The genetic dissection of chemotaxis in agrobacterium tumefaciens

Loake, Gary John

The Genetic Dissection of Chemotaxis in *Agrobacterium tumefaciens*

by

GARY JOHN LOAKE

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DECLARATION

I declare that all the experiments involved in this thesis submitted by me for the degree of Doctor of Philosophy are my own original work, except where due reference is made to coworkers, and has not been submitted for a degree at this or any other university.

For MUM and DAD

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ABBREVIATIONS

Amp = Ampicillin

Km = Kanamycin

Rif = Rifampicin

Cm = Chloramphenicol

Tc = Tetracycline

Sm = Streptomycin

Sp = Spectinomycin

A_{260} = Absorbance at 260nm

A_{280} = Absorbance at 280nm

bp = Base pair

EMS = Ethyl methane sulphonate

dCTP = deoxycytosine triphosphate

ATP = Adenosine triphosphate

KD = Kilodalton

LMP = Low melting point

SDS = Sodium dodecyl sulphate

No. = Number

Bact. = Bacteria

UV = Ultraviolet

T-DNA = Transfer DNA

Ti-plasmid = Tumour Inducing plasmid

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Abstract

A range of sugars, many of them characteristic of plant extracts were tested as potential chemoattractants for *Agrobacterium* . The results divided the sugars into 4 groups of attractants and indicated the presence of a highly sensitive chemotaxis system in *A.tumefaciens.*

Motility in *Agrobactewrium* consisted of long straight runs, with relatively few tumbles or stops. The propulsive mechanism seemed to resemble that of *Rhizobium.*

Methionine-starved methionine auxotrophs of *A.tumefaciens* , although fully motile, were non-chemotactic to sucrose or acetosyringone, unless supplemented with exogenous methionine. Neither ethionine nor α-methyl-DL-methionine could correct the non-chemotactic phenotype, while seleno-DL-methionine partially restored taxis. Pulse-labelling of *A.tumefaciens* with L-[methyl-^{3}H]-methionine in the presence of chloramphenicol, and an attractant resulted in the appearance of 2 radio-labelled proteins of approximately 55KDa. Thus, in *A.tumefaciens*, chemotactic responses may be associated with the transfer of methyl groups from methionine via S-adenosyl methionine to MCPs.

Using transposon mutagenesis a battery of *A.tumefaciens* chemotaxis mutants were generated and characterized. A number of mutated behavioural genes were isolated using the kanamycin resistant determinant of Tn5 as a positive selectable marker. Tn5 flanking sequences were used as probes to recover wild-type behavioural

genes from a gene library constructed in the cosmid pLAFR3. Behavioural genes were found to be clustered on the *A.tumefaciens* chromosome and to possess similarity with behavioural genes from *R.meliloti*.

Contents

CHAPTER 1

1.0 The *Rhizobiaceae*

1.1 The genus *Agrobacterium*

The genus *Agrobacterium* is a member of the family *Rhizobiaceae* (1). Morphologically, the Agrobacteria are Gram-negative rods with rounded ends, possessing 1-6 peritrichous flagella. They are mesophilic, with 28°C being the optimum temperature for their growth. Agrobacteria constitute a common component of the soil microflora and are especially found in association with plant roots (2). Although, Beijerinck and Van Deldan were the first to decribe *Agrobacterium* (3), perhaps the most poignant discovery was that of Smith and Townsend in 1907 (4) , which showed *Bacterium tumefaciens* to be the causal agent of Crown Gall, an infectious neoplastic transformation of plant cells (Fig.1.1). The genus *Agrobacterium* was created by Conn in 1942 (5) , after the members of the genus *Phytomonas* (*Pseudomonas*, *Xanthomonas* and *Corynebacteria*) had been reclassified. The genus has been sequentially expanded by the discovery of new members responsible for similar plant neoplastic diseases. It now contains four species; *A.tumefaciens* provoking Crown Gall (4) , *A.rhizogenes* Hairy Root (6) , *A.rubi* Cane Gall (7) , and the non-phytopathogenic *A.radiobacter* (8).

