in the plasmid pDUB1808. Regions corresponding to Tn5 are shown as thick lines.

Figure 3.13c is a map of the 5kb fragment of pDUB1905 with the Tn5 insertion sites of the three mutants marked.

Figure 3.11 a : Partial digestion of pAB1 for mapping of restriction sites.

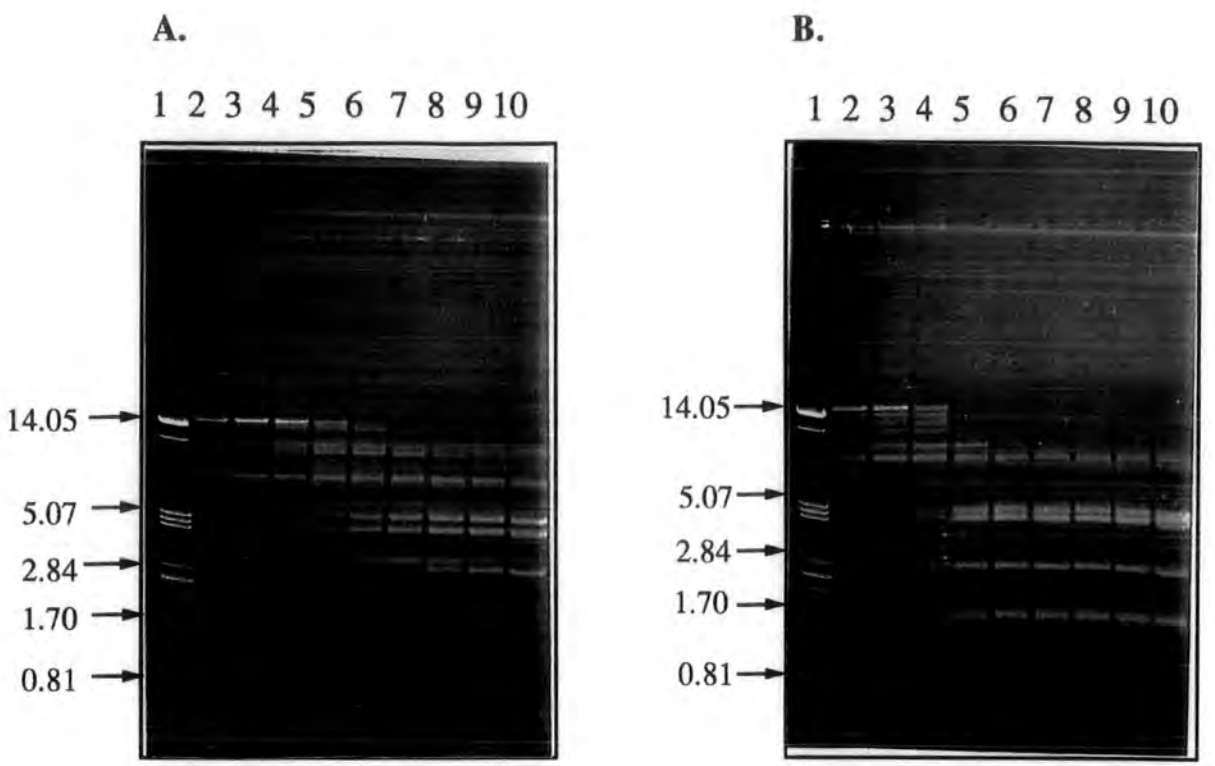


Figure 3.11 b : *Eco* RI map for part of pDUB1905 deduced from partial digestion.

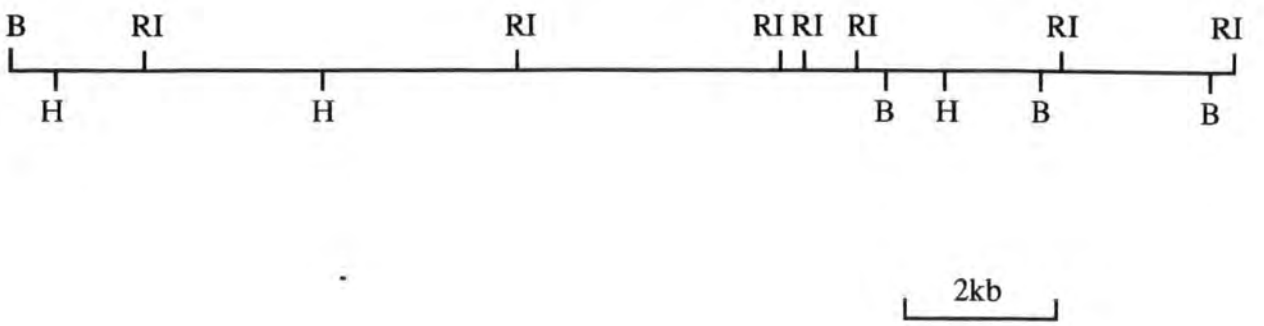


Figure 3.12 a : Mapping of pAB1 hybridisation with 3.3kb Tn5 flanking sequence from pDUB1808.

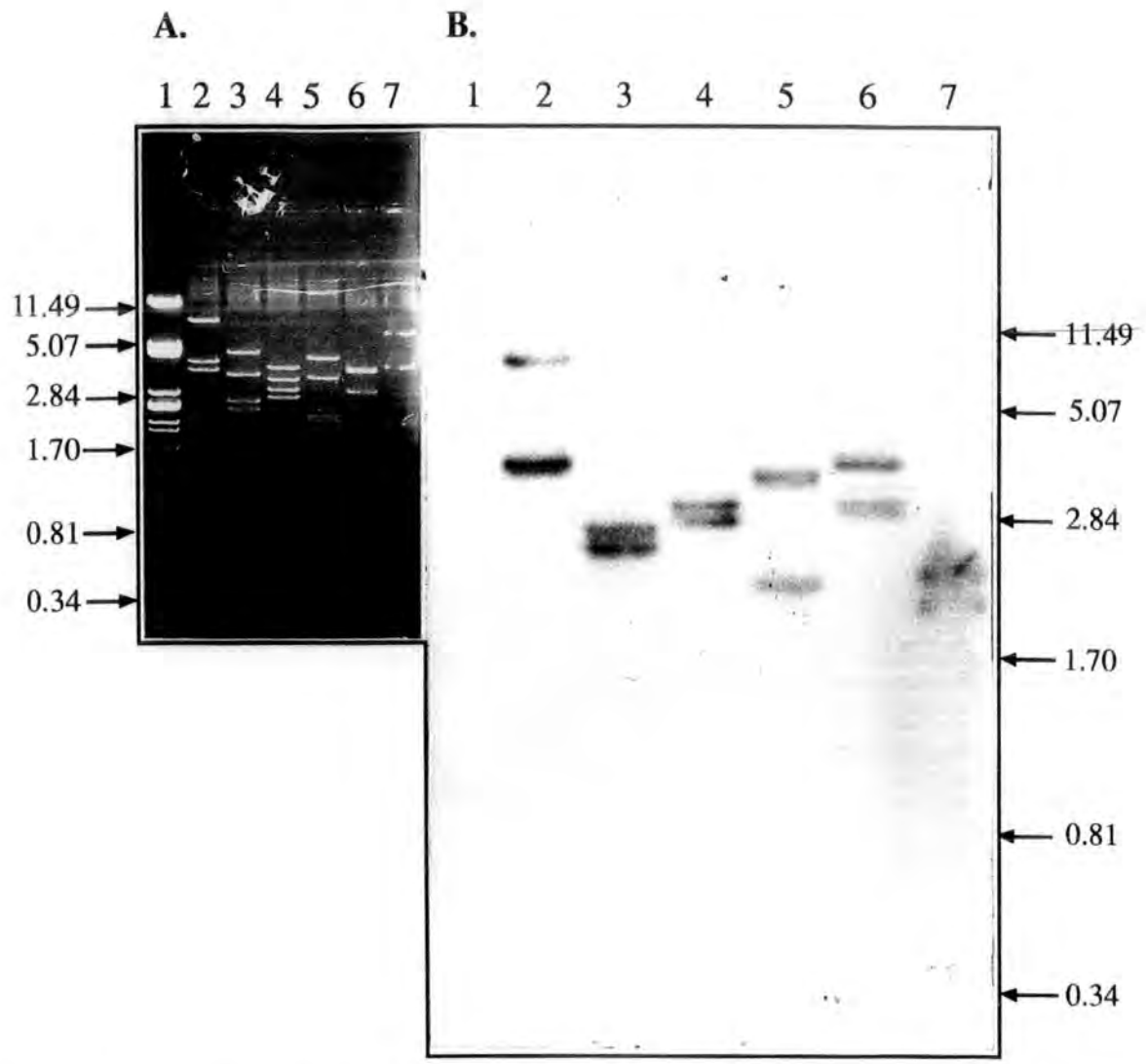


Figure 3.12 b : Map of pAB1 insert.

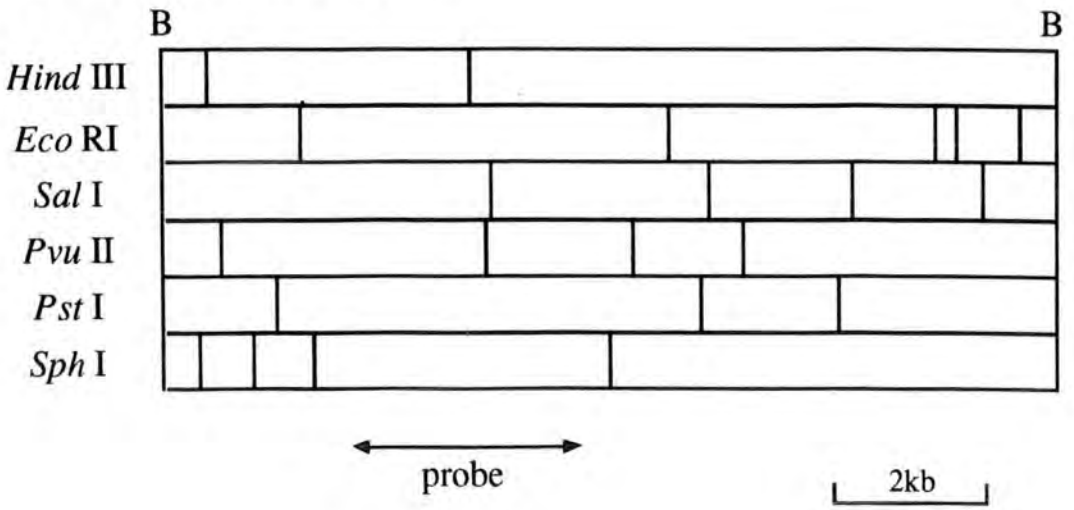


Figure 3.13 : Mapping of Tn5 insertion sites in pDUB1905.

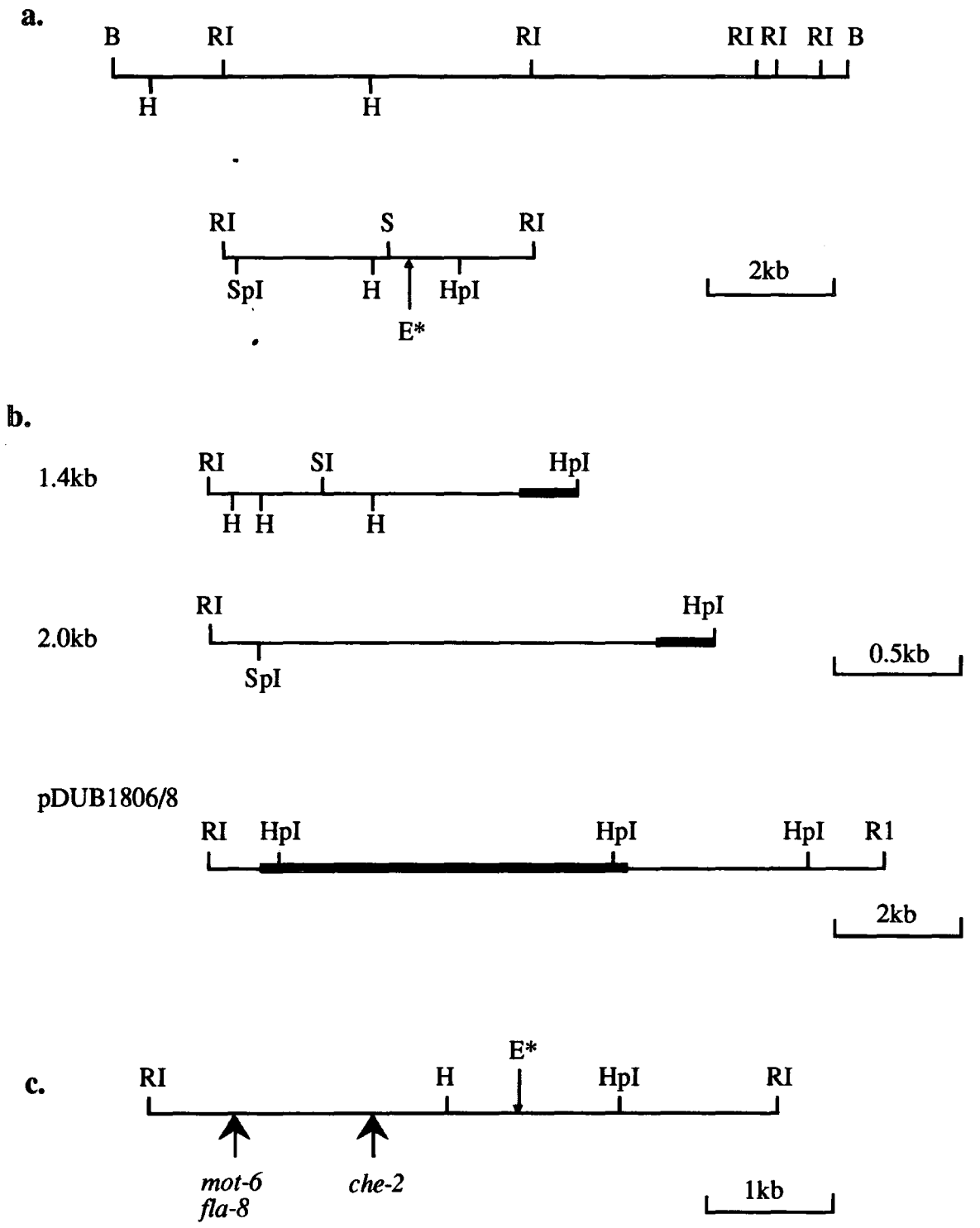


figure 3.13c. The exact locations of these two mutation sites are too close together on the chromosome to be distinguished by restriction analysis of any plasmid.

The two Tn5 flanking sequences from pDUB1802 were subcloned separately into *Eco* RI/*Sma* I digested pUC18 and analysis of restriction fragments using enzymes that cut in the polylinker of the vector carried out. Figure 3.13b shows restriction maps for both of these DNA sequences (see figure 3.10c for the data on the 1.4kb fragment). Two of the three *Hind* III restriction sites in this fragment were not expected to be present after results obtained with pAB1 and pDUB1905. The two *Hind* III sites closest to the *Eco RI* restriction site are probably cloning artefacts caused by mismatches during DNA replication, as they are not present in the wild type DNA. This is shown by the fact that neither a 450 or 550p fragment is apparent after *Hind* III digestion of pAB1- Figure 3.10c. The relative positions of the remaining *Hind* III site together with the *Sal* I site are in agreement with the chromosomal DNA maps and show that the Tn5 insertion in *che-2* is at the point shown in figure 3.13c. This is confirmed by the map obtained for the 2.0kb fragment, shown in figure 3.13b. The presumed position of an *Eco* RI star activity restriction site, which must have the sequence XAATTC to reform an *Eco* RI site in pDUB1802, is also illustrated in figure 3.13.

Mapping of three Tn5 insertion loci, responsible for motility mutants with different phenotypes, within a 1.2kb region of the *Agrobacterium* genome strongly suggests that the chemotaxis and motility genes of this bacterium are grouped together like those seen in *E. coli* and *R. meliloti*.

3.6 Clustering of motility genes in *Agrobacterium*.

Clustering of Tn5 insertion sites in the C58C[1] motility mutants and therefore probably also motility genes in *Agrobacteterium* was hinted at by the complementation data of Loake (1989) which showed that the presence of a single library cosmid, pDUB1900, in eight of the behavioural mutants restored motility. The hypothesis that behavioural genes were grouped together was supported by the complementation of three mutants with pDUB1905 as described earlier. DNA hybridisation studies were used to map Tn5 insertion loci in a number of motility mutants to try and definitely prove the close proximity of mutated sequences.

In addition to the behavioural mutant Tn5 flanking sequences isolated from *fla-3* (Loake, 1989) and the five mutants described in section 3.2, the regions flanking Tn5 have been isolated from all of the ten other non-motile mutants. Colony and Southern blot hybridisation experiments using these sequences as probes have localized the mutated sequences to a total of 7 cosmids and

the Tn5 insertion sites have been mapped for all but three of the mutants (*mot-5, tpc-17* and *fla-19*). The results show that twelve of the insertion sites are clustered together in two separate chromosomal regions.

The first of these overlaps the insert of pDUB1900, which was isolated using *fla-3* Tn5 flanking sequences (Loake, 1989). The presence of a number of mutant Tn5 insertion sites in this cosmid was demonstrated by flanking sequence hybridisation at high stringencies to digests of this cosmid (experiments carried out in association with those named in Shaw *et al.*, 1991). Analysis of the data obtained from these experiments enabled a restriction map of the chromosome overlapping this cosmid to be constructed for the enzymes *Eco* RI, *Bam* HI, *Hind* III and *Hpa* I (map constructed by Dr C.H.Shaw). A total of nine of the motility mutants, of both Fla$^-$ and Mot$^-$ phenotype, were caused by independent Tn5 insertions in an 18kb region of the chromosome (figure 3.14). This demonstrates that several genes required for motility in *Agrobacterium* are clustered together in the genome.

Data shown in previous sections shows that three more of the mutations are caused by disruption of a 5.0kb fragment present in pDUB1905, which indicates that region also encodes a cluster of motility genes.

Flanking sequences from *fla-7, tpc-17* and *fla-19* have been used for the isolation of five more cosmids from the library (pDUB1901-1904 and pDUB1906) which contain genes required for motility. It is unknown whether any other behavioural genes are present on these cosmids, as none of the other motility mutants contained Tn5 in their chromosomal inserts. Table 3.2 sumarises the complementation and flanking sequence hybridisation data that has been obtained so far.

3.7 Similarity between motility genes in *Agrobacterium* and *Rhizobium*

Agrobacterium and *Rhizobium* spp. possess homologous and functionally interchangeable genes that are required during the initial stages of the plant / bacteria interaction. The type of cell motility observed in the two genera are also very similar. Tn5 flanking sequences from several C58C^1 motility mutants were used in Southern blot analysis of chromosomal digests to assess the similarity of behavioural genes in these two Rhizobiaceae genera. Homologies to DNA from other motile bacteria was also investigated.

Chromosomal DNA from eight different motile strains (described in legends to figures 3.15-3.17) was isolated and purified by density gradient centrifugation. 5µg samples of DNA

Figure 3.14 : Map of the *A. tumefaciens fla-mot* cluster overlapped by pDUB1900.