Crown Gall is responsible for significant losses in agriculture and horticulture in Europe (9) , North America (10) , Australia (11) and Japan (12). Conversely, Hairy Root formation poses no threat to agriculture and plays an important role in the controlled induction of rooting in plants (13).

Both Crown Gall and Hairy Root cells are tumourous and are thus capable of autonomous growth, ie in the absence of phytohormones *in vitro* (14). Another characteristic peculiar to these cells is their synthesis and secretion of unusal metabolites called opines (15).

Agrobacterium pathogenicity is dependent upon the presence of a large tumour inducing (Ti) plasmid in *A.tumefaciens* (16,17) and a similar root inducing (Ri) plasmid in *A.rhizogenes* (18). A small discrete piece of DNA from these plasmids (the Transfer or T-DNA) is transferred from the bacterium to the plant cell genome in the process of transformation (19). It is the expression of the T-DNA that transforms plant cells to autonomous tumorigenic growth and opine secretion (20). The secreted opines can be catabolised by the specific virulent *Agrobacterium* strain that incited

Fig.1.1. Crown gall tumour induced by *Agrobacterium tumefaciens*.

tumour formation, but not by most other microflora (21). This was shown to be a Ti-plasmid borne trait (22,16). Thus, Agrobacteria possess a natural mechanism for 'genetically engineering' plants to provide them not only with an enviromental niche, but also a carbon and nitrogen source which they alone can utilise. The phrase 'genetic colonisation' (23) was coined for this mode of parasitism.

1.2. Attachment of *Agrobacterium* to plant cells

The first step in plant cell transformation has been proposed to be the attachment of *Agrobacterium* to susceptible plant cells exposed by wounding (24). Matthysse reported data that was consistent with the concept of a 'bacterial receptor' , possibly consisting of plant pectin and residing on susceptible plant cells (reviewed in (24)). The plant cell was demonstrated to play no active role in the attachment process, as *Agrobacterium* still bound heat treated or gluteraldehyde fixed plant cells (25). This initial attachment is loose and reversible, but is followed by the bacterial synthesis of cellulose fibrils, which results in the irreversible tight binding of *Agrobacterium* to susceptible plant cells (26). Matthysse genetically dissected these initial events with the isolation of cellulose fibril synthesising (*cel*) and attachment (*att*) mutants (27). Both classes of mutants mapped to a position around *trp-2* on the *Agrobacterium* chromosone, and exhibited a virulent or surprisingly avirulent phyenotype in the case of *cel* or *att* mutants respectively (27).

The *att* mutants possessed flagella, fimbriae, were fully motile, produced the normal complement of exopolysaccharides and exhibited no abnormalities in their lipopolysaccharide structure. Closer examination however revealed the absence of two polypeptides of 34 and 38KDa, which are present in the wild-type strain. Presumably, these polypeptides will be shown to play a pivotal role in the attachment of the bacterium to the plant cell wall.

1.3. The chromosomal virulence loci

Three other chromosomal loci distinct from *cel* and *att* have been defined. These loci *chv A*, *chv B* (28) and *psc A* (29) are deficient in both virulence and attachment. Mutants in these loci were isolated by virtue of their lack of fluorescence on calcofluor plates observed under U.V light. Both *chv A* and *chv B* map to an 11Kb chromosomal virulence region and define two transcriptional units A and B. *chv A* and *chv B*

mutants are resistant to *Agrobacterium* specific phages, lack flagella and are deficient in the synthesis, regulation or transcription of a small polysaccharide, a cyclic β-1,2-glucan, which is unique to the *Rhizobiaceae.* This polysaccharide, consisting of about 17-20 glucose residues, is predominately found in the periplasm but traces can also be detected in the media of *A.tumefaciens* cultures. The cellular and extracellular forms of β-1,2-glucan are absent in *chv B* mutants (30). The *chv A* mutants show no extracellular form of β-1,2-glucan and are presently being analysed for intracellular β-1,2-glucan (30).

pscA like *exoC* mutants of *Rhizobium meliloti* do not produce cellulose fibrils and are deficient in the synthesis of succinoglycan, the major exopolysaccharide of Agrobacteria (31).