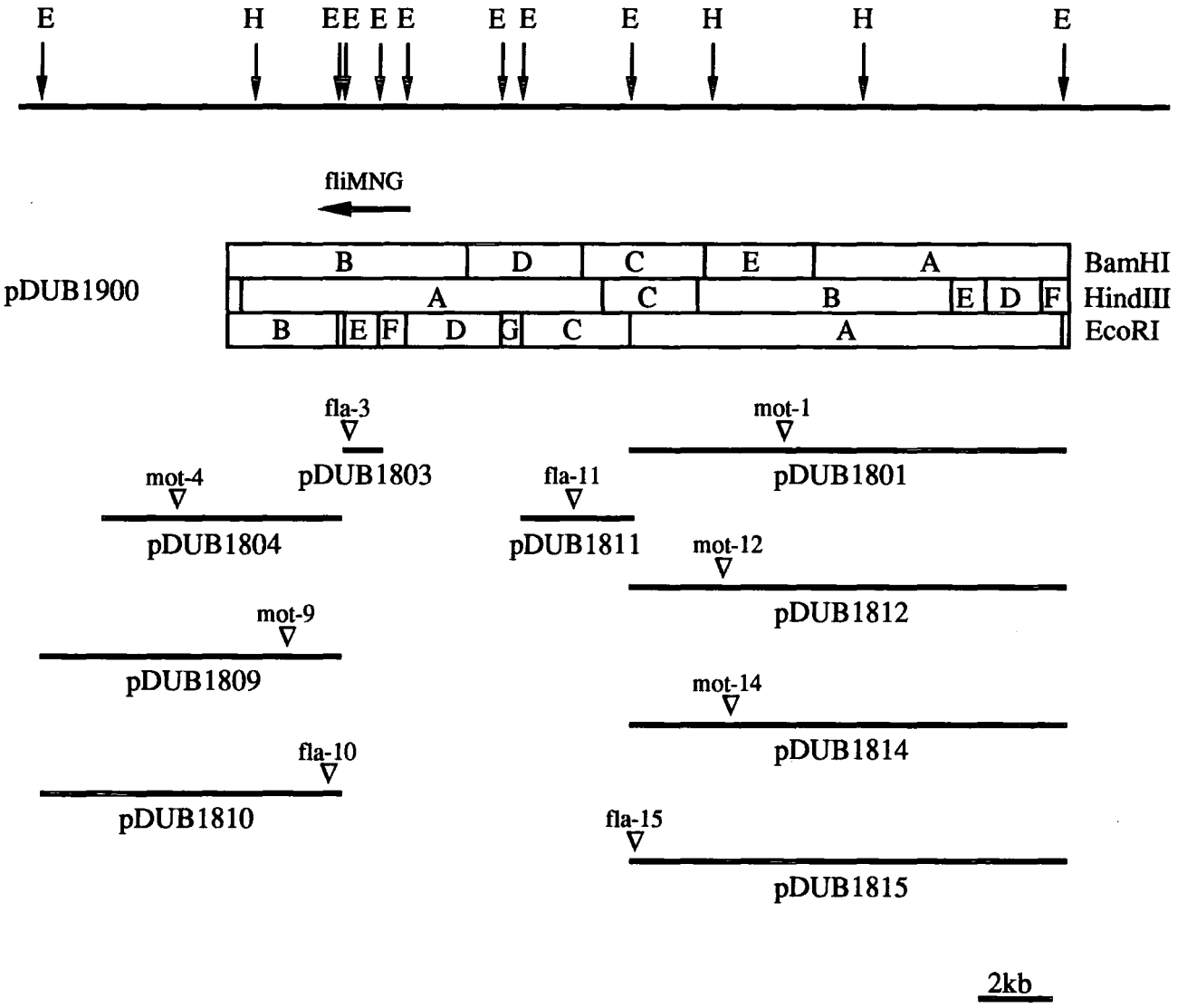


Fig. 1: Top line: EcoRI (E) and HpaI (H) sites within cluster 1 . Boxes denote BamHI, HindIII & EcoRI sites within the chromosomal insert in cosmid pDUB1900. Lines below that depict the extents of the cloned fragments isolated from various mutants, the Tn5 insertion site in each denoted by ∇ Horizontal arrow represents known extent of fliMNG operon.

Table 3.2 Properties of behavioural gene cosmids.

Mutant	Plasmid containing Tn5 flanking sequence	Flanking sequences hybridise to cosmid	Mutant complemented by cosmid
mot-1	pDUB1801	pDUB1900	pDUB1900
che-2	pDUB1802	pDUB1905	pDUB1905
fla-3	pDUB1803	pDUB1900	pDUB1900
mot-4	pDUB1804	pDUB1900	pDUB1900
mot-5	pDUB1905		
mot-6	pDUB1806	pDUB1905	pDUB1905
fla-7	pDUB1807	pDUB1901/2/3	
fla-8	pDUB1808	pDUB1905	pDUB1905
mot-9	pDUB1809	pDUB1900	pDUB1900
fla10	pDUB1810	pDUB1900	
fla-11	pDUB1811	pDUB1900	pDUB1900
mot-12	pDUB1812	pDUB1900	
mot-14	pDUB1814	pDUB1900	pDUB1900
fla-15	pDUB1815	pDUB1900	pDUB1900
tpc-17	pDUB1817	pDUB1904	ND
fla-19	pDUB1819	pDUB1906	ND

ND, not determined.

from different bacteria were digested to completion with *Eco* RI and separated by agarose gel electrophoresis before transfer to filters and hybridisation to radiolabelled flanking sequences from *che-2*, *mot-6*, *fla-7* and *fla-11*. Hybridisations were at 65°C and washes were carried out at 65°C in 0.1xSSC unless otherwise indicated

Tn5 flanking sequences from the *mot-6* mutation hybridised to *Agrobacterium* DNA only and demonstrated no homology to any of the other bacterial chromosomal DNA used. These were the only probes used that did not hybridise to any other samples. A combined 3.3 and 1.2kb probe from *Eco* RI/*Hpa* I digested pDUB1806 hybridised to a 5.0kb band in C58C^1 and GM19023 (figure 3.15). GM19023 is a C58C^1 derivative that has been cured of the 410kb cryptic plasmid by plasmid incompatibility (Rosenberg and Huguet, 1984). The identical hybridisation pattern observed in the C58C^1 and GM19023 lanes show that the Tn5 insertion in *mot-6* was not into the cryptic plasmid. No differences were seen between C58C^1 and GM19023

Figure 3.15 : Southern blot analysis of cloned *R. meliloti* behavioural genes and bacterial chromosoml DNA with sequences from pDUB1806.

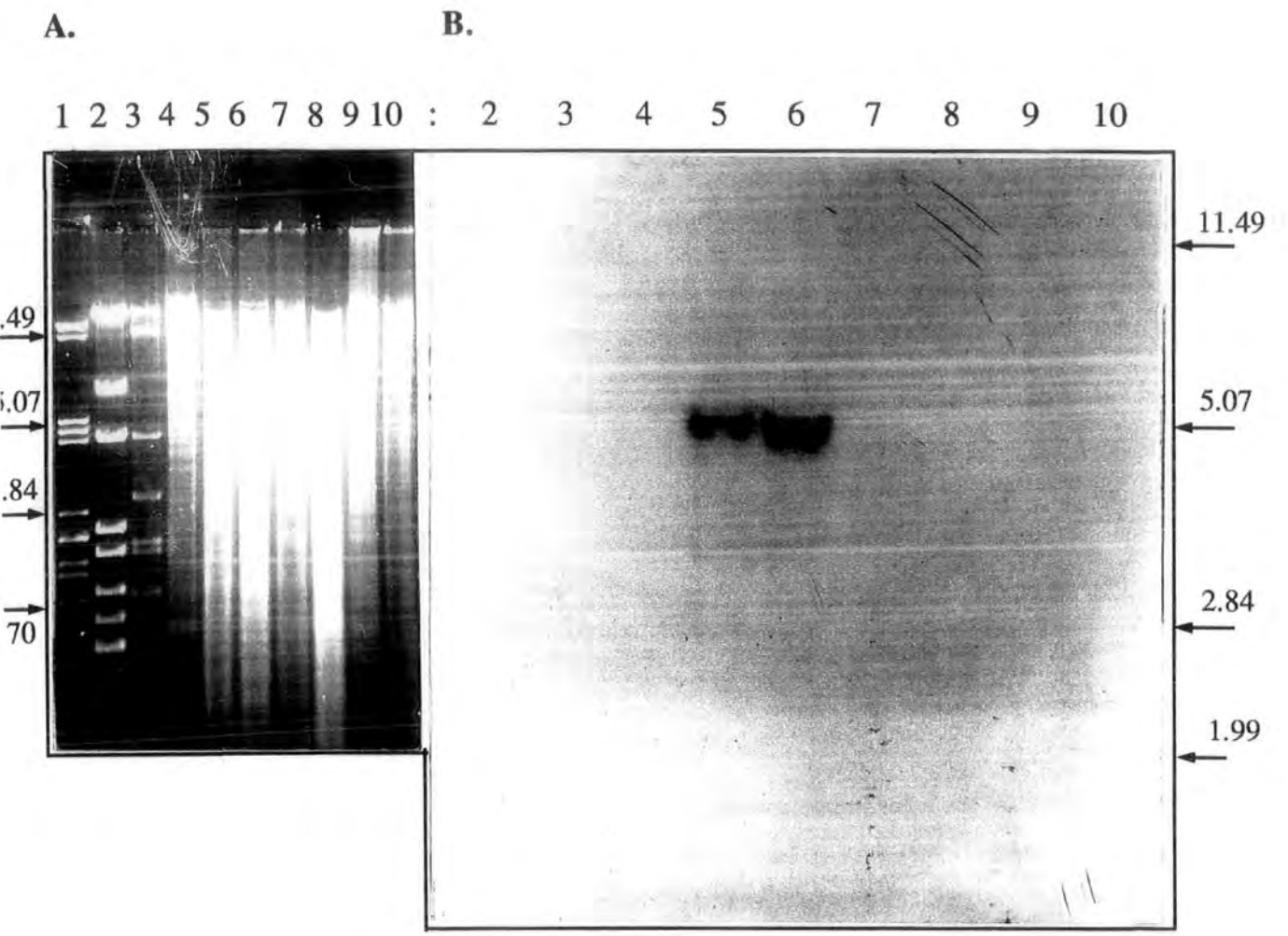


Figure 3.15 : Southern blot analysis of bacterial chromosomal DNA with Tn5 flanking sequences from mutant *mot-6*.

Panel A shows a photograph of agarose gel electrophoresis of DNA samples digested with *Eco* RI and panel B the resulting Southern blot after transfer of the DNA to nitrocellulose and hybridisation with radiolabelled 3.3 and 1.2kb fragments from *Eco* RI/*Hpa* I digested pDUB1806. Exposure of the X-ray film to the filter was for 64 hours at -80°C. The lanes contained plasmid and chromosomal DNA from the following sources.

Lane 1	*Pst* I cut λ-DNA
Lane 2	pRZ-2
Lane 3	pRZ-4
Lane 4	DH5α
Lane 5	C58C[1]
Lane 6	GM19023
Lane 7	*R. meliloti*
Lane 8	*R. leguminosarum* (cured of Sym-plasmid)
Lane 9	*P. reactans*
Lane 10	*P. talassii*

Figure 3.16 a : Homology between putative *Agrobacterium* motility genes and *Rhizobium meliloti* DNA.

Figure 3.16 a : Southern blot analysis of bacterial chromosomal DNA with Tn5 flanking sequences from pDUB1802.

Panel A shows the agarose gel electrophoresis of *Eco* R1 cut bacterial genomic DNA samples and panels B and C the resulting Southern blots after double-sided transfer of the DNA to nylon and hybridisation respectively to radiolabelled 1.4 and 2.0kb Tn5 flanking sequences prodced by *Eco* RI/*Hpa* I digestion of pDUB1802. The filters were exposed to the film for 96 hours at -80°C in both cases. The gel contained DNA from the following sources, with all genomic samples being cut by *Eco* RI.

Lane 1	*Pst* I cut λ-DNA
Lane 2	DH5α
Lane 3	C58C[1]
Lane 4	GM19023
Lane 5	*R. meliloti*
Lane 6	*R. leguminosarum* (cured)
Lane 7	*P. talassii*

Figure 3.16 b : Southern blot analysis of bacterial chromosomal DNA with Tn5 flanking sequence from pDUB1807.

The panel shows the data obtained after agarose gel electrophoresis of 5μg of digested total DNA from different bacterial genera, transfer of the DNA to nitrocellulose and hybridisation to the 2.1kb *Eco* RI/*Hpa* I fragment of pDUB1807. The filter was exposed to X-ray film for 84 hours at -80°C. The gel contained the following DNA samples.

Lane 1	λ-DNA	*Pst* I
Lane 2	DH5α	*Eco* RI
Lane 3	C58C[1]	*Eco* RI
Lane 4	C58C[1]	*Bam* HI
Lane 5	*R. meliloti*	*Eco* RI
Lane6	*R. meliloti*	*Bam* HI
Lane7	*R. leguminosarum* (cured)	*Eco* RI
Lane 8	*R. leguminosarum* biovar. *pisae*	*Eco* RI
Lane 9	*P. reactans*	*Eco* RI
Lane 10	*P. talassii*	*Eco* RI

Figure 3.17 : Southern blot analysis of the hybridisation of *fla-11* Tn5 flanking sequence with *R. meliloti* behavioural genes.

Panel A shows a photograph of agarose gel electrophoresis of *Eco* RI cut DNA samples and panels B, C and D the resulting Southern blots after transfer of the DNA to nylon, hybridization with pDUB1811 Tn5 flanking sequence and washing at different stringencies. The washing conditions were 2xSSC at 42°C - panel B, 2xSSC at 65°C - panel C and 0.1xSSC at 65°C - panel D. The filter was exposed to X-ray film for 16, 40 and 88 hours respectively. The gel contained DNA from the following sources.

Lane 1	*Pst* 1 cut λ-DNA
Lane 2	pRZ-2
Lane 3	C58C[1]
Lane 4	GM19023
Lane 5	*R. meliloti*
Lane 6	*R. leguminosarum* (cured)

Figure 3.16 a : Hybridisation of Tn5 flanking sequences from pDUB1802 to bacterial chromosomal DNA.

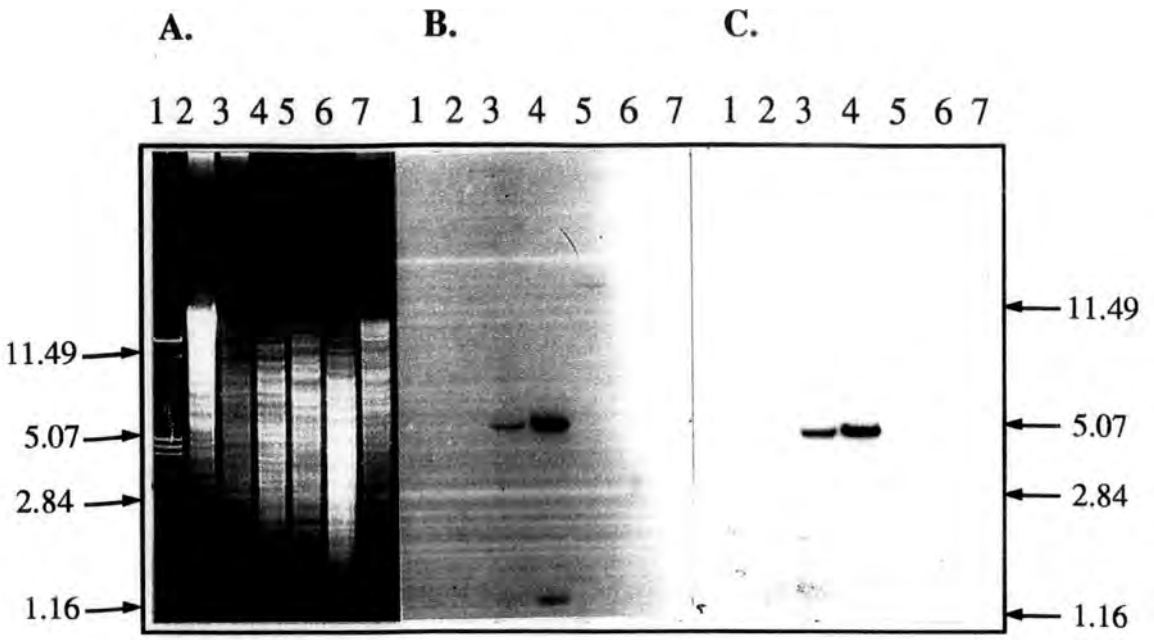


Figure 3.16 b : Hybridisation of Tn5 flanking sequence from pDUB1807 with bacterial chromosomal DNA.

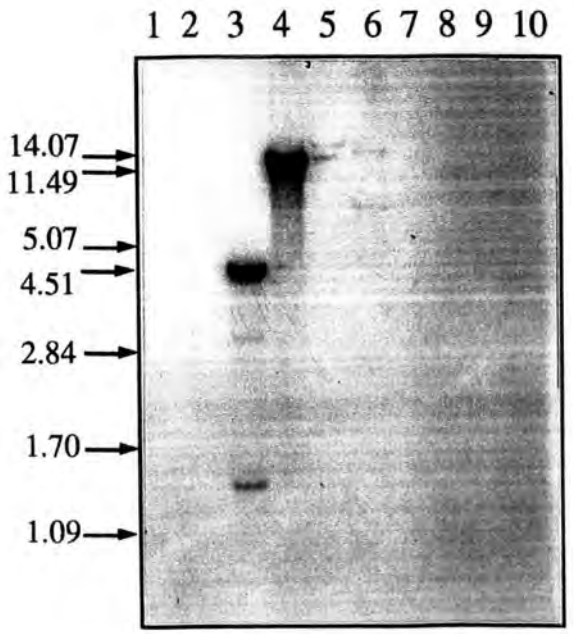
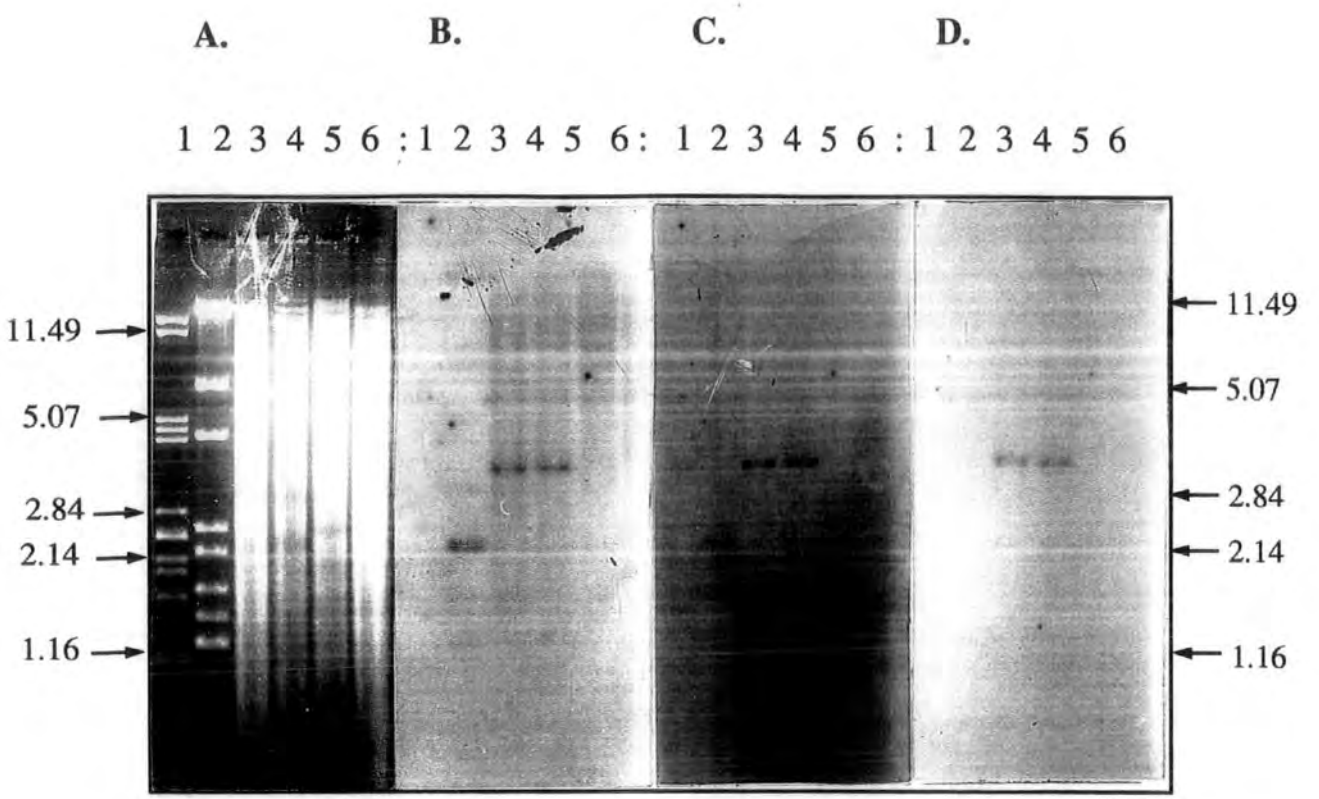


Figure 3.17 : Southern blot analysis of hybridisation of *fla-11* flanking sequence to plasmid containing cloned *R. meliloti* behavioural genes.



hybridisation in Southern blot analysis using any of the probes listed above, which shows that the Tn5 insertions responsible for loss of motility in these mutants were not in the cryptic plasmid.

Flanking sequences from *che-2* hybridised to the same 5.0kb *Eco* RI fragment in *Agrobacterium* DNA, but somewhat surprisingly also showed weak homolgy to a fragment of approximately 13kb in *R. meliloti* (figure 3.16a). The 3.3kb flanking sequence from *mot-6* overlaps regions of both pDUB1802 Eco RI/*Hpa* I fragments, so it is dificult to explain the difference in hybridisation to *Rhizobium* DNA. Hybridisation to *R. meliloti* DNA is also observed with the *fla-7* 2.1kb fragment produced on *Eco* RI/*Hpa* I digestion of pDUB1807 (figure 3.16b). Two *Eco* RI fragments approximately 14 and 17kb hybridised to the probe. A second gene related to that mutated by the Tn5 insertion in *fla-7* might exist in both *Agrobacterium* and *Rhizobium*. Two hybridising bands are seen in both types of DNA restriction used and there are no *Bam* HI or *Eco* RI sites in the 2.1 kb fragment used as the probe.

The 1.9kb Tn5 flanking sequence in pDUB1811 shares some homology with part of the cloned *R. meliloti* behavioural gene cluster present in the cosmid pRZ-2. Sequences in these two plasmids are not identical, as the probe was removed completely from pRZ-2 after high stringency washing at 65°C in 0.1xSSC (figure 3.17). The lack of hybridisation to genomic DNA from *R. meliloti* in the figure is probably due to the greatly differing amounts of target sequence in chromosomal and plasmid digests and reflects the limited homology between the *fla-11* flanking sequence and pRZ-2.

No hybridisation of any *Agrobacterium* motility mutant Tn5 flanking sequence has been observed with genomic DNA from *E. coli, R. leguminosarum* spp, *Pseudomonas reactans* or *Pseudomonas talassii* after high stringency washing at 65°C in 0.1xSSC.

These results demonstrate that some behavioural gene sequences are homologous in *R. meliloti* and *Agrobacterium* and the similarity does not extend to behavioural genes present in motile cells from several other bacterial genera. The proposed similarity between the agrobacterial and rhizobial chemotaxis systems is reinforced by observation of the patterns of motility together with immunological studies and oligonucleotide probe hybridisation, described later.

3.8 : Discussion.

The data presented describes the isolation of C58C^1 chromosomal DNA sequences which lead to an altered motility phenotype upon mutation by Tn5 insertion. Seven cosmids have now been isolated from a C58C^1 genomic library by hybridisation to Tn5 flanking sequences from behavioural mutants and two, pDUB1900 and 1905 have been characterised in more detail.

All of the cosmids were isolated by DNA hybridisation rather than complementation of behavioural phenotypes after transfer of the whole library to individual mutants. This potentially offered a quicker method to isolate cosmids containing motility genes and it was subsequently shown that the cosmids pDUB1900 and 1905 did restore wild type motility to a number of the mutants. The reasons why complementation was not a useful technique for the selection of either of these cosmids or pDUB1901-1903 from the library using the *fla-7* mutant are not known. Possible reasons for the failure of this method include loss of mobilization regions required by the transfer proteins of pRK2013 from the relevant cosmids; unequal amplification of potentially complementing cosmids in HB101 library cultures and deleterious effects of increased expression of the C58C^1 genes on the cosmids in the corresponding mutants.

Restriction digest analysis of the cosmids show that pDUB1900, 1901-1903 and 1905 do not overlap each other and hence there are at least three regions of the C58C^1 chromosome encoding genes required for wild-type motility. pDUB1900 and pDUB1905 contain sequences disrupted by Tn5 insertion in a number of different mutants, demonstrating that behavioural genes are clustered together in C58C^1, like those of *E. coli* and *R. meliloti*. It is almost certain that there are more genes required for chemotaxis and motility in *Agrobacterium* than those defined by the sixteen Tn5 motility mutants and it is probable that some are located on the cosmids already isolated. Saturation mutagenesis of the cosmids described above followed by introduction into the C58C^1 chromosome by homologous recombination will help map any more behavioural genes present in these plasmid inserts.

Comparison of the restriction maps of pDUB1900 and 1905 with those of the regions surrounding the *pscA* and *chvB* genes of *Agrobacterium* (Thomashow et al., 1987; Douglas *et al.*, 1985) show that none of the Tn5 insertions that map to these cosmids were in either the *pscA* or *chvB* gene, whose inactivation would also cause a loss of motility. Similarly the size of the Tn5 flanking sequences from pDUB1807 proves that the *fla-7* mutation was not in these two genes.

The cosmids pDUB1900 and pDUB1905 complement the mutations that map to them and restore wild-type motility. The occurrence of promotors and RNA transcript lengths from the inserts of these cosmids, responsible for phenotypic complementation, have not been investigated. The large chromosomal fragments selected during the constuction of the pLAFR-3 library (approximately 23kb) might be expected to retain the chromosomal transcription and translation signals for any genes or operons present in a cosmid and permit complementation. For mutations that map close to the *Eco* RI site of pLAFR-3, such as *che-2*, *mot-6* and *fla-8* in pDUB1905 (figure 3.9b and 3.13), the *lac* promotor of the vector may be the RNA transcription signal required for complementation. It has already been shown that this promotor is active in *Agrobacterium* strains (Chen & Winans, 1991).

The inability to obtain Rif/Km/Tc resistant colonies after mating of pDUB1901, 1902 or 1903 into the *mot-5* mutant in order to asess if the phenotype was complemented could have been due to the effects of over-expression of proteins encoded by these cosmids. It has already been noted that overproduction of the MotA protein in *E.coli* leads to significant decreases in growth rates (Blair & Berg, 1990) and it is possible that increased expression of a protein from cosmids containing a putative *Agrobacterium* motility cluster has a lethal effect on *mot-5* transconjugants.

The use of Tn5 flanking sequences as probes against chromosomal DNA of bacteria in other motile genera showed that some of the mutated genes of C58C^1 had homologous counterparts in *R. meliloti*. One of the flanking sequences from pDUB1811 hybridised to part of the *fla-che* region of *R. meliloti* cloned in pRZ-2 (figure 3.17). The 2.3kb *Eco* RI fragment of pRZ-2 to which the probe binds does not contain a defined motility mutation in *R. meliloti* (Ziegler *et al.*, 1986). The mutants used for the isolation of pRZ-2 by complementation were spontaneous mutants picked from the centre of a bacterial swarm It is therefore likely that other behavioural genes apart from those already localized to pRZ-2 exist in this region of the chromosome including the *R. meliloti* homologue of the *fla-11* gene of C58C^1. Complementation of *fla-11* with pRZ-2 would show functional as well as sequence homology between the two putative behavioural genes of the two strains.

Further homologies between C58C^1 motility genes and sequences present in motile bacteria from other genera have been revealed by sequencing of parts of pDUB1900 (Deakin and Shaw, unpublished data).

Deakin has shown that the protein encoded by the sequence mutated by Tn5 insertion in the *mot-1* mutation has 50% identity to the *flaA* and *flaB* genes of *R. meloloti* and homology to flagellins from several other bacterial strains. The *mot-1* mutant does possess flagella but they are perfectly straight, rather than the sinusoidal curve seen in wild-type cells. Tn5 insertion into the *mot-1* locus therefore causes aberrent flagella synthesis and the mutation has apparently occurred in a flagellin gene of *Agrobacterium* C58C^1, rather than a gene required for flagellar rotation. A sequence coding for a protein with 38% sequence similarity to the L-ring precursor of *Caulobacter crescentens* (product of the *flbN* gene) and 28% similarity to the L-ring of *Salmonella typhimurium* (FlagH) has also been identified in pDUB1900. Between these two sequences, which are on the *Eco* RI fragment E of pDUB1900 (see figure 3.14 p84) is a sequence encoding a protein which has 48% identity to FliZ of *B. subtitlis*. No function has been assigned to this protein, although mutants in *B. subtilis* are non-motile.

Shaw has also shown that sequences surrounding the Tn5 insertion sites in *fla-3* and *fla-10* encode a protein homologous to the FliG flagellar switch protein of *E. coli* and these mutations are in an operon, which includes the C58C^1 homologues of *fliM* and *fliN* also. *Agrobacterium*

tumefaciens therefore apparently contains a flagellar switch complex similar to that found in *E. coli*, even though its flagella rotate in the opposite direction.

Homology between genes disrupted in C58C^1 motility mutants and putative motility genes in other bacteria was only revealed in *R. meliloti* by Southern blot analysis using Tn5 flanking sequences with high stringency washing. The data described above shows that there is more similarity between the motility genes of different bacterial strains than revealed by the DNA hybridisation studies that have been carried out. Sequence analysis of the DNA adjacent to other Tn5 insertion sites will determine the extent of any further motility gene homologies and provide a clue to the function of the corresponding mutated genes in C58C^1. Complementation of characterised motility mutants from different bacterial genera, especially those of *R. meliloti* and *E. coli* will show functional as well as sequence conservation in proteins required for bacterial motility and may provide a method for mapping further behavioural functions to the cosmids already isolated from the C58C^1 library.

CHAPTER 4

Investigation of the occurrence of methyl-accepting chemotaxis proteins in *Agrobacterium tumefaciens* C58C1.

Proteins related to the four methyl-accepting chemotaxis proteins of *E. coli* have been shown to be present in a number of bacterial genera from different families of the eubacteria and it is possible that most , if not all, motile bacteria utilise methyl-accepting proteins in at least some of their tactic responses. *Agrobacterium* C58C^1 chemoattraction towards sucrose requires methionine, or more specifically S-adenosyl methionine (Loake, 1989) which provides some evidence that a methylation-dependent chemotaxis system exists in *Agrobacterium* for at least some chemoeffectors. This chapter describes experiments carried out to investigate further the possibility that proteins homologous to *E. coli* methyl-accepting chemotaxis proteins (MCP's) exist in *Agrobacterium* C58C^1.

Three different experimental approaches were used. These were DNA hybridisation studies using synthetic oligonucleotides, *in vivo* methyl labelling of proteins and immunological screening with an antibody raised against the *E. coli* MCP Tar.

4.1. Oligonucleotide probe hybridisation.

The four methyl accepting taxis proteins present in *E. coli*, Tar, Tap, Trg and Tsr, are closely related with a large number of absolutely or highly conserved residues in their amino acid sequences. Homology between the proteins is particularly extensive in the cytoplasmic regions required for intracellular signalling and regions that become methylated (Hazelbauer *et al.*, 1990). The conserved sequences among the proteins mean that oligonucleotides can be used to probe chromosomal DNA for the presence of sequences encoding MCP homologous domains.

Comparison of the sequences of the four proteins (Bollinger *et al.*, 1984; Krikos *et al.*, 1983) revealed an absolutely conserved sequence of 18 amino acids (residues 371-388 in Tsr) in a region that functions in signal output to the intracellular chemotactic proteins. The DNA sequences coding for these amino acids in the Tar, Tap and Tsr genes were used for the design of oligonucleotide probes for the first 12 residues in the conserved sequence, as outlined in figure 4.1.

Of the 36 nucleotides coding for these amino acids 30 are identical. Differences between the sequences are the result of different codon usage and occur at the third base of six codons. Three of the third-base ambiguities showed a consensus sequence, where two of the genes contained the same nucleotide whilst the other three, coding for threonine or alanine, had different bases present in all three sequences.

Figure 4.1 : Design of MCP Oligonucleotide Probes.

A. Conserved sequences in MCP genes used in probe design.

```
      I  A  F  Q  T  N  I  L  A  L  N  A  A  V  E  A  A  R
tar   ATTGCCTTCCAGACTAATATCCTCGCGCTGAATGCCGCGGTTGAAGCCGCGCGT
tap   ATTGCTTTCCAGACCAATATTCTGGCCCTGAATGCGGCAGTGGAAGCGGCTCGC
trg   ATTGCTTTCCAGACGAATATTCTGGCACTGAATGCTGCCGTTGAAGCCGCGCGC
```

Consensus sequence.

ATTGC<u>T</u>TTCCAGAC • AATAT<u>T</u>CT<u>G</u>GC • CTGAATGC • GC • GT<u>T</u>GAAGC<u>C</u>GC<u>G</u>CGC

(Underlined bases occur in two of the sequences, dots mean no consensus is observed).

B. Sequences of synthetic oligonucleotides corresponding to the first 12 residues.

```
Oligo A :  ATTGCTTTCCAGACGAATATTCTGGCGCTGAATGC
                 C          C       C  C  C
                            T             A
```

Oligo B : ATTGCTTTCCAGAC<u>G</u>AATATTCTGGC<u>G</u>CTGAATGC

(Underlined bases have no consensus among the three sequences).

Two oligonucleotide probes each of 35 nucleotides, were synthesized containing only the first two bases corresponding to the last alanine in order to reduce the number of different sequences present. Oligonucleotide A was a degenerate probe, with a mixture of relevant bases being used at ambiguous positions during synthesis. The probe therefore consisted of a mixture of 72 different 35mer oligonucleotides, as depicted in 4.1b. Oligonucleotide probe B was a single 35mer, consisting of the consensus sequence from the first 35 bases, with G inserted at the two places where all three sequences were different.

These sequences were synthesized without a 5' phosphate and were labelled directly with [γ-^{32}P]-dATP before use in DNA hybridisdation studies.

Characterisation of oligonucleotide hybridisation to *E. coli* MCP genes.

In order to determine the usefulness of the MCP oligonucleotide probes and assess the stringency of Southern blot washing required to produce meaningful results with C58C^{1} DNA, probe A was hybridised to chromosomal digests of the *E. coli* strain DH5α. Both of the oligonucleotides used were 35 bases long and use of the empirical formula T_d (^{o}C) = 2 (A + T) + 4(G +C) to determine the dissociation temperature and hence hybridisation temperature (T_d - 12^{o}C) gave an unfeasibly high temperatures for hybridisation between 86 and 94^{o}C. The probes were evidently too long for this formula to be useful and hybridisation and washing of Southern blot filters was therefore carried out at a standard temperature of 65^{o}C used for Southern blot analysis.

5μg samples of DH5α chromosomal DNA were digested to completion with *Eco* RI, *Hind* III, *Pst* I and *Sal* I before electrophoresis and transfer to a nylon filter. After hybridisation to radiolabelled probe A for 16 hours at 65^{o}C, the filter was washed in 1xSSC at 65^{o}C - see figure 4.2. There were no restriction sites for the enzymes used to digest the DNA within the degenerate oligonucleotides present in probe A, so it might be expected that there would be four bands in each lane, corresponding to the separate MCP genes. However two of the genes, *tar* and *tap*, are adjacent to each other in the same operon (Slocum and Parkinson, 1983) and should have been on the same DNA fragments in the digests used.

Figure 4.2 demonstrates that probe A hybridised strongly to three restriction fragments in *E.coli* DNA, and that washing in 1xSSC at 65^{o}C was stringent enough to remove almost all hybridisation to non-MCP sequences by the degenerate oligonucleotide probe. In each lane, there were two or three relatively weakly hybridising bands and one strongly hybridising species. Presumably two of the less intense bands in each case contained the *trg* and *tsr* genes, and *tar* and *tap* were together on the single strongly hybridising fragments. The intense band in the *Sal* I

Figure 4.2 : Southern blot analysis of *E.coli* chromosomal digests with probe A.

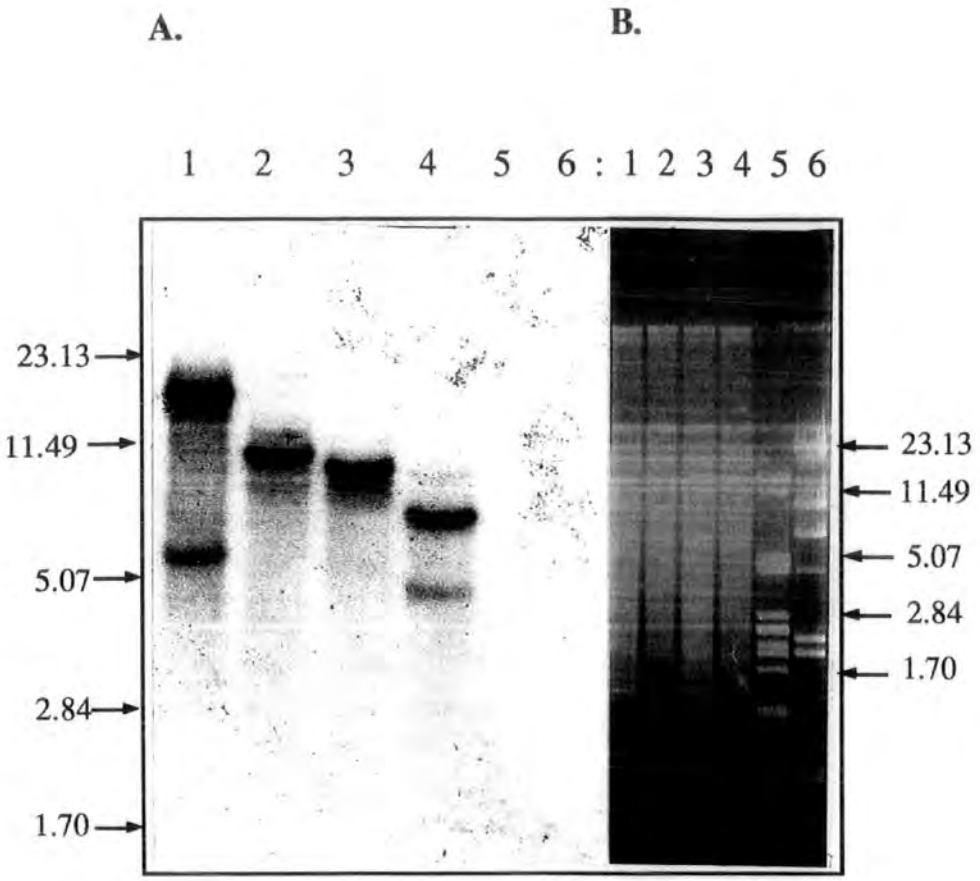


Figure 4.2 : Southern blot analysis of *E. coli* DNA with oligonucleotide probe A.

Panel B shows a photograph of gel elctrophoresis of chromosomal DNA digests and panel A the resulting Southern blot after transfer of the DNA to nylon and hybridisation with radiolabelled oligonucleotide probe A. The X-ray film was exposed to the filter for 160 hours at -80°C. The gel contained the following DNA samples.

Lane 1	DH5α	*Eco* RI
Lane 2	DH5α	*Hind* III
Lane 3	DH5α	*Pst* I
Lane 4	DH5α	*Sal* I
Lane 5	λ-DNA	*Pst* I
Lane 6	λ-DNA	*Hind* III

digest, approximately 7.5kb in size, was the same size as the *Sal* I fragment containing the *tar* and *tap* described by Slocum and Parkinson (1983), but the 16kb *Eco* RI fragment seen in figure 4.2 was not. On subsequent washing of the filter with 0.1xSSC at 65°C, all of the probe was removed from the filter.

Probe A hybridised strongly to the 9.8kb *Eco* RI insert in pAK108 (Boyd *et al.*, 1981) which contains *tar* and *tap* (data not shown), but in this case washing with 0.1xSSC at 65°C did not remove the probe completly. This is presumably because of the greatly increased number of target sequence copies in plasmid compared to chromosomal DNA digests.

These results demonstrated that probing of chromosomal digests with probe A followed by washing at 65°C in 1xSSC detected specific hybridisation to DNA fragments containing sequences that could encode the 12 amino acid MCP-conserved region.

Hybridisation to *Agrobacterium* and *Rhizobium* DNA.

Oligonucleotide probe A was used initially in hybridisation to DNA from other bacteria because it was a degenerate probe rather than an *E. coli* consensus sequence. 5μg of DNA from DH5α, *A. tumefaciens* GM19023, *R. meliloti* and *R.leguminosarum* (cured of Sym plasmid) were digested to completion with *Eco* RI, separated on a 0.6% agarose gel and transferred to a nylon filter. Hybridisation to radiolabelled probe A and washing stringency were the same as for the Southern blot anlysis of DH5α DNA, described above.

Probe A hybridized to a total of nine *Eco* RI fragments in GM19023, with three bands, of 5.0, 7.2 and 7.4kb, hybridising especially strongly (figure 4.3). The *E. coli Eco* RI fragments that strongly bound probe A in this experiment were 6.0, 15.0 and 17.0 kb in size, giving the same result as that found earlier (figure 4.2). Three fragments with sizes of 2.2, 3.0 and 9.5kb hybridized to probe A in *R.meliloti* DNA and perhaps somewhat surprisingly in view of this no homologies with any *R. leguminosarum* fragments were discovered.

The consensus oligonucleotide probe B was also end labelled and used to probe a duplicate Southern blot, containing identical DNA samples (figure 4.4). The filter in this instance was washed in 1xSSC at 50°C after hybridisation at 50°C for 16 hours.

At this lower stringency, essentially the same hybridisation pattern to *E. coli* and C58C1 DNA samples was observed as for probe A, with two extra bands of 1.0 and 1.8kb in the *Agrobacterium* lane. The lower stringency used in this experiment presumably allowed probe B to bind to sequences with some mismatches in them, to give almost the same result as the degenerate probe. When the filter was washed again with 1xSSC at 65°C only four fragments of 1.6, 2.8, 4.4 and 7.2kb remained hybridised to the probe in the GM19023 lane (data not shown).

Figure 4.3 : Southern blot analysis of bacterial chromosomal DNA digests with oligonucleotide probe A.

Panel A shows a photograph of the agarose gel electrophoresis of chromosomal DNA and panel B the resulting blot after the DNA was transferred to nylon and hybridised to radiolabelled probe A. The film was exposed to X-ray film for 210 hours at -80°C. The gel contained DNA from the following sources

Lane 1	λ-DNA	*Pst* I
Lane 2	DH5α	*Eco* RI
Lane 3	GM19023	*Eco* RI
Lane 4	*R. meliloti*	*Eco* RI
Lane 5	*R.leguminosarum* (cured)	*Eco* RI

Figure 4.3 : Southern blot analysis of bacterial chromosomal DNA digests with oligonucleotide probe A.