Recently, similar DNA sequences to *chvA* , *chv B* and *psc A* have been shown to exist in *Rhizobium trifoli* , *Rhizobium leguminosarum* , *R.meliloti* and *Rhizobium phaseoli* but to be absent in *Pseudomonas* species (32,33). Dylan and coworkers isolated the *R.meliloti* sequences similar to both *chv A* and *chv B* and designated them *ndv A* and *ndv B* respectively (34). Transposon mutagenesis and marker exchange of these genes produced *R.meliloti* mutants that provoked nodule like structures on alfalfa, were devoid of bacteroids, lacked infection threads and could not fix nitrogen. *R.meliloti* cosmid clones complementing *ndv A* and *ndv B* were also found to complement *A.tumefaciens* *chv A* and *chv B* mutants respectively to wild-type attachment and virulence phenotypes. The same relationship was also found to exist between *exoC* and *pscA* mutants (33).

Therefore, at present there are three loci essential for virulence in *A.tumefaciens* which are interchangeable with genes essential for symbiosis in *R.meliloti.* This suggests a general role for cell surface components in the mediation of the initial stages in plant-microbe interactions.

Winans and coworkers have recently presented evidence for a fourth chromosomal virulence gene, *chv D* (35). This gene is proposed to have a regulatory role in T-DNA transfer.

1.4. The Tumor-inducing (Ti) and Root-inducing (Ri) plasmids

The Ti-plasmid of *A.tumefaciens* and the Ri-plasmid of *A.rhizogenes* are essential for tumour induction (16,17). They are usually about 190-240 Kb in size and present in 1-3 copies per cell (Fig.1.4).

Tumours and hairy roots induced by Agrobacteria produce a characteristic set of opines, which can only be degraded by virulent Agrobacteria (20). The synthesis of opines in tumours and hairy roots and their subsequent catabolism by Agrobacteria is encoded by genes present on the Ti (Ri) plasmid (19,36). Based on the opines produced in the tumours, Ti-plasmids can be placed into four groups and Ri-plasmids into three groups. Octopine (N2(D-1-carboxyethyl)-1-arginine) is found in tumours incited by the Octopine Ti-plasmid, eg. pTiB6. Nopaline (N2(D-1,3-dicarboxypropyl)-1-arginine) is found in tumours formed by the nopaline Ti-plasmid, eg. pTiC58. Strains which produce neither nopaline or octopine were initially termed null-type, however, this class now contains two different types of strains; Leucinopine (N2(L-1, 3-dicarboxypropyl)-1-leucine) and D,L-succinamopine (asparaginopine) (N2(D-1, 3-dicarboxypropyl)-1-asparagine) found in tumours formed by leucinopine (eg. pTi542) and asparaginopine (eg. pTiEU6) Ti-plasmid harbouring strains respectively. Hairy Roots induced with a mannopine Ri-plasmid (eg. pRiTR7) contain mannopine (N2(1-D-mannityl)1-glutamine). Agropine Ri-plasmids (eg. pRiA4) are responsible for the formation of agropine (1-2′-β-lactone of mannopine) besides mannopine in Hairy Root. Strains with a third class of Ri-plasmids (eg. pRi2659) provoke Hairy Root with cucumopine (structure unkown). Another class of opines, the agrocinopines, which are phosphorylated sugar derivitives (37), are present in tumours and Hairy Roots. Agrocinopine A is produced by nopaline and succinamopine Ti and agropine and mannopine Ri; agrocinopine C by leucinopine Ti and mannopine Ri harbouring *Agrobacterium* strains.

Restriction endonuclease maps of Ti and Ri plasmids from each class are distinct, however within a class such maps may be similar or even identical. The wide host range (WHR) octopine Ti plasmids form a very homogeneous group (38), as do the mannopine Ri and the agropine Ri plasmids (39). The nopaline Ti-plasmids form a heterogeneous group (38), although some nopaline Ti-plasmids (pTiT37) are very similar to succinamopine Ti-plasmids (pTiEU6).