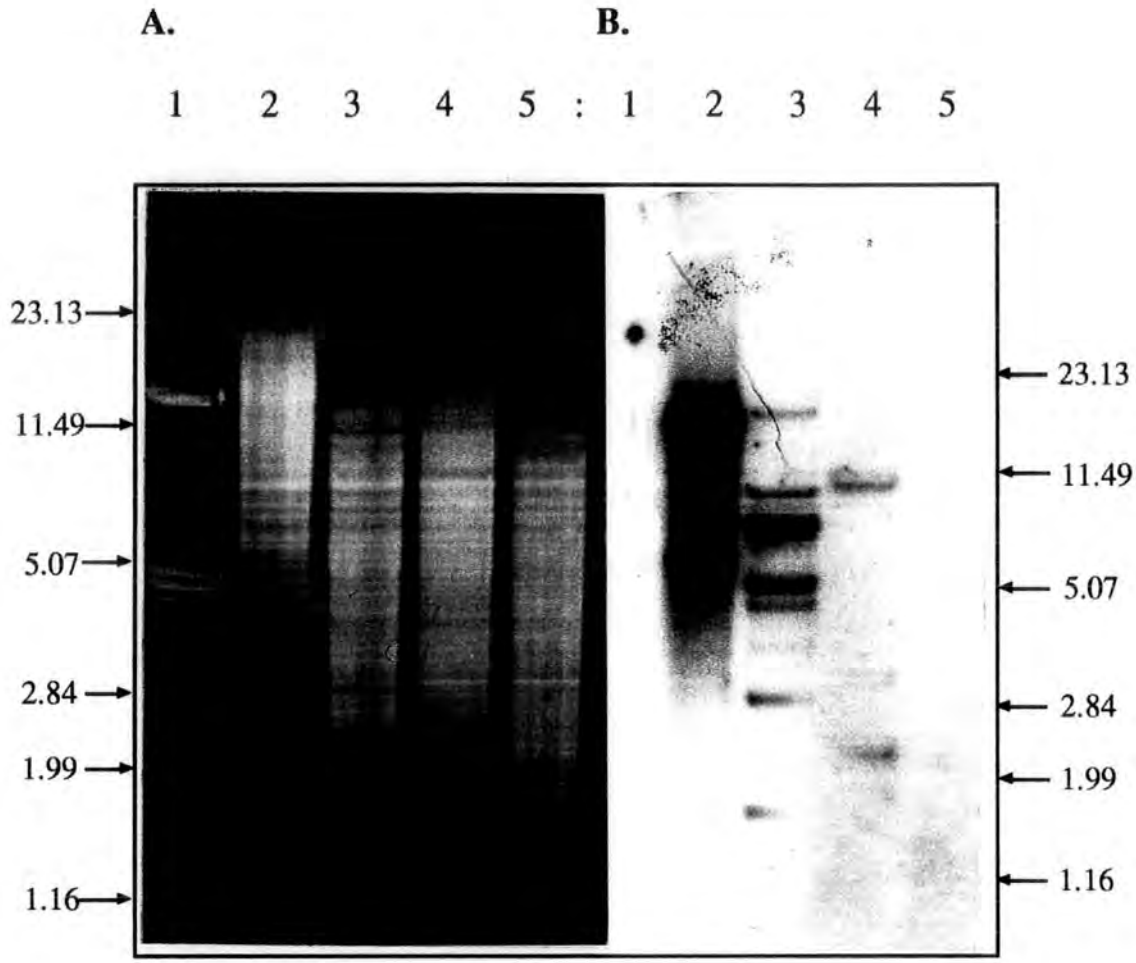
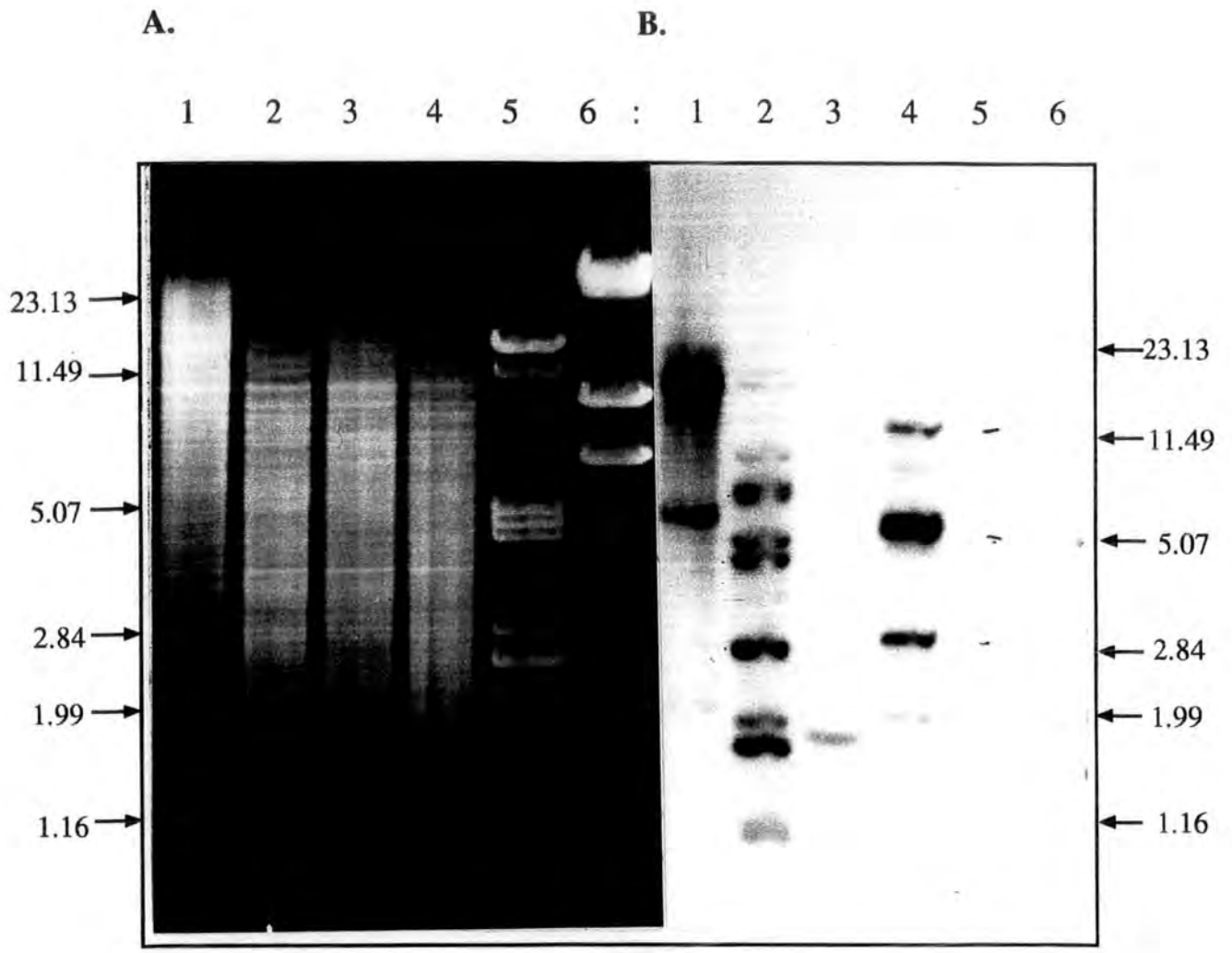


Figure 4.4 : Southern blot analysis of bacterial chromosomal DNA with oligonucleotide probe B.

Panel A shows a photograph of the agarose gel electrophoresis and panel B the resulting Southern blot after transfer of the DNA to nylon and hybridisation to radiolabelled probe B. The filter was exposed to X-ray film for 24 hours at -80°C. The gel contained the following DNA samples.

Lane 1	DH5α	*Eco* RI
Lane 2	GM19023	*Eco* RI
Lane 3	*R. meliloti*	*Eco* RI
Lane 4	*R. leguminosarum* (cured)	*Eco* RI
Lane 5	λ-DNA	*Pst* I
Lane 6	λ-DNA	*Hind* III

Figure 4.4 : Southern blot analysis of chromosomal DNA's with probe B.



The same sized fragments revealed in figure 4.3 must have hybridised with a subset of the degenerate probe A which had similar sequences to that present in probe B.

No differences were observed in the hybridisation of either oligonucleotide probe to C58C^1 and GM19023 chromosomal digests (data not shown), which means no homologous sequences were present on the cryptic plasmid of *A. tumefaciens*.

The two *Rhizobium* lanes produced very different results with the two probes. The consensus probe B only hybridised to one 1.9kb *R. meliloti* fragment at 50°C (figure 4.4) and was removed completely by higher stringency washing at 65°C (data not shown). Conversely, probe B hybridised to nine fragments in the *R. leguminosarum* lane, as opposed to none binding probe A. Three of the fragments in this lane, with sizes of 2.9, 5.1, and 11.5kb (marked on photograph in figure 4.4) still bound probe B after washing in 1xSSC at 65°C. The differences between the two probes in *Rhizobium* DNA hybridisation could have been due to the different hybridisation temperatures employed in the experiments, but washing at the same stringency should have produced the same result. It is more likely that the difference was due to the amounts of probe containing the consensus sequence (1.4% for probe A compared to 100% for probe B) and longer exposures of the filter used in figure 4.3 would probably have revealed hybridisation of probe A to *R. leguminosarum* DNA.

These experiments demonstrate that both degenerate and consensus sequence oligonucleotides homologous to part of *E.coli* MCP genes hybridise to a number of chromosomal sequences in both *Agrobacterium tumefaciens* and *Rhizobium* spp., at stringencies that allow specific hybridisation to the MCP genes of *E.coli*. This suggests that proteins with homolgy to a short region of the MCPs in enteric bacteria could be encoded in all three bacterial strains studied. It is not yet known if transcription and translation occurs at the sequences that hybridise to the probes.

In the *E. coli* genome, MCP genes are clustered together with genes required for motility and chemotaxis. This raised the possibility that the MCP homologous sequences in *Agrobacterium* were contained in the C58C^1 library cosmids isolated becuse they overlapped Tn5 insertion loci that produced a non-motile phenotype. However none of the six library cosmids described in chapter 3 hybridised with probe A, even after low stringency probing and washing at 43°C.

4.2. *In vivo* methylation experiments with C58C^1.

Methyl accepting chemotaxis proteins in *E. coli* are characterised by changes in their methylation states after addition of chemoattractants or repellents, which are responsible for

adaptation of the bacteria to their new chemical environment. Methylation of these proteins and changes in methylation levels on the addition of chemostimuli can be studied by *in vivo* labelling experiments using L-[methyl ^{3}H]-methionine. Tritium incorporated into cell components in the presence of a protein synthesis inhibitor, and revealed by SDS-PAGE followed by fluorography is due to methylation via the S-adenosyl methionine pathway. Addition of attractants or repellents before cell lysis should alter the methylation patterns observed if any labelled proteins function like the MCPs of enteric bacteria.

Using this method, the four *E. coli* MCPs are labelled to produce methylated proteins of45-65kDa and the procedure has been used to show the presence of methyl accepting taxis proteins in *Pseudomonas putida* (Harwood, 1989) and *Halobacterium halobium* (Alam *et al.*,1989). *In vivo* labelling experiments using C58C^{1} were carried out with the addition of different compounds before cell lysis to try and prove that MCPs exist in *Agrobacterium.*

In vivo labelling of *Agrobacterium* using tritiated methionine.

Initially 200μgml^{-1} chloramphenicol was used as the protein synthesis inhibitor but this proved to be unsatisfactory, as all of the proteins in the gel were labelled. The apparent lack of inhibition of protein synthesis was not due to the activity of a resistance gene, as agar plates containing 25μgml^{-1} chloramphenicol did not support growth of the C58C^{1} Rif resistant strain used in the labelling studies. The presence of 200μgml^{-1} puromycin did inhibit protein synthesis, measured by ^{3}H leucine incorporation into proteins (eg figure 4.5, lane 2) and this was used in all subsequent methylation experiments.

After *in vivo* methylation experiments over a time course, carried out as described in materials and methods and using 4% formalin and 10% TCA to precipitate proteins, a protein of approximately 45kDa was labelled in C58C^{1} (figure 4.5). The protein was labelled in a time-dependent manner, with maximal methylation occuring after approximately 45 minutes (data not shown). Lower molecular mass methylated components (of apparent M_r 12-20 kDa) were observed as a series of diffuse or tight bands in this and several other labelling experiments. The methylation pattern in this region was variable and no changes to it were observed on addition of any chemostimulus. Further investigation of these labelled compounds was not undertaken, but they might be oligosaccharide derivatives, like those of apparent M_r 17-29 kDa observed during *Halobacterium halobium* methylation (Alam *et al.*, 1989).

No methylated proteins were detected in *E. coli* using 4% formalin and 10% TCA (figure 4.5, lane 1). This was probably due to inadequate lysis of the cells, as labelled proteins of approximately 60kDa in DH5α and 45kDa in C58C^{1} were observed using the same labelling conditions when the cells were lysed by boiling in SDS sample buffer (figure 4.6). Boiling in

Figure 4.5 : *In vivo* methylation of proteins in C58C1.

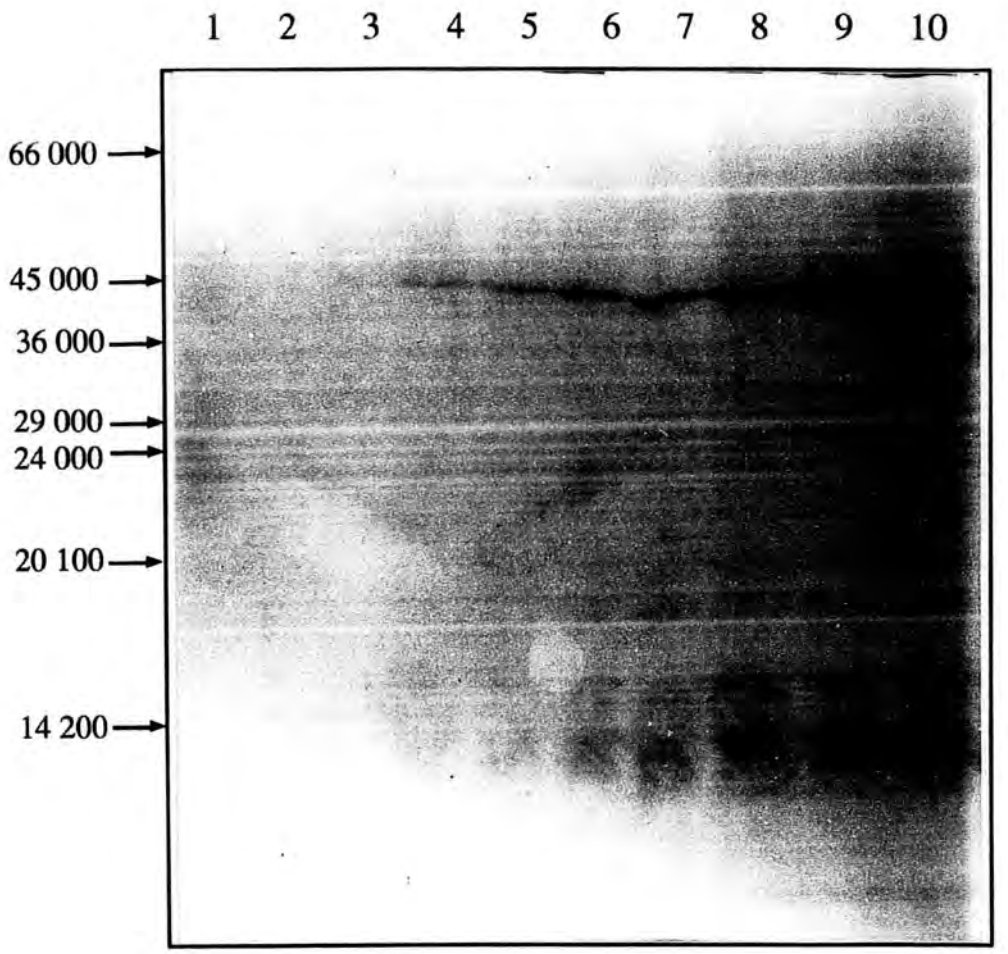


Figure 4.5 : *In vivo* labelling of proteins in C58C^1 with L-[methyl-^{3}H] methionine.

The panel shows a photograph of the fluorograph resulting from *in vivo* labelling of proteins in C58C^1 liquid culture. Labelled cells were lysed, the proteins precipitated and separated on a 12% polyacrylamide gel before fluorography. The dried gel was exposed to X-ray film for 72 hours at room temperature. The lanes contained protein samples from cells treated in the following manner before lysis.

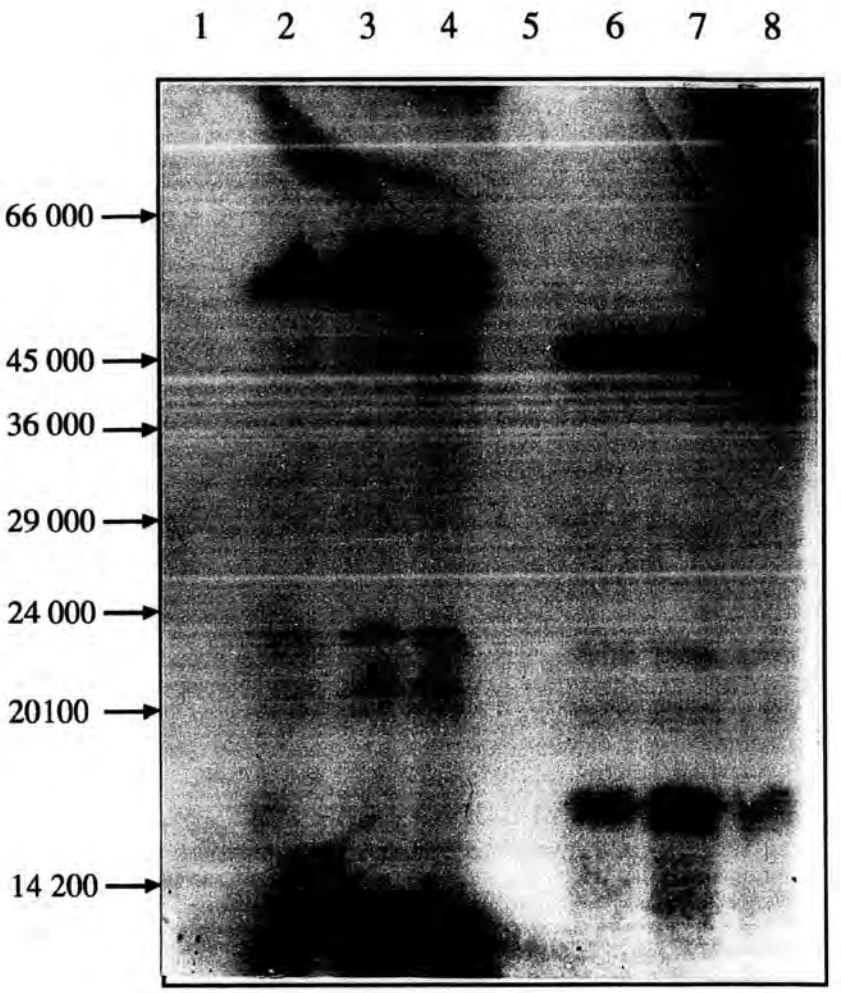
Lane	Strain	Label	Time	Addition
1	DH5α	^{3}H met	30	-
2	C58C^1	^{3}H leu	30	-
3	C58C^1	^{3}H met	5	-
4	"	"	10	-
5	"	"	15	-
6	"	"	20	-
7	"	"	30	-
8	"	"	40	-
9	"	"	40	Phenol 3 min.
10	"	"	40	Phenol 5 min.

Figure 4.6 : *In vivo* methylation of DH5α and C58C[1] cells - methylation pattern after lysis of cells by boiling in sample buffer.

The panel shows a photograph of the fluorograph resulting from *in vivo* labelling of DH5α and C58C[1] cells in liquid culture. Labelled cells were pelleted in a microfuge and boiled for 5 minutes in 40μl of sample buffer. 40μl of fresh sample buffer was then added and the protein sample boiled a further 5 minutes before separation on a 12% polyacrylamide gel followed by fluorography. The dried gel was exposed to X-ray film for 160 hours at room temperature. The lanes contained protein samples from cells treated in the following manner before cell lysis.

Lane	Strain	Label	Time	Addition
1	DH5α	^{3}H leu	30	-
2	"	^{3}H met	30	-
3	"	"	60	-
4	"	"	60	Phenol 5 min
5	C58C[1]	^{3}H leu	30	-
6	"	^{3}H met	30	-
7	"	"	60	-
8	"	"	60	Phenol 5 min

Figure 4.6 : *In vivo* methylation of DH5α and C58C1 - methylation pattern after lysis by boiling in sample buffer.



sample buffer did not allow sharp resolution of labelled proteins in either bacteria however, and accurately timed termination of the labelling rection was difficult to achieve.

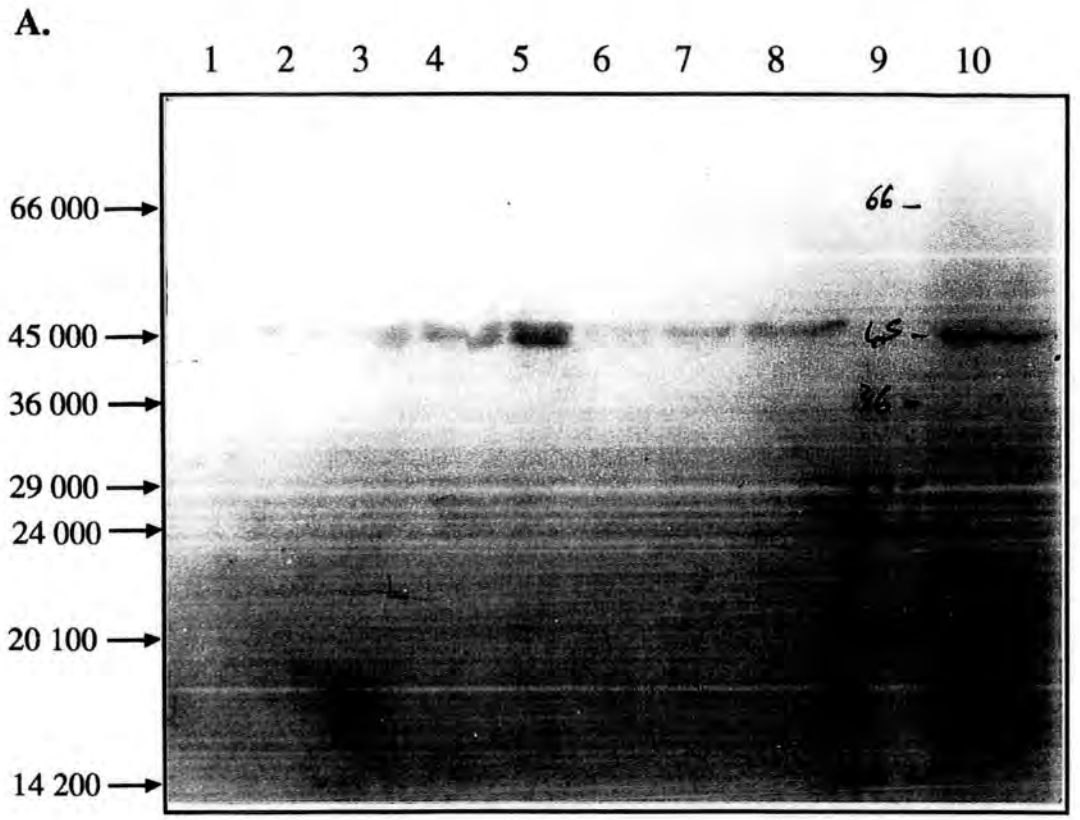
Use of formalin and 10% TCA often resulted in the loading of unequal amounts of protein and it was found that lysis with 1% SDS and 15% TCA was a more reliable method. For subsequent experiments with C58C^1, the labelling reactions were stopped by the addition of 1% SDS and 15% TCA. The reagents used to stop the labelling and precipitate protein apparently had no effect on experimental results, as only one or two bands of approximately 45kDa were observed in either case. The number of labelled bands that appeared in this region of the gels was not consistent, with either one or two labelled products being observed (figure 4.7).

Phenol was added to a concentration of 0.1% at timed intervals before cell lysis to see if it had any effect on protein methylation in C58C^1. Phenol was initially chosen as the first chemostimulant to try and elicit possible methylation changes because it is a complex chemoeffector in *E. coli*, capable of causing motility changes via all four MCPs if they are expressed individually. It acts as an attractant for wild type cells, with induced methylation of Tar and Tsr (Imae *et al.*, 1987), but there is also simultaneous repellent signalling, mediated by Trg, Tap and Tsr which results in demethylation of at least the Trg protein (Yamamoto *et al.*, 1990). The addition of phenol did cause changes in methylation of the 45kDa protein or proteins although again its effect was variable. It either increased the extent of methylation (figures 4.5 and 4.7b), decreased it (figure 4.7a) or apparently had no effect (figure 4.8, lane 7). Phenol induced methylation or demethylation reactions continued for the whole of the five minute period before cell lysis.

If the labelled protein at 45kDa was a methyl accepting chemotaxis protein, it would be expected that some of the known chemoattractants for *Agrobacterium* would induce changes in methylation. A number of attractants were added, at the concentration giving the largest response in chemotaxis assays, 5 minutes before cell lysis to see if they had any effect on the methylation patterns observed. The attractants 10^{-3}M arginine, 10^{-3}M valine and 10^{-6}M sucrose did not change the methylation pattern observed (figure 4.8). The presence of aspartate + serine, both at 10^{-3}M, or 1% L-broth (data not shown) or the addition the of 0.01% HCl also did not alter the banding patterns observed (figure 4.8).

The nature of the ^{3}H methyl bond to the cellular components labelled in the polyacrylamide gel used for the fluorograph in figure 4.8 was investigated using the diffusion assay method of Chelsky *et al*, (1984). Methyl ester linkages, such as those to glutamate residues in the MCPs of *E. coli* and *H. halobium*, are base-labile and ^{3}H methyl esters can be quantified by scintillation counting of the volatile methanol released. Lanes 8 and 9 in the dried gel were cut into 2mm slices from top to bottom and the ^{3}H methanol release assayed after 24 hours incubation at 37^{o}C in

Figure 4. 7 : Time dependent labelling of C58C1 with tritiated L-methyl methionine - changes caused by the addition of phenol.



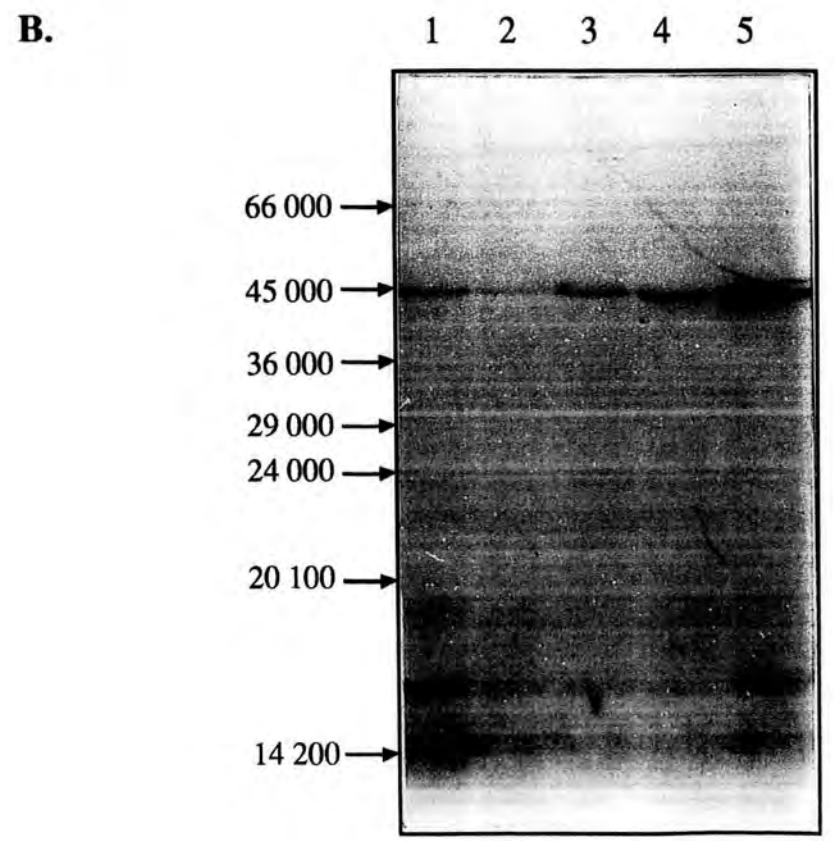


Figure 4.7 : *In vivo* labelling of C58C[1] proteins with L-[methyl-^{3}H] methionine: differential methylation patterns upon addition of phenol.

Panels A and B show photographs of the fluorographs resulting from labelling experiments in which phenol was added to some cells before cell lysis. Proteins were precipitated, resuspended in sample buffer and separated on a 12% polyacrylamide gel before fluorography. The dried gels were exposed to the X-ray film for 72 hours (panel A) and 336 hours (panel B) at room temperature. The gel contained protein samples from bacteria labelled with L-[methyl-^{3}H] methionine and treated in the following way before lysis. (For panel A, lane 9 = molecular weight protein standards)

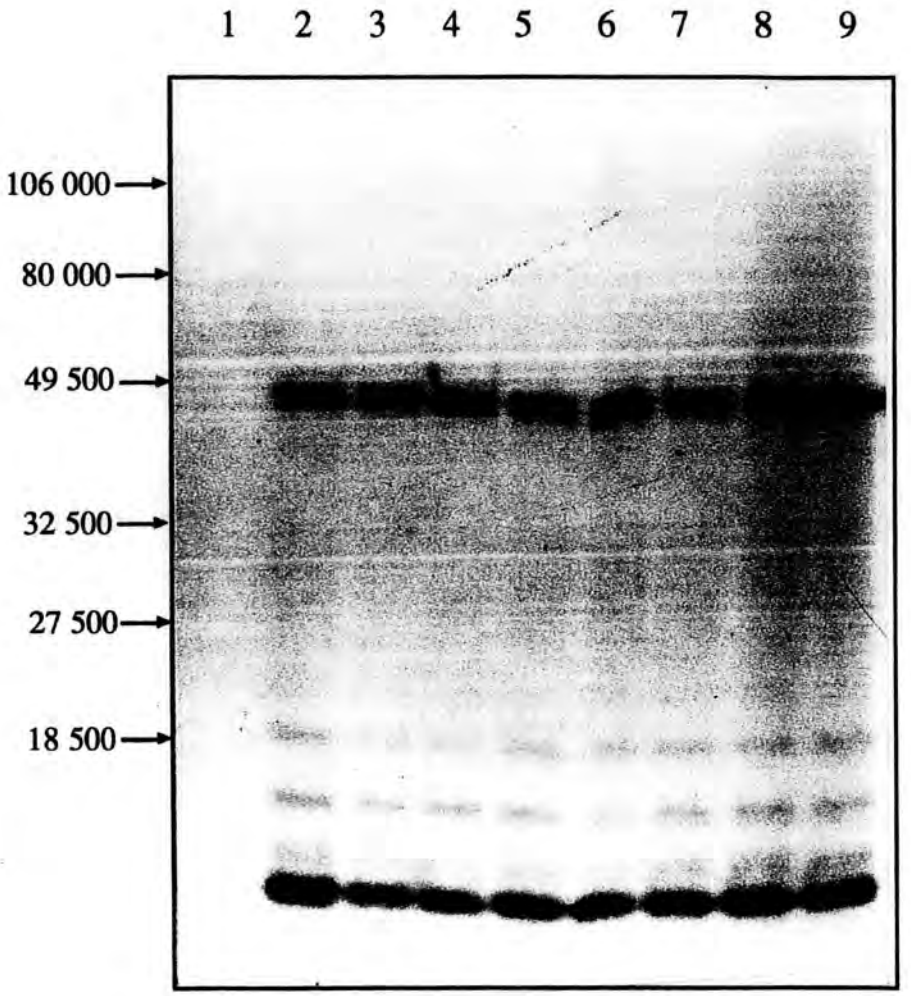
A	Lane	Time	Addition
	1	10	-
	2	15	-
	3	20	-
	4	30	-
	5	45	-
	6	60	-
	7	60	Phenol 1 min.
	8	60	Phenol 3 min.
	10	60	Phenol 5 min.
B.	1	50	-
	2	60	-
	3	60	Phenol 1 min
	4	60	Phenol 3 min.
	5	60	Phenol 5 min.

Figure 4.8 : Methylation of proteins in C58C[1] with possible attractants and repellents added.

The panel shows a photograph of the fluorograph resulting from labelling of C58C[1] with L-[methyl-^{3}H] methionine for 40 minutes and the effect of attractant or repellent addition 5 minutes before cell lysis. The precipitated bacterial proteins were separated on a 10% Schägger gel which was treated with sodium salicylate before drying. The dried gel was exposed to X-ray film for 120 hours at room temperature. Lane 1 contained protein from cells labelled with tritiated leucine for 40 minutes and the other lanes protein from cells labelled with tritiated methionine. The following compounds were added to the bacteria before lysis.

Lane 2	-
Lane 3	arginine
Lane 4	valine
Lane 5	sucrose
Lane 6	aspartate/serine
Lane 7	phenol
Lane 8	HCl
Lane 9	-

Figure 4.8 : *In vivo* methylation of proteins in C58C1 after addition of possible attractants or repellents.



1M sodium hydroxide. No significant differences were observed between any gel slice, which indicated that the label in the gel was not present in a base-labile form. This suggested that the label was not covalently linked to cellular components in the gel as a methyl ester group.

4.3. MCP antibody binding studies.

Methyl accepting proteins that are part of the tactic systems of several Gram positive and negative bacteria show structural similarities and bind antibodies raised against MCPs from *E. coli* (Nowlin *et al*, 1985; Hazelbauer *et al*, 1990). An antibody raised against Tar, described in Mutoh *et al*, (1986), was used in Western blot analysis of total protein extracts to see if a structurally homologous protein to this *E. coli* MCP exists in *Agrobacterium*.

25 and 50μg samples of a protein extract made from DH5α and C58C^1 were separated on a 10% Schägger gel and the proteins transferred to nitrocellulose in a liquid transfer Western blot apparatus. After staining with Ponceau S to allow marking of protein size standards, the filter was screened with a 1/500 dilution of the Tar antibody, which had been adsorbed with an *E. coli* acetone powder. Using a horseradish peroxidase-linked secondary antibody developed with diaminobenzidine and cobalt chloride, two proteins approximately 60kDa in size were bound by the antibody in both *E. coli* and *Agrobacterium* (figure 4.9). It is not known if the two cross-reacting bands in either of the bacteria are the same protein with different methylation levels, or two different proteins.

4.4. Discussion.

Bacteria use both methylation dependent and independent chemotaxis systems to respond to a wide range of stimuli within their environment. Methylation independent systems, such as the phosphotransferase carbohydrate chemotaxis system in *E. coli*, are often involved in responding to growth limiting factors such as carbohydrates or oxygen and it is possible that the methylation dependent systems may be used to attain sites for optimal growth rates in favourable environments. *Agrobacterium tumefaciens* C58C^1 possesses a highly sensitive chemotaxis system (Loake *et al.*, 1988) that enables it to move towards, and colonise favourable niches around wounded plants, but it is not yet known whether this behaviour utilises a methylation dependent or independent chemotaxis system.

The methionine requirement for C58C^1 chemotaxis towards sucrose (Loake, 1989) provides good evidence that methylation has a role to play in attraction towards at least this compound, but subsequent experiments do not yet provide proof that MCPs exist in the organism. The experiments described in this work with both oligonucleotide probes and

Figure 4.9 : Western blot analysis of *Agrobacterium* proteins wih *E. coli* Tar antibody.

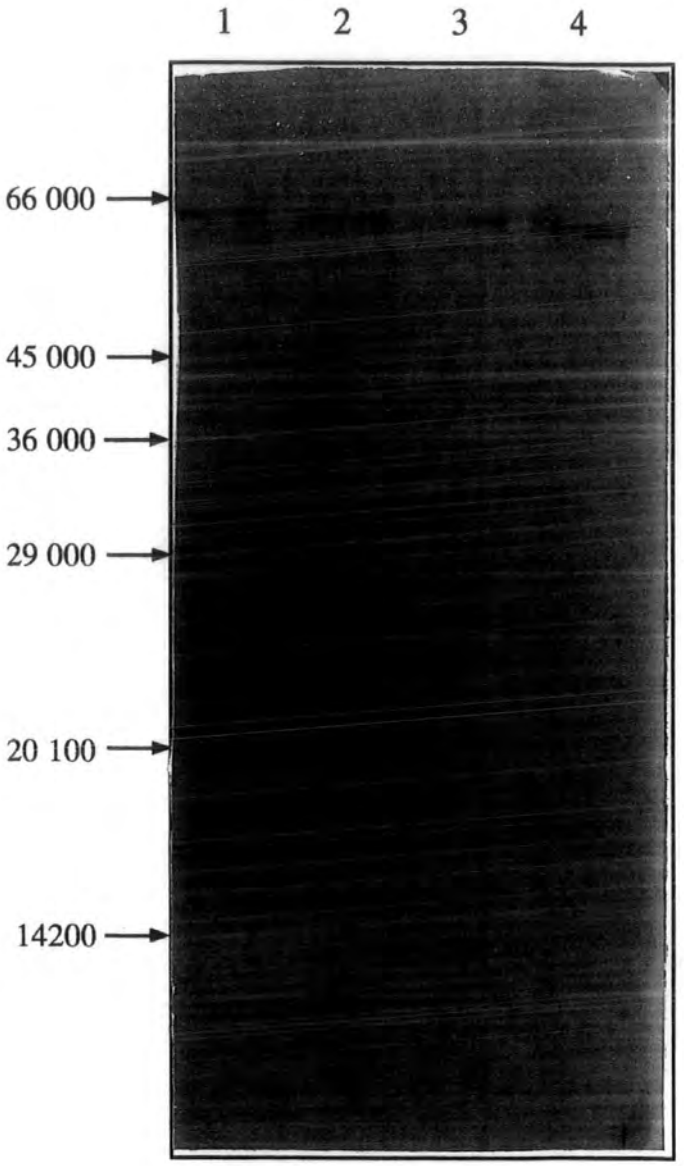


Figure 4.9 : Western blot analysis of *E. coli* and *Agrobacterium* proteins with Tar antibody.

The panel opposite shows a photograph of a Western blot developed using a horseradish peroxidase linked secondary antibody with diaminobenzidine and cobalt chloride. The primary antibody was a Tar antibody in a 1/500 dilution. The protein samples separated by polyacrylamide gel electrophoresis using a 12% Schägger gel and transferred to a nitrocellulose filter using liquid transfer apparatus were as follows.

Lane 1	DH5α	25μg
Lane 2	DH5α	50μg
Lane 3	C58C[1]	25μg
Lane 4	C58C[1]	50μg

immunoblot screening suggest that MCP homologous proteins exist in *Agrobacterium*, but unequivocal data in the form of chemoeffector induced changes in protein methylation levels has not been obtained.

Hybridisation of the oligonucleotide probes at high stringencies to a number of *Eco* RI chromosomal fragments in both *Agrobacterium* and *Rhizobium* showed that both genera have DNA homologies to a small part of the *E. coli* MCP genes. It is not known whether these sequences are transcribed or any RNA read in the right frame to produce the same amino acid sequence as that found in MCPs. Hybridisation of the same *Eco* RI fragments to oligonucleotides designed to bind to other conserved regions of the MCPs in *E. coli* would provide strong evidence that the fragments revealed in this work do encode methyl accepting chemotaxis proteins. Isolation of DNA from C58C^1 that is homologous to the oligonucleotides is complicated by the occurrence of MCP genes in all commonly used bacterial hosts. The presence of all four MCP genes in HB101 meant that the pLAFR-3 library could not be screened directly and in order to be able to clone the oligonucleotide binding sequences, the library will have to be transferred to a host strain lacking MCP genes, such as HCB437 (Wolfe *et al.*, 1987).

In enteric bacteria, methyl accepting chemotaxis proteins are methylated in a time dependent manner due to methyl group turnover onto the protein, and changes are observed on addition of chemostimuli. The data shown in figure 4.7 therefore appears to show the presence of a methyl accepting chemotaxis protein, with time dependent methylation and changes in methylation states of the labelled 45kDa protein after the addition of phenol. The two bands running close together in the gel could represent two proteins of similar molecular weight that are both methylated in C58C^1, or be the same protein with different numbers of methyl groups attached. A number of different labelled products of approximately 60kDa are seen in methylation experiments with *E. coli* cells containing only one MCP gene (see Lee and Imae, 1990 for example of Tar) and these reflect various methylation states. The reason why the presence of a single methyl group causes a significant change in mobility on polyacrylamide gels is unknown, but it is thought to affect SDS binding to the protein (G. Hazelbauer, personal communication).

The methylation patterns observed before and after addition of phenol, both in respect of the number of labelled bands and the overall net effect of phenol (either increase or decrease in methylation levels) were not consistently reproducible. This suggests that the labelled protein(s) approximately 45kDa in size is not a methyl accepting chemotaxis protein but a protein that is labelled under the conditions employed, with the amount and changes in methylation reflecting the general metabolic state of the cell. The inability to elicit changes in methylation of this protein with compounds such as sucrose, for which it has been shown that C58C^1 requires the presence of S-adenosyl methionine for chemoattraction, supports the view that the 45kDa protein is not an MCP.

During *in vivo* methylation experiments in *E. coli*, the translation elongation factor EF-Tu also becomes methylated and is visible on fluorographs of SDS polyacrylamide gels (See figure 4.6 - lanes 2-4). This protein is 45kDa in *E. coli* and can be mono and dimethylated at the Lys_{56} residue. The labelled bands of 45kDa seen in C58C^1 could be the *Agrobacterium* elongation factor with different numbers of methyl groups attached, but the reason for phenol induced changes in methylation states of this protein is unclear.

The protein labelled with ^3H after *in vivo* methylation is clearly not the same as that reacting to the Tar antibody, unless cleavage of the 60kDa cross-reacting protein occurs during cell lysis. In *E. coli* MCP proteins there is a site in the linker region, between the transmembrane spanning region 2 and one of the sequences that become methylated, that is extremely sensitive to proteolysis (Hazelbauer et al., 1990). It is thought that this proteolysis-sensitive site occurs in an exposed loop or hinge between two structurally independent domains of the transducers. The C58C^1 45kDa protein may be the same protein as that bound by the Tar antibody if cleavage is occurring during cell lysis at similar sites in putative MCP's in *Agrobacterium*. Proteolysis of MCP-like proteins in C58C^1 to produce a 45kDa protein would explain why the bands of that size in C58C^1 samples (lanes 6-8 in figure 4.6) are so much more intense than those in the DH5α lanes and of similar intensity to the MCP proteins labelled in DH5α, approximately 60kDa in size. Addition of protease inhibitors before cell lysis would allow investigation of the possibility that cleavage of *Agrobacterium* MCP homologues had occurred before protein separation on gels, leading to a false estimate of the size of the labelled protein in C58C^1.