Tumour morphology is also a Ti-plasmid determined trait. Octopine Ti-plasmids

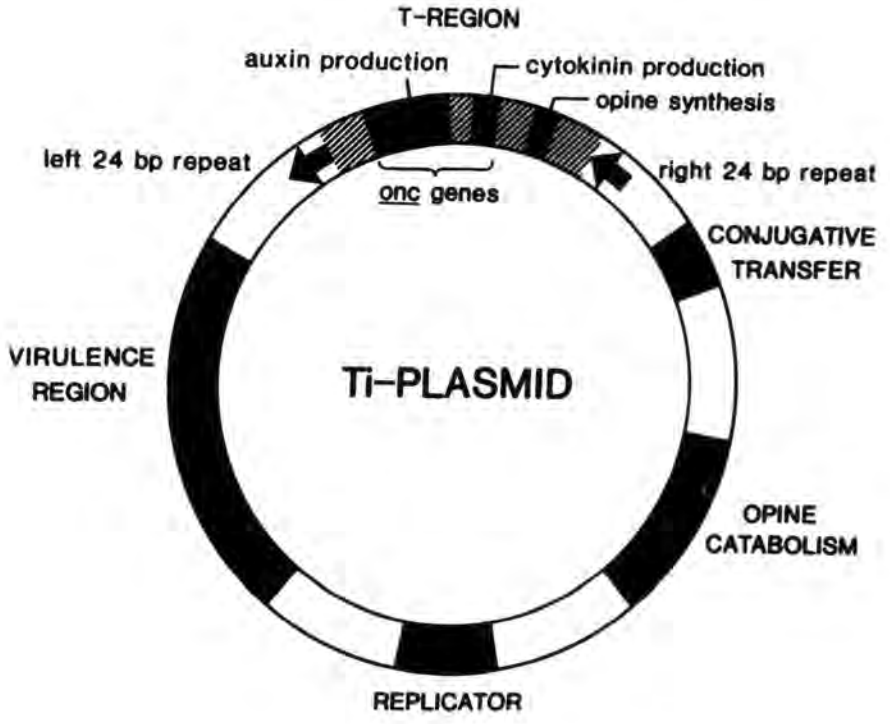


Fig.1.4. Genetic map of an Octopine Ti plasmid. (From Melchers and Hooykaas. (1987)).

form rough tumours with adventitious roots on *Kalanchoe diagremontiana.* Leucinopine Ti-plasmids also produce rough tumours, however adventious roots are absent. In stark contrast nopaline and succinamopine Ti-plasmids form smooth tumours, which occasionally produce adventitious shoots. Ri-plasmids exhibit more subtle tumour morphology variations, root formation by agropine Ri-plasmids is usually slightly more pronounced than that observed in mannopine Ri-plasmids for example (40). In addition agropine Ri-plasmids show a wider host range than mannopine Ri-plasmids.

Sensitivity to bacteriocins is mediated by a locus on nopaline and succinamopine Ti-plasmids (41). The bacteriocin agrocin 84 is a phosphorylated sugar derivitive with structural homology to agrocinopine A (42), whose biosynthesis is encoded by the plasmid pAgK84 found in *A.radiobacter* strain K84 (43). Agrocin 84 enters nopaline and succinamopine Ti-plasmid harbouring strains via the agrocinopine A uptake system encoded by these plasmids, and once in the cell it inhibits both protein synthesis and DNA replication (44).

Beaty and coworkers have shown a locus on nopaline Ti-plasmids termed *Tzs* distinct from the T-DNA, encodes a dimethylallyltransferase, which acts in the first step of cytokinin biosynthesis to produce the phytohormone transzeatin (45).