If the labelled protein of 45kDa in C58C^1 was the Tar antibody-binding *Agrobacterium* MCP homologue which was cleaved during cell lysis, the inability to show attractant-induced changes in methylation could have been because too small chemotactic stimuli were applied for the time period before cell lysis. The length of adaptation time, and extent of methylation changes in *E. coli* increase with increasing concentrations of chemostimuli added to the cells. If a similar phenomenon occurred in C58C^1, it is possible that the cells could have adapted to the new concentrations of chemoeffectors in the media and returned to an unstimulated type of movement, reversing any methylation changes that had occurred. MCP labelling experiments with higher concentrations of chemostimuli or shorter time periods than five minutes before cell lysis, to see if the methylation pattern of the 45kDa protein changes, might help to show if this was the case.

The binding of Tar antibody to a protein in C58C^1 shows that structurally related proteins of a similar mass to *E.coli* MCPs exist in *Agrobacterium*, which widens still further the number of bacterial genera that contain proteins with similarites to these signal transduction proteins. The result obtained with C58C^1 was the same as that previously reported when *Rhizobium meliloti* proteins were screened using an antibody raised against the Trg MCP (Hazelbauer *et al.*, 1990). Both Agrobacteria and Rhizobia therefore contain proteins that are structurally homologous to the

well-characterised enteric bacterial MCP proteins, but *in vivo* labelling of these proteins with chemo-effector induced changes in methylation has not yet been demonstrated in these genera. It is possible that the *Rhizobiaceae* have developed a different system of chemotactic adaptation which does not involve methylation, but it seems more likely that definitive data demonstrating the presence of enteric bacterial MCP-like proteins will be obtained with the correct labelling conditions. An *in vivo* labelling protocol has now been worked out for *Agrobacterium* and results from further labelling experiments suggested above will help to show if the labelled 45kDa protein is an MCP or the *Agrobacterium* homologue of Ef-Tu.

The similarity between the signal transduction proteins of Agrobacteria and Rhizobia suggested by immunoscreening and labelling experiments is also demonstrated by hybridisation of the MCP oligonucleotide probes. This demonstrates that related sequences, possibly coding for MCP like proteins, exist in both genera. Isolation of library cosmids in an *E. coli* strain gutted for all four MCP's may provide a simple way to isolate MCP homologues from C58C^1 and *Rhizobium* spp.

Conclusion

This work describes experiments carried out to characterise further the *Agrobacterium* C58C^1 motility mutants constructed by Loake (1989). Isolation of Tn5 flanking sequences and their use as probes in DNA hybridisation studies provided a method for the isolation and mapping of the sites of Tn5 insertion in the mutants. Flanking sequences have been cloned from all 16 of the truly non-chemotactic mutants (measured in capillary asays using L-broth as an attractant) and cosmids overlapping the Tn5 insertion sites of 14 of these mutants have now been isolated. Behavioural functions in *A. tumefaciens* are clustered together on the chromosome, as in other motile bacteria.

The restriction mapping of two regions of the C58C^1 chromosome (pDUB1900 and 1905), containing a total of twelve Tn5 insertion sites that lead to altered motility, will facilitate the molecular analysis of motility genes in *A. tumefaciens*. It is almost certain that other genes, as yet undefined by the motility mutants, exist in C58C^1 and it is highly likely that some are present on the cosmids already isolated. Saturation mutagenesis of the mapped regions of pDUB1900 and 1905 is now possible, which might define further genes required for chemotaxis in C58C^1 as well as providing information about transcriptional organization and control.

Southern blot analysis suggests that some of the mutated behavioural genes in C58C^1 have homologous counterparts in *R. meliloti,* and this homology does not extend to other motile bacterial genera. Recent sequencing data however shows that homologies between motility genes in C58C^1 and other bacteria are more extensive than revealed by the DNA hybridisation studies. Complementation analysis of characterised motility mutants, especially those of *E. coli* and *R. meliloti,* using the C58C^1 library cosmids described can be used to study functional conservation between the chemotactic systems of different bacterial genera.

Oligonucleotide probe hybridisation and Western blot analysis demonstrate that proteins homologous to the MCP signal transduction proteins of enteric bacteria exist in *A. tumefaciens* and *R. meliloti*. An *in vivo* protein labelling system for C58C^1 was developed but did not provide definite proof that MCP's are present. The further labelling experiments suggested may demonstrate that such proteins occur in *A. tumefaciens*. Screening of the C58C^1 library with an MCP oligonucleotide probe, whose hybridisation has been characterised in this work, is a potential strategy for isolation of the MCP-homologous genes of *Agrobacterium*.

The data presented here provides the basis for a detailed study of the molecular biology underlying motiltity and chemotaxis in *A. tumefaciens* and should enable the characterisation of further behavioural genes in the future.

REFERENCES

Abouhamad, W.N., Manson, M., Gibson, M.M. and Higgins, C.F. (1991) Peptide transport and chemotaxis in *E. coli* and *S. typhimurium* - characterisation of the dipeptide permease (DPP) and the dipeptide binding protein. *Molec. Microbiol.* **5:** 1035-1047.

Adler, J. (1973) A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. *J. Gen. Microbiol.* **74:** 77-91.

Adler, J. (1975) Chemotaxis in bacteria. *Ann. Rev. Biochem.* **44:** 341-356.

Aguilar, J.M.M., Ashby, A.M., Richards, A.J.M., Loake, G.J., Watson, M.D. and Shaw, C.H. (1988) Chemotaxis of *Rhizobium leguminosarum* biovar *phaseoli* towards flavenoid inducers of the symbiotic nodulation genes. *J. Gen. Microbiol.* **134:** 2741-2746.

Alam, M., Lebert, M., Oesterhelt, D. and Hazelbauer, G.L (1989) Methyl accepting taxis proteins in *Halobacterium halobium*. *EMBO. J.* **8:** 631-639.

Ames, P. and Bergman, K. (1981) Competitive advantage provided by bacterial motility in the formation of nodules by *Rhizobium meliloti*. *J. Bacteriol.* **148:** 728-729.

Ames, P., Schluderberg, S.A. and Bergman, K. (1980) Behavioural mutants of *Rhizobium meliloti*. *J. Bacteriol.* **141:** 722-727.

Ankenbauer, R.G. and Nester, E.W. (1990) Sugar mediated induction of *Agrobacterium tumefaciens* virulence genes: structural specificity and activities of monosaccharides. *J. Bacteriol.* **172:** 6442-6446.

Armitage, J.P., Gallagher, A. and Johnston, A.W.B. (1988) Comparison of the chemotactic behaviour of *Rhizobium leguminosarum* with and without the nodulation plasmid. *Molec. Microbiol.* **2:** 743-748.

Arnosti, D.N. and Chamberlin, M.J. (1989) Secondary sigma factor controls transcription of flagellar and chemotaxis genes in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **86:** 830-834

Ashby, A.M., Watson, M.D., Loake, G.J. and Shaw, C.H. (1988) Ti-plasmid specified chemotaxis of *Agrobacterium tumefaciens* C58C^1 towards *vir* - inducing phenolic compounds and soluble factors from monocotyledonous and dicotyledonous plants. *J. Bacteriol.* **170:** 4181-4187.

Ashby, A.M., Watson, M.D. and Shaw, C.H. (1987) A Ti-plasmid determined function is responsible for chemotaxis of *Agrobacterium tumefaciens* towards the plant wound product acetosyringone. *FEMS Microbiol. Letts.* **41:** 189-192.

Bashan, Y. and Levanony, H. (1987) Horizontal and vertical movement of *Azospirillum brasilense* Cd in the soil and along the rhizosphere of wheat and weeds in controlled and field environments. *J. Gen. Microbiol.* **133:** 3473-3480.

Beauchamp, C.J., Chilton, W.S., Dion, P. and Antoun, H. (1990) Fungal catabolism of crown gall opines. *Appl. Env. Microbiol.* **56:** 150-155.

Beijerinck, M.W. and Van Delden, A. (1902) Ueber die assimilation des freien stickstoffs durch bakterien. *Zentralblatt fur bakteriologie, parasitenkunde und infektionskrankheiten Abt 2* **9:** 3-43.

Berg, H.C. and Brown, D.A. (1972) Chemotaxis in *Escherichia coli* analysed by three dimensional tracking. *Nature* **239:** 500-504.

Berg, H.C. and Tedesco, P.M. (1975) Transient response to chemotactic stimuli in *Escherichia coli. Proc. Natl. Acad. Sci. USA* **72::** 3235-3239.

Bergman, K., Gulash-hoffee, M., Hovestadt, R.E., Larosiliere, R.C., Ronco, P.G. and Su, L. (1988) Physiology of behavioural mutants of *Rhizobium meliloti* : evidence for a dual chemotaxis pathway. *J. Bacteriol.* **170:** 3249-3254.

Beringer, J.E., Beynon, J.L., Buchanan-Wollaston, A.V. and Johnston, A.W.B. (1978) Transfer of the drug resistance transposon Tn5 to *Rhizobium. Nature* **276:** 633-634.

Bernaerts, M.J. and De Ley, J. (1963) A biochemical test for crown gall bacteria. *Nature* **197:** 406-407.

Bhuvaneswari, T.V., Bhagwat, A. and Bauer, W.D. (1981) Transient susceptibility of root cells in four common legumes to nodulation by rhizobia. *Plant Physiol.* **68:** 1114-1119.

Binns, A.N. and Thomashow, M.F. (1988) Cell biology of *Agrobacterium* infection and transformation of plants. *Ann. Rev. Microbiol.* **42:** 575-606.

Blair, D.F. and Berg, H.C. (1990) The MotA protein of *E. coli* is a proton conducting component of the flagellar motor. *Cell* **60:** 439-449.

Bollinger, J., Park, C., Harayama, S. and Hazelbauer, G.L. (1984) Structure of the Trg protein: homologies with and differences from the other sensory transducers of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **81:** 3287-3291.

Bomhoff, G., Klapwijk, P.M., Kester, H.C.M., Schilperoort, R.A., Hernalsteens, J.P. and Schell, J. (1976) Octopine and nopaline synthesis and breakdown genetically controlled by a plasmid of *Agrobacterium tumefaciens*. *Mol. Gen. Genet.* **145:** 177-181.

Borkovich, K.A. and Simon, M.I. (1990) The dynamics of protein phosphorylation in bacterial chemotaxis. *Cell* **63:** 1339-1348.

Borkovich, K.A., Kaplan, N., Hess, J.F. and Simon, M.I. (1989) Transmembrane signal transduction in bacterial chemotaxis involves ligand dependent activation of phosphate group transfer. *Proc. Natl. Acad. Sci. USA* **86:** 1208-1212.

Bourret, R.B., Hess, J.F., Borkovich, K.A., Pakula, A.A. and Simon, M.I. (1989) Protein phosphorylation in chemotaxis and 2-component regulatory systems of bacteria. *J. Biol. Chem.* **264:** 7085-7088.

Boyd, A., Kendall, L. and Simon, M.I. (1983) Structure of the serine receptor in *Escherichia coli*. *Nature* **301:** 623-626.

Boyd, A., Krikos, A. and Simon, M (1981) Sensory transducers of *E. coli* are encoded by homologous genes. *Cell* **26:** 333-343.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.

Bradley, D.E., Douglas, C.J. and Peschon, J. (1984) Flagella specific bacteriophages of *Agrobacterium tumefaciens* : Demonstration of virulence of non-motile strains. *Can. J. Microbiol.* **30:** 676-681.

Braun, A.C. (1958) A physiological basis for the autonomous growth of the crown gall tumour cell. *Proc. Natl Acad. Sci. USA* **44:** 344-349.

Buchanan-Wollaston, V., Pasiatore, J.E. and Cannon, F. (1987) The *mob* and *ori* T mobilization functions of a bacterial plasmid promote its transfer to plants. *Nature* **328:** 172-175.

Buchman, I., Marner, F.J., Schroeder, G., Waffenschmidt, S. and Schroeder, J. (1985) Tumour genes in plants: T-DNA encoded cytokinin biosynthesis. *EMBO. J.* **4:** 853-859.

Caetano-Anolles, G., Cristestes, D.K. and Bauer, W.D. (1988a) Chemotaxis of *Rhizobium meliloti* to the plant flavone luteolin requires functional nodulation genes. *J. Bacteriol.* **170:** 3164-3169.

Caetano-Anolles, G., Wall, L.G., Demicheli, E.M., Bauer, W.D. and Favelukes, G. (1988b) Role of motility and chemotaxis in efficiency of nodulation by *Rhizobium meliloti. Plant Physiol.* **86:** 1288-1235.

Cangelosi, G.A., Ankenbauer, R.G. and Nester, E.W. (1990a) Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. *Proc. Natl. Acad. Sci. USA* **86:** 6708-6712.

Cangelosi, G.A., Hung, L., Puvanesarajah, V., Staley, G., Ozga, A.D., Leigh, J.A. and Nester, E.W. (1987) Common loci for *Agrobacterium tumefaciens* and *Rhizobium meliloti* exopolysaccharide synthesis and their roles in plant interactions. *J. Bacteriol.* **169:** 2086-2091.

Cangelosi, G.A., Martinetti, G., Leigh, J.A., Lee, C.C., Theines, C. and Nester, E.W. (1989) Role of *Agrobacterium tumefaciens* ChvA protein in export of β-1,2-glucan. *J. Bacteriol.* **171:** 1609-1615.

Cangelosi, G.A., Martinetti, G. and Nester, E.W. (1990b) Osmosensitivity phenotypes of *Agrobacterium tumefaciens* mutants that lack periplasmic β-1,2-glucan. *J. Bacteriol.* **172:** 2172-2174.

Chang, C-C., Chen, C-M., Adams, D.R. and Trost, B.M. (1983) Leucinopine, a characteristic compound of some crown gall tumours. *Proc. Natl. Acad. Sci. USA* **80:** 3573-3576.

Chelsky, D., Gutterson, N.I. and Koshland, D.E. Jr (1984) A diffusion assay for detection and quantitation of methyl-esterified proteins on polyacrylamide gels. *Anal. Biochem.* **141:** 143-148.

Chen, C-Y. and Winans, S.C. (1991) Controlled expression of the transcriptional activator gene *virG* in *Agrobacterium tumefaciens* by using the *Escherichia coli lac* promoter. *J. Bacteriol.* **173:** 1139-1144.

Chet, I., Zilberstein, Y. and Henis, Y. (1973) Chemotaxis of *Pseudomonas lachrymans* to plant extracts and to water droplets collected from the leaf surfaces of resistant and susceptible plants. *Physiol. Plant Pathol.* **3:** 473-479.

Chilton, W.S., Tempe, J., Matzke, M. and Chilton, M-D. (1984) Succinamopine: a new crown gall opine. *J. Bacteriol.* **157:** 357-362.

Chilton, M-D., Drummond, M.H., Merlo, D.J., Sciaky, D. and Montoya, A.L. (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell* **11:** 263-271.

Chilton, M-D., Saiki, R.K., Yadav, N., Gordon, M.P. and Quetier, F. (1980) T-DNA from *Agrobacterium* Ti-plasmid is in the nuclear DNA fraction of crown gall tumour cells. *Proc. Natl. Acad. Sci. USA* **77:** 4060-4064.

Christie, P.J., Ward, J.E., Gordon, M.P. and Nester, E.W. (1989) A gene required for transfer of T-DNA to plants encodes an ATPase with autophosphorylating activity. *Proc. Natl. Acad. Sci. USA* **86:** 9677-9681.

Christie, P.J., Ward, J.E., Winans, S.C. and Nester, E.W. (1988) The *Agrobacterium tumefaciens virE2* gene product is a single stranded DNA binding protein that associates with the T-DNA. *J. Bacteriol.* **170:** 2659-2667.

Citovsky, V., Wong, M.L. and Zambryski, P. (1989) Co-operative interaction of *Agrobacterium* VirE2 protein with single-stranded DNA: implications for the T-DNA transfer process. *Proc. Natl. Acad. Sci. USA* **86:** 1193-1197.

Clarke, L. and Carbon, J. (1976) A colony bank containing synthetic colE1 hybrid plasmids representative of the entire *E. coli* genome. *Cell* **9:** 91-99.

Close, T.J., Rogowsky, P.M., Kado, C.I., Winans, S.C., Yanofsky, M.F. and Nester, E.W. (1987) Dual control of the *Agrobacterium tumefaciens* Ti-plasmid virulence genes. *J. Bacteriol.* **169:** 5113-5118.

Colbère-Garapin, F., Horodniceanu, F., Kourilsky, P. and Garapin, A.C. (1981) A new dominant hybrid selective marker for higher eukaryotic cells. *J. Mol. Biol.* **150:** 1-4.

Conn, H.J. (1942) Validity of the *Alcaligenes*. *J. Bacteriol.* **44:** 353-360.

Cooley, M.B., D'Souza, M.R. and Kado, C.I. (1991) The *virC* and *virD* operons of the *Agrobacterium* Ti-plasmid are regulated by the *ros* chromosomal gene : analysis of the cloned *ros* gene. *J. Bacteriol.* **173:** 2608-2616.

Cuppels, D.A. (1988) Chemotaxis by *Pseudomonas syringae* pv. tomato. *Appl. Env. Microbiol.* **54:** 629-632.

Currier, W.W. and Strobel, G.A. (1976) Chemotaxis of *Rhizobium* spp. to plant exudates. *Plant. Physiol.* **57:** 820-823.

De Cleene, M. (1979) Crown gall: economic importance and control. *Zentralblatt fur bakteriolgie, parasitenkunde, infektionskrankheiten und hygiene, Abt 2* **134:** 551-554.

Depicker, A., Van Montagu, M. and Schell, J. (1978) Homologous DNA sequences in different Ti-plasmids are essential for oncogenicity. *Nature* **275:** 150-153.

De Weger, L.A., Van der Vlugt, C.I.M., Wijfjes, A.H.M., Bakker, P.A.H.M., Schippers, B. and Lugtenberg, B. (1987) Flagella of a plant growth stimulating *Pseudomonas fluorescens* strain are required for colonization of potato roots. *J. Bacteriol.* **169:** 2769-2773.

Ditta, G., Stanfield, S., Corbin, D. and Helsinki, D.R. (1980) Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti. Proc. Natl. Acad. Sci. USA* **77:** 7347-7351.

Douglas, C.J., Staneloni, R.J., Rubin, R.A. and Nester, E.W. (1985) Identification and genetic analysis of an *Agrobacterium tumefaciens* chromosomal virulence region. *J. Bacteriol.* **161:** 850-860.

Durrenberger, F., Crameri, A., Hohn, B. and Koukolikova-Nicola, Z. (1989) Covalently bound VirD2 protein of *Agrobacterium tumefaciens* protects the T-DNA from exonucleolytic degradation. *Proc. Natl. Acad. Sci. USA* **86:** 9154-9158.

Dylan, T., Ielpi, L., Stanfield, S., Kashyap, L., Douglas, C., Yanofsky, M., Nester, E.W., Helinski, D.R. and Ditta, G. (1986) *Rhizobium meliloti* genes required for nodule development are related to chromosomal virulence genes of *Agrobacterium tumefaciens. Proc. Natl. Acad. Sci. USA* **83:** 4403-4407.

Ebert, P.R., Ha, S.B. and An, G. (1987) Identification of an essential upstream element in the nopaline synthase promotor by stable and transient assays. *Proc. Natl. Acad. Sci. USA* **84:** 5745-5749.

Eisenbach, M. (1990) Functions of the flagellar modes of rotation in bacterial motility and chemotaxis. *Molec. Microbiol.* **4:** 161-167.

Eisenbach, M., Wolf, A., Welch, M., Caplan, S.R., Lapidus, I.R., Macnab, R.M., Aloni, H. and Asher, O. (1990) Pausing and speed fluctuation of the bacterial flagellar motor and their relation to motility and chemotaxis. *J. Mol. Biol.* **211:** 551-563.

Ellis, J.G., Kerr, A., Petit, A. and Tempe, J. (1982) Conjugal transfer of nopaline and agropine Ti-plasmids : the role of agrocinopines. *Mol. Gen. Genet.* **186:** 269-274.

Figurski, D.H. and Helinski, D.R. (1979) Replication of an origin containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc. Natl. Acad. Sci. USA* **76:** 1648-1652.

Gaworzewska, E.T. and Carlile, M.J. (1982) Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. *J. Gen. Microbiol.* **128:** 1179-1188.

Gegner, J.A. and Dahlquist, F.W. (1991) Signal transduction in bacteria : CheW forms a reversible complex with the protein kinase CheA. *Proc. Natl. Acad. Sci. USA* **88:** 750-754.

Genetello, C., Van Larebeke, N., Holsters, M., Depicker, A., Van Montagu, M. and Schell, J. (1977) Ti-plasmids of *Agrobacterium* as conjugative plasmids. *Nature* **265:** 561-563.

Ghai, J. and Das, A. (1989) The *virD* operon of *Agrobacterium tumefaciens* Ti-plasmid encodes a DNA-relaxing enzyme. *Proc. Natl. Acad. Sci. USA* **86:** 3109-3113.

Götz, R., Limmer, N., Ober, K. and Schmitt, R. (1982) Motility and chemotaxis of *Rhizobium* with complex flagella. *J. Gen. Microbiol.* **128:** 789-798.

Götz, R. and Schmitt, R. (1987) *Rhizobium meliloti* swims by unidirectional rotation of right handed flagellar helices. *J. Bacteriol.* **169:** 3146-3150.

Goy, M.F., Springer, M.S. and Adler, J. (1977) Sensory transduction in *Escherichia coli* : role of a protein methylation reaction in sensory adaptation. *Proc. Natl. Acad. Sci. USA* **74:** 4964-4968.

Grubl, G., Vogler, A.P. and Lengeler, J.W. (1990) Involvement of the histidine protein (HPr) of the phosphotransferase system in chemotactic signalling of *Escherichia coli* K-12. *J. Bacteriol.* **172:** 5871-5876.

Gulash, M., Ames, P., Larosiliere, R.C. and Bergman, K. (1984) Rhizobia are attracted to localized sites on legume roots. *Appl. Env. Microbiol.* **48:** 149-152.

Harlow, E. and Lane, D. (1988) *Antibodies a Laboratory Manual.* Cold Spring Harbour Laboratory Publications.

Harwood, C.S. (1989) A methyl accepting protein is involved in benzoate taxis in *Pseudomonas putida*. *J. Bacteriol.* **171:** 4603-4608.

Hawes, M.C. (1990) Living plant cells released from the root cap: a regulator of microbial populations in the rhizosphere? *Plant Soil.* **129:** 19-27.

Hawes, M.C. and Smith, L.Y. (1989) Requirement for chemotaxis in pathogenicity of *Agrobacterium tumefaciens* on roots of soil-grown pea plants. *J. Bacteriol.* **171:** 5668-5671.

Hawes, M.C., Smith, L.Y. and Howarth, A.J. (1988) *Agrobacterium tumefaciens* mutants deficient in chemotaxis to root exudates. *Molec. Plant-Microbe Interac.* **1:** 182-186.

Hazelbauer, G.L. (1988) The bacterial chemosensory system. *Can. J. Microbiol.* **34:** 466-474.

Hazelbauer, G.L., Yaghmai, R., Burows, G.G., Baumgartner, J.W., Dutton, D.P. and Morgan, D.G. (1990) Transducers: transmembrane receptor proteins involved in bacterial chemotaxis. In: *Biology of the Chemotactic Response. Society for General Microbiology Symposium 46* pp107-134. J.P. Armitage and J.S. Lackie (Eds.) Cambridge University Press.

Heinrich, D. and Hess, D. (1985) Chemotactic attraction of *Azospirillum lipoferum* by wheat roots and characterisation of some attractants. *Can. J. Microbiol.* **31:** 26-31.

Helman, J.D. and Chamberlin, M.J. (1987) DNA sequence analysis suggests that expression of flagellar and chemotaxis genes in *Escherichia coli* and *Salmonella typhimurium* is controlled by an alternative sigma factor *Proc. Natl. Acad. Sci. USA* **84:** 6422-6424.

Herrera-Estrella, A., Van Montagu, M. and Wang, K. (1990) A bacterial peptide acting as a plant nuclear targeting signal: the amino-terminal portion of the *Agrobacterium* VirD2 protein directs a β-galactosidase fusion protein to the tobacco nucleus. *Proc. Natl. Acad. Sci. USA* **87:** 9534-9537.

Hess, J.F., Bourret, R.B. and Simon, M.I. (1988a) Histidine phosphorylation and phosphoryl group transfer in bacterial chemotaxis. *Nature* **336:** 139-143.

Hess, J.F., Oosawa, K., Kaplan, N. and Simon, M.I. (1988b) Phosphorylation of three proteins in the signalling pathway of bacterial chemotaxis. *Cell* **53:** 79-87.

Hirsch, A.M., Wilson, K.J., Jones, J.D.G., Bang, M., Walker, V.V. and Ausubel, F.M. (1984) *Rhizobium meliloti* nodulation genes allow *Agrobacterium tumefaciens* and *Escherichia coli* to form pseudonodules on alfalfa. *J. Bacteriol.* **158:** 1133-1143.

Holsters, M., Villaroel, R., Gielen, J., Seurinck, J., De Greve, H., Van Montagu, M. and Schell, J. (1983) An analysis of the boundaries of the octopine TL-DNA in tumours induced by *Agrobacterium tumefaciens. Mol. Gen. Genet.* **190:** 35-41.

Hooykaas, P.J.J., Hofker, M., den Dulk-Ras, H and Schilperoort, R.A (1984) A comparison of virulence determinants in an octopine Ti-plasmid, a nopaline Ti-plasmid and an Ri-plasmid by complementation analysis of *Agrobacterium tumefaciens* mutants. *Plasmid* **11:** 195-205.

Hooykaas, P.J.J., Klapwijk, P.M., Nuti,, M.P., Schilperoort, R.A. and Rorsch, A. (1977) Transfer of the *Agrobacterium tumefaciens* Ti-plasmid to avirulent agrobacteria and to *Rhizobium ex planta. J. Gen. Microbiol.* **98:** 477-484.

Hooykaas, P.J.J., Van Brussel, A.A.N., den Dulk-Ras, H., Van Slogteren, G.M.S. and Schilperoort, R.A. (1981) Sym-plasmid of *Rhizobium trifolii* expressed in different rhizobial species and *Agrobacterium tumefaciens. Nature* **291:** 351-353.

Howard, E. and Citovsky, V. (1990) The emerging structure of the T-DNA complex. *Bioessays* **12:** 103-108.

Howard, E.A., Winsor, B.A., De Vos, G. and Zambryski, P. (1989) Activation of the T-DNA transfer process in *Agrobacterium* results in the generation of a T-strand protein complex: tight association of VirD2 with the 5' ends of T-strands. *Proc. Natl. Acad. Sci. USA* **86:** 4017-4021.

Howie, W.J., Cook, R.J. and Weller, D.M. (1987) Effects of soil matric potential and cell motility on wheat root colonisation by fluorescent pseudomonads suppressive to take-all. *Phytopathol.* **77:** 286-292.

Huang, M.W., Cangelosi, G.A., Halperin, W. and Nester, E.W. (1990a) A chromosomal *Agrobacterium tumefaciens* gene required for effective signal transduction. *J. Bacteriol.* **172:** 1814-1822.

Huang, Y., Morel, P., Powell, B. and Kado, C.I. (1990b) VirA, a coregulator of Ti-specified virulence genes is phosphorylated *in vitro. J. Bacteriol.* **172:** 1142-1144.

Iino,T., Komeda, Y., Kutsukake, K., Macnab, R.M., Matsumura, P., Parkinson, J.S., Simon, M.I. and Yamaguchi, S. (1988) New unified nomenclature for the flagellar genes of *Escherichia coli* and *Salmonella typhimurium*. *Microbiol. Rev.* **52:** 533-535.

Imae, Y., Oosawa, K., Mizuno, T., Kihara, M. and Macnab, R.M. (1987) Phenol: a complex chemoeffector in bacterial chemotaxis. *J. Bacteriol.* **169:** 271-379.

Inon de Iannino, N. and Ugalde, R.A. (1989) Biochemical characterisation of avirulent *Agrobacterium tumefaciens chvA* mutants: synthesis and excretion of β-1,2-glucan. *J.Bacteriol.* **171:** 2842-2849.

Jen, G. and Chilton, M-D. (1986) The right border region of pTiT37 T-DNA is intrinsically more active than the left border region in promoting T-DNA transformation. *Proc. Natl. Acad. Sci. USA* **83:** 3895-3899.

Jin, S., Komari, T., Gordon, M.P. and Nester, E. W. (1988) Genes responsible for the supervirulent phenotype of *Agrobacterium tumefaciens* strain A281. *J. Bacteriol.* **169:** 4417-4425.

Jin, S., Prusti, R.K., Roitsch, T., Ankenbauer, R.G. and Nester, E.W. (1990a) Phosphorylation of the VirG protein of *Agrobacterium tumefaciens* by the autophosphorylated VirA protein: essential role in biological activity of VirG. *J. Bacteriol.* **172:** 4945-4950.

Jin, S., Roitsch, T., Ankenbauer, R.G., Gordon, M.P. and Nester, E.W. (1990b) The VirA protein of *Agrobacterium tumefaciens* is autophosphorylated and is essential for *vir* gene regulation. *J. Bacteriol.* **172:** 525-530.

John, M.C. and Amasino, R.M. (1988) Expression of an *Agrobacterium* Ti-plasmid gene involved in cytokinin biosynthesis is regulated by virulence loci and induced by plant phenolic compounds. *J. Bacteriol.* **170:** 790-795.

Jorgensen, R.A., Rothstein, J. and Reznikoff, W.S (1979) a restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance *M.G.G.* **177:** 65-72.

Kanemoto, R.H., Powell, A.T., Akiyoshi, D.E., Regier, D.A., Kerstetter, R.A., Nester, E.W., Hawes, M.C. and Gordon, M.P. (1989) Nucleotide sequence and analysis of the plant inducible locus *pinF* from *Agrobacterium tumefaciens*. *J. Bacteriol.* **171:** 2506-2512.

Kennedy, B.W. and Alcorn, S.M. (1980) Estimates of USA crop losses to prokaryotic plant pathogens. *Plant Dis.* **64:** 674-676.

Kerr, A. (1969) Crown gall of stone fruit 1: isolation of *Agrobacterium tumefaciens* and related species. *Aust. J. Biol. Sci.* **22:** 111-116.

Kerr, A. (1974) Soil microbiological studies on *Agrobacterium radiobacter* and biological control of crown gall. *Soil Sci.* **118:** 168-172.

Kerr, A., Manigault, P. and Tempe, J. (1977) Transfer of virulence *in vivo* and *in vitro* in *Agrobacterium. Nature* **265:** 560-561.

Kerr, A. and Panagopoulos, C.G. (1977) Biotypes of *Agrobacterium radiobacter* var. *tumefaciens* and their biological control. *Phytopathol. Z.* **90:** 172-179.

Kersters K. and DeLey J. (1982) Family Rhizobiaceae - genus *Agrobacterium.* In *Bergeys Manual of Systematic Bacteriology.* Vol 1 9th edition. N.R.Krieg (Ed) Williams and Wilkins, Baltimore.

Krikos, A., Mutoh, N., Boyd, A. and Simon, M.I. (1983) Sensory transducers of *E. coli* are composed of discrete structural and functional domains. *Cell* **33:** 615-622.

Krupski, G., Götz, R., Ober, K., Pleir, E. and Schmitt, R. (1985) Structure of complex flagellar filaments in *Rhizobium meliloti. J. Bacteriol.* **162:** 361-366.

Kuldau, G.A., De Vos, G., Owen, J., McCafrey, G. and Zambryski, P. (1990) The *virB* operon of *Agrobacterium tumefaciens* pTiC58 encodes 11 open reading frames. *Mol. Gen. Genet.* **221:** 256-266.

Kuo, S.C. and Koshland, D.E. (1987) Roles of *cheY* and *cheZ* gene products in controlling flagellar rotation in bacterial chemotaxis of *Escherichia coli. J. Bacteriol.* **169:** 1307-1314.

Kushner, S.R. (1978) An improved method for transformation of *E. coli* with colE1 derived plasmids. In: *Genetic engineering* H.W. Boyer and A.S. Nicosia (Eds.) Elsevier Holland.

Laemmli, E.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.

Lapidus, I.R., Welch, M. and Eisenbach, M. (1988) Pausing of flagellar rotation is a component of bacterial motility and chemotaxis. *J. Bacteriol.* **170:** 3627-3632.

Larsen, S.H., Adler, J., Gargus, J.J. and Hogg, R.W. (1974a) Chemomechanical coupling without ATP: the source of energy for motility and chemotaxis in bacteria. *Proc. Natl. Acad. Sci. USA* **71:** 1239-1243.

Larsen, S.H., Reader, R.W., Kort, E.N., Tso, W. and Adler, J. (1974b) Change in direction of flagellar rototion is the basis of the chemotactic response in *Escherichia coli*. *Nature* **249:** 74-77.

Lee, L. and Imae, Y. (1990) Role of threonine residue in ligand recognition of the Tar chemoreceptor in *Escherichia coli*. *J. Bacteriol.* **172:** 377-382

Lianhui, Z. and Kerr, A. (1991) A diffusible compound can enhance conjugal transfer of the Ti-plasmid in *Agrobacterium tumefaciens*. *J. Bacteriol.* **173:** 1867-1872.

Liddell, C.M. and Parke, J.L. (1989) Enhanced colonization of pea taproots by a fluorescent pseudomonad biocontrol agent by water infiltration into soil. *Phytopathology* **79:** 1327-1332.

Lippincott, B.B. and Lippincott, J.A. (1969) Bacterial attachment to a specific wound site is an essential stage in tumour initiation by *Agrobacterium tumefaciens*. *J. Bacteriol.* **97:** 620-628.

Lippincott, J.A., Lippincott, B.B. and Starr, M.P. (1981) The genus *Agrobacterium*. In: *The Prokaryotes*. pp842-855. M.P. Starr, H. Stolp, H.G. Trueper, A. Balows and H.G. Schlegel (Eds.) Springer -Verlag.

Lippincott, B.B., Whatley, M.W. and Lippincott, J.A. (1977) Tumour induction by *Agrobacterium* involves attachment of the bacterium to a site on the host plant cell wall. *Plant Physiol.* **59:** 388-390.

Liu, J.D. and Parkinson, J.S. (1989) Role of CheW protein in coupling membrane receptors to the intracellular signalling system of bacterial chemotaxis. *Proc. Natl. Acad. Sci. USA* **86:** 8703-8707.

Liu, J.D. and Parkinson, J.S. (1991) Genetic evidence for interaction between CheW and Tsr proteins during chemoreceptor signalling by *Escherichia coli*. *J. Bacteriol.* **173:** 4941-4951.

Loake, G.J. (1989) The genetic dissection of chemotaxis in *Agrobacterium tumefaciens*. PhD thesis, University of Durham.

Loake G.J., Ashby, A.M. and Shaw, C.H. (1988) Attraction of *Agrobacterium tumefaciens* C58C^1 towards sugars involves a highly sensitive chemotaxis system. *J. Gen. Microbiol.* **134:** 1427-1432.

Lupas, A. and Stock, J. (1989) Phosphorylation of an N-terminal regulatory domain activates the CheB methylesterase in bacterial chemotaxis. *J. Biol. Chem.* **264:** 17337-17342.

McBride, K. and Knauf, V.C. (1988) Genetic anlysis of the *virE* operon of the *Agrobacterium* Ti-plasmid pTiA6. *J. Bacteriol.* **170:** 1430-1437.

Macnab, R.M. (1987a) Flagella. In: *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology Vol 1 pp70-83. F.C. Neidhart, J. Ingraham, K.B. Low, B. Magasanik, M. Schaechter and H.E. Umbarger (Eds.) American Society For Microbiology Publications, Washington D.C..

Macnab, R.M. (1987b) Motility and chemotaxis. In: *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology Vol 1 pp732-759. F.C. Neidhart, J. Ingraham, K.B. Low, B. Magasanik, M. Schaechter and H.E. Umbarger (Eds.) American Society For Microbiology Publications, Washington D.C.

Macnab, R.M. (1990) Genetics, structure and assembly of the bacterial flagellum. In: *Biology of the Chemotactic Response, Society for General Microbiology Symposium 46* pp77-106. J.P. Armitage and J.M. Lackie (Eds.) Cambridge University Press.

Macnab, R.M. and Koshland, D.E. Jr (1972) The gradient sensing mechanism in bacterial chemotaxis. *Proc. Natl. Acad. Sci. USA* **69:** 2509-2512.

Macnab, R.M. and Ornston, M.K. (1977) Normal to curly flagellar transitions and their role in bacterial tumbling: stabilization of an alternative quaternary structure by mechanical force. *J. Mol. Biol.* **112:** 1-30.

McNally, D.F. and Matsumura, P. (1991) Bacterial chemotaxis signalling complexes - formation of a CheA/CheW complex enhances autophosphorylation and affinity for CheY. *Proc. Natl. Acad. Sci. USA* **88:** 6269-6273.

Mandel, M., and Higa, A. (1970) Calcium dependent bacteriophage DNA infection of *E. coli*. *J. Mol. Biol.* **98:** 503-517.

Manson, M.D., Tedesco, P., Berg, H.C., Harold, F.M. and Van der Drift C. (1977) A protonmotive force drives bacterial flagella. *Proc. Natl. Acad. Sci. USA* **74:** 3060-3064.

Marks, J.R., Lynch, T.J., Karlinsey, J.A. and Thomashow. (1987) *Agrobacterium tumefaciens* virulence locus *pscA* is related to the *Rhizobium meliloti exoC* locus. *J. Bacteriol.* **169:** 5835-5837.

New, P.B. and Kerr, A (1972) Biological control of crown gall: field measurements and glasshouse experiments. *J. Appl. Bacteriol.* **35:** 279-287.

Ninfa, E.G., Stock, A., Mowbray, S. and Stock, J. (1991) Reconstitution of the bacterial chemotaxis signal transduction system from purified components. *J. Biol. Chem.* **266:** 9764-9770.

Nowlin, D.M., Nettleton, D.O., Ordal, G.W. and Hazelbauer, G.L. (1985) Chemotactic transducer proteins of *E. coli* exhibit homology with methyl accepting proteins from distantly related bacteria. *J. Bacteriol.* **163:** 262-266.

Okamoto, S., Yamamoto, A.T., Ito, K., Takebe, I. and Machida, Y. (1991) Localization and orientation of the VirD4 protein of *Agrobacterium tumefaciens* in the cell membrane. *Mol. Gen. Genet.* **228:** 24-32.

Oosawa, K., Hess, J.F. and Simon, M.I. (1988) Mutants defective in bacterial chemotaxis show modified protein phosphorylation. *Cell* **53:** 89-96.

Otten, L.A.B.M., Piotrowicik, G., Hooykaas, P.J.J. Dubois, M., Szegedi, E. and Schell, J. (1985) Identification of an *Agrobacterium tumefaciens* pTiB6S3 *vir* region fragment that enhances the virulence of pTiC58. *Mol. Gen. Genet.* **199:** 189-193.

Parke, D., Ornston, L.N. and Nester, E.W. (1987) Chemotaxis to plant phenolic inducers of virulence genes is constitutively expressed in the absence of the Ti-plasmid in *Agrobacterium tumefaciens. J. Bacteriol.* **169:** 5336-5338.

Parkinson, J.S. (1976) *CheA*, *CheB* and *CheC* genes of *Escherichia coli* and their role in chemotaxis. *J. Bacteriol.* **126:** 758-770.

Parkinson, J.S. (1981) Genetics of bacterial chemotaxis. In: *Genetics as a Tool in Microbiology. Society for General Microbiology Symposium 31* pp265-290. S.W. Glover and D.A. Hopwood (Eds.) Cambridge University Press.

Parkinson, J.S. (1988) Protein phosphorylation in bacterial chemotaxis. *Cell* **53:** 1-2.

Pazour, G.J. and Das, A. (1990a) VirG, an *Agrobacterium tumefaciens* transcriptional activator, initiates translation at a UUG codon and is a sequence specific DNA binding protein. *J. Bacteriol.* **172:** 1241-1249.

Pazour, G.J. and Das, A. (1990b) Characterisation of the VirG binding site of *Agrobacterium tumefaciens. Nucl. Acids Res.* **18:** 6909-6913.

Peralta, E.G., Hellmiss, R. and Ream, W (1986) Overdrive, a T-DNA transmission enhancer on the *A. tumefaciens* tumour inducing plasmid. *EMBO. J.* **5:** 1137-1142.

Peralta, E.G. and Ream, W. (1985) T-DNA border sequences required for crown gall tumorigenesis. *Proc. Natl. Acad. Sci. USA* **82:** 5112-5116.

Peters, K.N., Frost, J.W. and Long, S.R. (1986) A plant flavone, luteolin induces expression of *Rhizobium meliloti* nodulation genes. *Science* **233:** 977-980.

Petit, A., Delhaye, S., Tempe, J. and Morel, G. (1970) Recherches sur les guanidines des tissues de crown gall. Mise en evidence d'une relation biochemique entre les souches d'*Agrobacterium* et les tumeurs qu'elles indusient. *Physiol. Veg.* **8:** 205-213.

Petit, A., Tempe, J., Kerr, A., Holsters, M., Van Montagu, M. and Schell, J. (1978) Substrate induction of conjugative activity of *Agrobacterium tumefaciens* Ti-plasmid. *Nature* **271:** 570-571.

Pleier, E and Schmitt, R. (1989) Identification and sequence analysis of two related flagellin genes in *Rhizobium meliloti. J. Bacteriol.* **171:** 1467-1475.

Postma, P.W. and Lengeler, J.W. (1985) Phosphoenol pyruvate : carbohydrate phospho-transferase system of bacteria. *Micobiol. Rev.* **49:** 232-269.

Pueppke, S.G. and Hawes, M.C. (1985) Understanding the binding of bacteria to plant surfaces. *Trends Biotech.* **3:** 310-313.