The Ti-plasmid region specifying replication and incompatability has been cloned (46). Incompatability studies have placed the nopaline, octopine and succinamopine Ti-plasmids in the same group, inc-Rh-1 (47). The leucinopine Ti-plasmids belong to a different group inc-Rh-2, together with certain non Ti-plasmids encoding nopaline catabolic functions (47). The agropine Ti-plasmids make-up a third group inc-Rh-3 (48).

The Ti and Ri-plasmids are present in less than one percent of the total *Agrobacterium* soil population. Both plasmids are however conjugative, their *tra* genes being inducible by specific opines (49); octopine, agrocinopine A and agrocinopine C for octopine, nopaline and leucinopine Ti-plasmids respectively (50,51). Therefore, at the site of infection the Ti-plasmid can be rapidly mobilised and consequently the genes specifing virulence and opine catabolism disseminated. The inducers of the Ri-plasmid *tra* genes have still to be elucidated.

In addition to Ti and Ri-plasmids Agrobacteria also possess cryptic 'megaplasmids' (52). Due to their large size these plasmids have proved notoriously difficult to isolate and subsequently still wait characterisation.

1.5. The T-DNA

Using southern blot analysis a specific piece of the Ti-plasmid was discovered to be integrated in to the nuclear DNA of every tumour line tested, this DNA fragment was termed the T-DNA (53) (Fig.1.5). In order for transformation to occur the T-DNA must integrate into a region of the plant genome which is transcriptionally active. From one to a dozen copies of the T-DNA, either complete or truncated may be found integrated. These T-DNA's may be found at the same locus as a tandem (54) or inverted (55) repeat, or at different loci on the same or a different chromosome (56). The T-DNA of the Ri-plasmid is thought to behave in a similar fashion. The T-DNA is bordered by imperfect 24bp direct repeats which play an essential role in transfer. The sequences of the border repeats essential for T-DNA transfer in the nopaline (57) and octopine (58) Ti-plasmids and the agropine Ri-plasmid (59) have been determined. A consensus sequence compiled from eight different border repeats reveals two conserved sequences of 12 and 7bp with a variable 5bp region between them (60). Within the T-region itself sequences may be found which possess similarity to the border repeats (61), the significance of these pseudo-repeats with respect to T-DNA transfer remains to be elucidated. T-DNA has been isolated from tumour cell lines and the regions around the T-DNA-plant-DNA left and right junctions have been analysed (58). It was discovered there was more variation at the left-end than the right-end junctions; two-thirds of the right-end junctions were found to be lying within 11bp of the right-border repeat, however, two-thirds of the left end-junctions were only within 217bp of the left-border repeat (62). For the right repeat a maximum of the left 3bp were found integrated into the plant genome compared with up to the right 12bp of the left repeat (57).

Nopaline and succinamopine Ti-plasmids and mannopine Ri-plasmids have only one T-DNA region, however, octopine and leucinopine Ti-plasmids and agropine Ri-plasmids have two. These two T-DNA regions are called TL and TR respectively, they are both terminated by the 24bp border repeats and separated from each other by a lengh of DNA called the TC-region. The TL and TR are usually around 13.5 and 6-7kb in size respectively for octopine Ti-plasmids (53), this compares to 23kb for the single T-DNA region present in nopaline Ti-plasmids (54). Analysis of transformed plant cells has revealed that they may receive the TL or TR-DNA or both (63). The