Puvanesarajah, V., Schell, F.M., Stacey, G., Douglas, C.J. and Nester, E.W. (1985) Role for 2-linked-β-D-glucan in the virulence of *Agrobacterium tumefaciens. J. Bacteriol.* **164:** 102-106.

Raymundo, A.K. and Ries, S.M. (1980) Chemotaxis of *Erwinia amylovora. Phytopathol.* **70:** 1066-1069.

Ream, W. (1989) *Agrobacterium tumefaciens* and interkingdom genetic exchange. *Ann. Rev Phytopathol.* **27:** 583-618.

Rice, M.S. and Dahlquist, F.W. (1991) Sites of deamidation and methylation in Tsr, a bacterial chemotaxis sensory transducer. *J. Biol. Chem.* **266:** 9746-9753.

Rogwsky, P.M., Close, T.J., Chimera, J.A., Shaw, J.J. and Kado, C.I. (1987) Regulation of the *vir* genes of *Agrobacterium tumefaciens* plasmid pTiC58. *J. Bacteriol.* **169:** 5101-5112.

Roitsch, T., Wang, H., Jin, S. and Nester, E.W. (1990) Mutational analysis of the VirG protein, a transcriptional activator of *Agrobacterium tumefaciens* virulence genes. *J. Bacteriol.* **172:** 6054-6060.

Ronson, C.W., Nixon, B.T. and Ausubel, F.M. (1987) Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. *Cell* **49:** 579-581.

Rosenberg, C and Huguet, T. (1984) The pAtC58 plasmid of *Agrobacterium tumefaciens* is not essential for tumour induction. *Mol. Gen. Genet.* **196:** 533-536.

Rossignol, G and Dion, P (1985) Octopine, nopaline and octopinic acid utilisation in *Pseudomonas*. *Can. J. Microbiol.* **31:** 68-74.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual* 2nd Edition. Cold Spring Harbour Laboratory Press, New York.

Sanders, D.A., Gillece-Castro, B.L., Stock, A.M., Burlinghame, A.L. and Koshland, D.E. Jr (1989) Identification of the site of phosphorylation of the chemotaxis response regulator protein. *J. Biol. Chem.* **264:** 21770-21778.

Schägger, H. and von Jagow, G. (1987) Tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1-100kDa. *Anal. Biochem.* **166:** 368-379.

Schell, J., Van Montagu, M., De Beuckeleer, M., De Block, M., Depicker, A., De Wilde, M., Engler, G., Genetello, C., Hernalsteens, J.P., Holsters,M., Seurinck, J., Silva, B., Van Vliet, F. and Villarroel, R. (1979) Interactions and DNA transfer between *Agrobacterium tumefaciens,* the Ti-plasmid and the plant host. *Proc. R. Soc. Lond. B* **204:** 251-266.

Scher, F.M., Kloepper, J.W. and Singleton, C.A. (1985) Chemotaxis of fluorescent *Pseudomonas* spp. to soybean seed exudates *in vitro* and in the soil. *Can. J. Microbiol.* **31:** 570-574.

Scher, F.M., Kloepper, J.W., Singleton, C., Zaleska, J. and Laliberte, M. (1988) Colonization of soybean roots by *Pseudomonas* and *Serratia* species: relationship to bacterial motility, chemotaxis and generation time. *Phyopathology* **78:** 1055-1059.

Schroeder, G., Waffenschmidt, S., Weiler, E.W. and Schroeder, J. (1984) The T-region of the Ti-plasmid codes for an enzyme synthesizing indole-3-acetic acid. *Eur. J. Biochem.* **138:** 387-391.

Schroth, M.N., Weinhold, A.R., McCan, A.H., Hildebrand, D.C. and Ross, N. (1971) Biology and control of *Agrobacterium tumefaciens*. *Hilgardia* **40:** 537-552.

Segall, J.E., Block, S.M. and Berg, H.C. (1986) Temporal comparisons in bacterial chemotaxis. *Proc. Natl. Acad. Sci. USA* **83:** 8987-8991.

Shaw, C.H., Ashby, A.M., Brown, A.P., Royal, C., Loake, G.J. and Shaw, C.H. (1988a) VirA and VirG are the Ti-plasmid functions required for chemotaxis of *Agrobacterium tumefaciens* towards acetosyringone. *Molec. Microbiol.* **2:** 413-417.

Shaw, C.H., Ashby, A.M., Loake, G.J. and Watson, M.D. (1988b) One small step: the missing link in crown gall. In: *Oxford Surveys of Plant Molecular and Cell Biology* Vol **5:** 177-183. B.J. Miflin (Ed.) Oxford University Press.

Shaw, C.H., Loake, G.J., Brown, A.P. and Garrett, C.S. (1989) Molecular biology of chemotaxis in *Agrobacterium*. In: *Molecular Signals in Microbe-Plant Symbiotic and Pathogenic Systems*. B. Lugtenberg (Ed.) Springer-Verlag KG Berlin.

Shaw, C.H., Loake, G.J., Brown, A.P., Garrett, C.S., Deakin, W., Alton, G., Hall, M., Jones, S.A., O.Leary, M. and Primavesi, L. (1991) Isolation and characterisation of behavioural mutants and genes of *Agrobacterium tumefaciens*. *J. Gen. Microbiol.* **137:** 1939-1953.

Shaw, C.H., Watson, M.D., Carter, G.H. and Shaw, C.H. (1984) The right hand copy of the nopaline Ti-plasmid 25bp repeat is required for tumour formation. *Nucl. Acids Res.* **12:** 6031-6041.

Shimoda, N., Toyada-Yamamoto A., Nagamine, J., Usami, J., Katayama, M., Sakagami, Y. and Machida. Y. (1990) Control and expression of *Agrobacterium vir* genes by synergistic actions of phenolic signal molecules and monosaccharides. *Proc. Natl. Acad. Sci. USA* **87:** 6684-6688.

Shioi, J., Tribhuwan, R.C., Berg, S.T. and Taylor, B.L. (1988) Signal transduction in chemotaxis to oxygen in *Escherichia coli* and *Salmonella typhimurium*. *J. Bacteriol.* **170:** 5507-5511.

Silverman, M. and Simon, M. (1974a) Flagellar rotation and the mechanism of bacterial motility. *Nature* **249:** 73-74.

Silverman, M. and Simon, M. (1974b) Characterisation of *Escherichia coli* flagellar mutants that are insensitive to catabolite repression. *J. Bacteriol.* **120:** 1196-1203.

Simon, R., Priefer, U. and Puhler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Biotechnology* **1:** 784-791.

Simon, R., Quandt, J. and Klipp, W. (1989) New derivatives of transposon Tn5 suitable for mobilization of replicons, generation of operon fusions and induction of genes in Gram-negative bacteria. *Gene* **80:** 161-169.

Slocum, M.K. and Parkinson, J.S. (1983) Genetics of the methyl accepting chemotaxis proteins in *Escherichia coli* : organization of the *tar* region. *J. Bacteriol.* **155:** 567-577.

Smith, J.M., Rowsell, E.H., Shioi, J. and Taylor, B.L. (1988) Identification of a site of ATP requirement for signal processing in bacterial chemotaxis. *J. Bacteriol.* **170:** 2698-2704.

Smith, E.F. and Townsend, C.O. (1907) A plant tumour of bacterial origin. *Science* **25:** 671-673.

Soberon, X., Covarrubias, L. and Bolivar, F (1980) Construction and characterisation of new cloning vehicles IV : deletion derivatives of pBR322 and pBR325. *Gene* **9:** 287-305.

Soby, S. and Bergman, K. (1983) Motility and chemotaxis of *Rhizobium meliloti* in soil. *Appl. Env. Microbiol.* **46:** 995-998.

Sockett, R.E., Armitage, J.P and Evans, M.C.W. (1987) Methylation independent and methylation dependent chemotaxis in *Rhodobacter sphaeroides* and *Rhodospirillum rubrum*. *J. Bacteriol.* **169:** 5808-5814.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98:** 503-517.

Spencer, P.A. and Towers, G.H.N. (1988) Specificity of signal compounds detected by *Agrobacterium tumefaciens*. *Phytochemistry* **27:** 2781-2785.

Springer, M.S., Goy, M.F. and Adler, J. (1977) Sensory transduction in *Escherichia coli* : two complementary pathways of information processing that involve methylated proteins. *Proc. Natl. Acad. Sci. USA.* **74:** 3312-3316.

Springer, W.R. and Koshland, D.E. Jr (1977) Identification of a protein methyltransferase as the *cheR* gene product in the bacterial sensing system. *Proc. Natl. Acad. Sci. USA* **74:** 533-537.

Stachel, S.E., Messens, E., Van Montagu, M and Zambryski, P. (1985) Identification of the signal molecules produced that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature* **318:** 624-629.

Stachel, S.E. and Nester, E.W. (1986) The genetic region and transcriptional organisation of the A6 Ti-plasmid of *Agrobacterium tumefaciens*. *EMBO. J.* **5:** 1445-1454.

Stachel, S.E., Nester, E.W. and Zambryski, P. (1986) A plant cell factor induces *Agrobacterium tumefeciens vir* gene expression. *Proc. Natl. Acad. Sci. USA* **83:** 379-383.

Stachel, S.E. and Zambryski, P. (1986a) *Agrobacterium tumefaciens* and the susceptible plant cell: a novel adaptation of extracellular recognition and DNA conjugation. *Cell* **47:** 155-157.

Stachel, S.E and Zambryski, P. (1986b) VirA andVirG control the plant induced activation of the T-DNA transfer processes of *Agrobacterium tumefaciens*. *Cell* **46:** 325-333.

Staskawicz, B., Dahlbeck, D., Keen, N. and Napoli, C. (1987) Molecular characterisation of cloned avirulence genes from Race 0 and Race 1 of *Pseudomonas syringae* pv *glycinea*. *J. Bacteriol.* **169:** 5789-5794.

Steck, T.R., Morel, P. and Kado, C.I. (1988) *vir* box sequences in *Agrobacterium tumefaciens* pTiC58 and A6. *Nucl. Acids Res.* **16:** 8736.

Stewart, R.C. and Dahlquist, F.W. (1987) Molecular components of bacterial chemotaxis. *Chem. Rev.* **87:** 997-1025.

Stock, A.M. and Stock, J.B. (1987) Purification and characterisation of the CheZ protein of bacterial chemotaxis. *J. Bacteriol.* **169:** 3301-3311.

Stock, J. (1990) Role of protein carboxyl methylation in bacterial chemotaxis. In: *Protein Methylation* pp275-284. Paik, W.K. and Kim, S. (Eds.) CRC press inc. Boca Raton Fl.

Stock, J.B. and Koshland, D.E. Jr (1978) A protein methylesterase involved in bacterial sensing. *Proc. Natl. Acad. Sci. USA* **75:** 3659-3663.

Stock, J.B., Ninfa, A.J. and Stock, A.M. (1989) Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol. Rev.* **53:** 450-490.

Stock, J.B., Stock, A.M. and Mottonen, J.M. (1990) Signal transduction in bacteria. *Nature* **344:** 395-400.

Sutherland, I.W. (1985) Biosynthesis and composition of Gram-negative bacterial extracellular and wall polysaccharides. *Ann. Rev. Microbiol.* **39:** 243-270.

Taylor, B.L. and Lengeler, J.W. (1990) Transductive coupling by methylated transducing proteins and permeases of the Phosphotransferase system in bacterial chemotaxis. In: *Membrane Transport and Information Storage* pp69-90 Alan R. Liss (Ed.).

Tempe, J. and Goldman, A. (1982) Occurrence and biosynthesis of opines. In: *Molecular Biology of Plant Tumours* pp428-450 G. Kahl and J. Schell (Eds.) Academic Press New York.

Thomashow, M.F., Hugly, S., Bucholz, W.G. and Thomashow, L.S. (1986) Molecular basis for the auxin-independent phenotype of crown gall tumour tissues. *Science* **231:** 616-618.

Thomashow, M.F., Karlinsey, J.E., Marks, J.R. and Hurlbert, R.E. (1987) Identification of a new virulence locus in *Agrobacterium tumefaciens* that affects polysaccharide composition and plant cell atachment. *J.Bacteriol.* **169:** 3209-3216.

Thomashow, M.F., Panagopoulos, C.G., Gordon, M.P. and Nester, E.W. (1980) Host range of *Agrobacterium tumefaciens* is determined by the Ti-plasmid. *Nature* **283:** 794-796

Toro, N., Datta, A., Carmi, O.A., Young, C., Prusti, R.K. and Nester, E.W. (1989) The *Agrobacterium tumefaciens* VirC1 gene product binds to overdrive, a T-DNA transfer enhancer. *J. Bacteriol.* **171:** 6845-6849.

Toro, N., Datta, A., Yanofsky, M. and Nester, E.W. (1988) Role of the overdrive sequence in the T-DNA border cleavage in *Agrobacterium. Proc. Natl. Acad. Sci. USA* **85:** 8558-8562.

Tremblay, G., Cagliardo, R., Chilton, W.S. and Dion, P. (1987) Diversity among opine utilising bacteria: identification of coryneform isolates. *Appl. Env. Microbiol.* **53:** 1519-1524.

Uttaro, A.D., Cangelosi, G.A., Germia, R.A., Nester, E.W. and Ugalde, R.A. (1990) Biochemical characterisation of avirulent *exoC* mutants of *Agrobacterium tumefaciens. J. Bacteriol.* **172:** 1640-1646.

Valdivia, R.H., Wang, L. and Winans, S.C. (1991) Characterisation of a putative periplasmic transport system for octopine accumulation encoded by *Agrobacterium tumefaciens* Ti-plasmid pTiA6. *J. Bacteriol.* **173:** 6398-6405.

Van der Werf, P. and Koshland, D.E. Jr (1977) Identification of a γ-glutamyl ester in bacterial membrane protein involved in chemotaxis. *J. Biol. Chem.* **252:** 2793-2795.

Van Larebeke, N., Engler, G., Holsters, M., Van der Elsacker, S., Zaenen, I., Schilperoort, R.A. and Schell, J. (1974) Large plasmid in *Agrobacterium tumefaciens* essential for crown gall inducing ability. *Nature* **252:** 167-170.

Van Larebeke, N., Genetello, C., Schell, J., Schilperoort, R.A., Hermans, A.K., Hernalsteens, J.P. and Van Montagu, M. (1975) Acquisition of tumour inducing ability by non-oncogenic *Agrobacterium* as a result of plasmid transfer. *Nature* **255:** 742-743.

Van Montagu, M. and Schell, J. (1979) The plasmids of *Agrobacterium tumefaciens*. In: *Plasmids of Medical, Environmental and Commercial Importance*. K.N. Timmis and A. Puhler (Eds.) Elsevier/North Holland Biomedical Press.

Van Onckelen, H., Prinsen, E., Inze, D., Rudelsheim, P., Van Lijsebettens, M., Folgin, A., Schell, J., Van Montagu, M. and De Gref, J. (1986) *Agrobacterium* T-DNA gene-1 codes for tryptophan 2-monooxygenase activity in tobacco crown galls. *Febs. Lett.* **198:** 357-360.

Van Veen, R.J.M., den Dulk-Ras, H., Schilperoort, R.A. and Hooykaas, P.J.J. (1987) Chromosomal nodulation genes: Sym-plasmid containing *Agrobacterium* strains need chromosomal virulence genes *chvA* and *chvB* for nodulation. *Plant Mol. Biol.* **8:** 105-108.

Van Veen, R.J.M., den Dulk-Ras, H., Bisseling, T., Schilperoort, R.A. and Hooykaas, P.J.J. (1988) Crown gall tumour and root nodule formation by the bacterium *Phyllobacterium myrsinacearum* after the introduction of an *Agrobacterium* Ti-plasmid or a *Rhizobium* Sym-plasmid. *Mol. Plant-Microbe Interact.* **1:** 231-234.

Velumbathi, K., Krishnan, M., Gould, J.H., Smith, R.H. and Gelvin, S.B. (1989) Opines stimulate induction of the *vir* genes of the *Agrobacterium tumefaciens* Ti-plasmid. *J. Bacteriol.* **171:** 3696-3703.

Wang, K., Genetello, C., Van Montagu, M. and Zambryski, P. (1987) Sequence context of the T-DNA border repeat element determines its relative activity during T-DNA transfer to plant cells. *Mol. Gen. Genet.* **210:** 338-346.

Wang, K., Herrera-Estrella, L., Van Montagu, M. and Zambryski, P. (1984) Right 25bp terminus sequences of the nopaline T-DNA is essential for and determines direction of T-DNA transfer from *Agrobacterium* to the plant genome. *Cell* **38:** 35-41.

Ward, J.E., Dale, E.M. and Binns, A.N. (1991) Activity of the *Agrobacterium* T-DNA transfer machinery is affected by *virB* gene products. *Proc. Natl. Acad. Sci. USA* **88:** 9350-9354.

Ward, J.E., Dale, E.M., Nester, E.W. and Binns, A.N. (1990) Identification of a VirB10 protein aggregate in the inner membrane of *Agrobacterium tumefaciens*. *J. Bacteriol.* **172:** 5200-5210.

Waters, V.L., Hirata, I.C.H., Pansegrau, W., Lanka, E. and Guiney, D.G. (1991) Sequence identity in the nick regions of *IncP* plasmid transfer origins and T-DNA borders of *Agrobacterium tumefaciens*. *Proc. Natl. Acad. Sci. USA* **88:** 1456-1459.

Watson, B., Currier, T.C., Gordon, M.P., Chilton, M-D. and Nester, E.W. (1975) Plasmid required for virulence of *Agrobacterium tumefaciens*. *J. Bacteriol.* **123:** 255-264.

Whatley, M.H., Bodwin, J.S., Lippincott, B.B and Lippincott, J.A. (1976) Role for *Agrobacterium* cell envelope lipopolysaccharide in infection site attachment. *Infect. Immun.* **13:** 1080-1083.

White, F.F. and Nester, E.W. (1980) Hairy root plasmid encodes virulence traits in *Agrobacterium rhizogenes*. *J. Bacteriol.* **141:** 1134-1141.

White, P.R. and Braun, A.C. (1942) A cancerous neoplasm of plants - autonomous bacteria-free crown gall tissue. *Cancer Res.* **2:** 597-617.

Willmitzer, L., De Beuckeleer, M., Lemmers, M., Van Montagu, M. and Schell, J. (1980) DNA from the Ti-plasmid is present in the nucleus and absent from plastids of plant crown gall cells. *Nature* **287:** 359-361.

Willmitzer, L., Dhaese, P., Schreier, P.H., Schmalenbach, W., Van Montagu M. and Schell, J. (1983) Size, location and polarity of T-DNA encoded transcripts in nopaline crown gall tumours: common transcripts in octopine and nopaline tumours. *Cell* **32:** 1045-1056.

Winans, S.C. (1990) Transcriptional induction of an *Agrobacterium* regulatory gene at tandem promoters by plant released phenolic compounds, phosphate starvation and acidic growth media. *J. Bacteriol.* **172:** 2433-2438.

Winans, S.C. (1991) An *Agrobacterium* two component regulatory system for the detection of chemicals released from plant wounds. *Molec. Microbiol.* **5:** 2345-2350.

Winans, S.C., Kerstetter, R.A. and Nester, E.W. (1988) Transcriptional regulation of the *virA* and *virG* genes of *Agrobacterium tumefaciens*. *J. Bacteriol.* **170:** 4047-4054.

Wolfe, A.J., Conley, M.P., Kramer, T.J. and Berg, H.C. (1987) Reconstitution of signalling in bacterial chemotaxis. *J. Bacteriol.* **169:** 1878-1885.

Yadav, N.S., Vanderleyden, J., Bennet, D.R., Barnes, W.M. and Chilton, M-D. (1982) Short direct repeats flank the T-DNA on a nopaline Ti-plasmid. *Proc. Natl. Acad. Sci. USA* **79:** 6332-6326.

Yamamoto, K., Macnab, R.M. and Imae, Y. (1990) Repellent response functions of the Trg and Tap chemoreceptors of *Escherichia coli*. *J. Bacteriol.* **172:** 383-388.

Yanisch-Perron, C., Vierra, J. and Messing, J. (1985) Improved M13 cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. *Gene* **33:** 103-119.

Zaenen, I., Van Larebeke, N., Teuchy, H., van Montagu, M. and Schell, J. (1974) Supercoiled circular DNA in crown gall inducing *Agrobacterium* strains. *J. Mol. Biol.* **86:** 109-127.

Zambryski, P. (1988) Basic processes underlying *Agrobacterium* mediated DNA transfer to plant cells. *Ann. Rev. Genet.* **22:** 1-30.

Zhulin, I.B., Tretyakova, S.E. and Ignatiov, V.V. (1988) Chemotaxis of *Azospirillum brasilense* towards compounds typical of plant-root exudates. *Folia Microbiologica* **33:** 277-280.

Ziègler, R.J., Peirce, C. and Bergman, K. (1986) Mapping and cloning of the *fla-che* region of the *Rhizobium meliloti* chromosome. *J. Bacteriol.* **168:** 785-790.

Zorreguieta, A., Geremia, R.A., Cavaignac, S., Cangelosi, G.A., Nester, E.W. and Ugalde, R.A. (1988) Identification of the product of an *Agrobacterium tumefaciens* chromosomal virulence gene. *Mol. Plant-Microbe Interact.* **1:** 121-127.