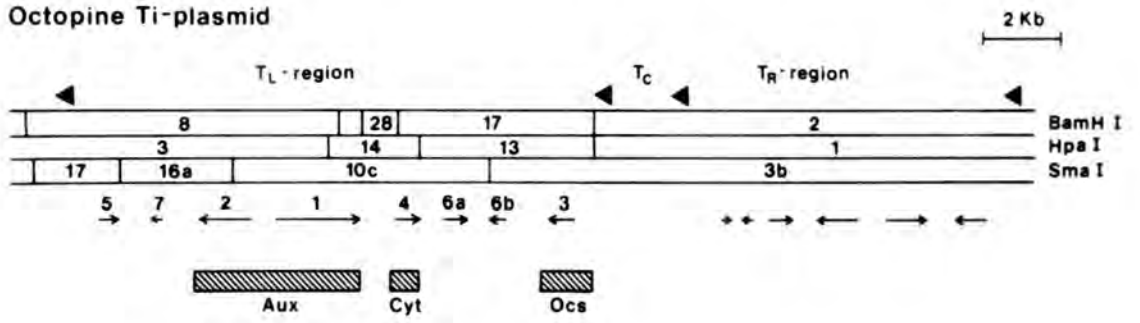


Fig.1.5. Physical map of the T-region of the octopine Ti plasmid. The TL-region, TC-region and TR-region segments are surrounded by 24bp direct border repeats (arrow heads). Open reading frames on TL-DNA and TR-DNA are indicated by black arrows. The T-DNA loci *aux* , *cyt* and *ocs* are boxed. (From Barker et al. (1984)).

TC-region has never been found as an independent integrated unit. In one tumour line however, the TC-region is present as a unit comprising in addition the TR and TL-region (64).

1.6. T-DNA encoded genes

Transcript maps have been compiled for the octopine Ti TL DNA (65), the octopine TR DNA (66), the nopaline T-DNA (67) and the agropine Ri TL and TR DNA (68). The data shows the T-DNA encodes a number of genes whose total is dependent on the specific T-DNA species. For the octopine TL-DNA, the best studied, eight transcripts were defined. Transposon mutagenesis of different T-DNA species failed to induce phenotypic changes in the bacterial host, thus it was postulated that T-DNA genes may be expressed specifically in the plant. Subsequently, three T-DNA loci were defined for the TL-DNA of the octopine Ti-plasmid which altered host range and/or tumour morphology (69). These mutants exhibited changes in tumour morphology on tobacco and *Kalanchoe* and were avirulent on tomato (69). One of the mutants produced root outgrowths from tumours on *Kalanchoe* and tobacco and could be restored to virulence on tomato by the addition of cytokinin. The other two mutants produced shoot outgrowths from tumours on *Kalanchoe* and tobacco and could be restored to virulence on tomato by the addition of auxin. This data suggested that T-DNA loci were specifying functions for the synthesis of plant growth hormone compounds resembling the mode of action of cytokinins and auxins, thus these loci were called *cyt* and *aux* respectivly. The product of the *cyt* gene was subsequently shown to catalyse the reaction of isopentenyl- pyrophosphate and AMP to isopentenyl-AMP, a compound with cytokinin activity (70). The *cyt* gene was thus renamed *ipt* , in view of the isopentenyl transferase activity of its gene product. Similar work with the *aux* gene products showed they converted tryptophan to indole acetic acid (IAA), an auxin. The *aux-1* gene product was found to possess monooxygenase activity in converting tryptophan to indole acetamide (IAM) (71). IAM then acted as the substrate for the *aux-2* gene product, an amide hydrolase which converted IAM to IAA (72). The *aux* loci were therefore renamed *iaaM* and *iaaH* respectively. Genes which possessed similarity to *ipt* , *iaaM* and *iaaH* were also found in the T-region of nopaline and succinamopine Ti-plasmids (73).

These plant growth promoting genes may be considered analogous to mammalian oncogenes in that they too induce uncontrolled proliferative growth and can be carried by a gene vector; the Ti (Ri) plasmid as opposed to a virus for mammalian oncogenes. Therefore, the *ipt* , *iaaM* and *iaaH* genes can be considered to be plant *onc* genes.

The 6b gene of the TL-region of the octopine Ti-plasmid also possesses *onc* gene activity. On *Kalanchoe* only mutations in the *ipt* , *iaaM* and *iaaH* genes lead to avirulence, suggesting the presence of only three *onc* genes. On *Nicotiana* however, an intact 6b gene even in the presence of mutated *iaaH* and *ipt* genes is sufficient for tumourigenesis (73). Therefore, gene 6b seems to possess some species-specific *onc* activity.

The T-DNA in addition to its plant growth pertubation properties also encodes genes which allow the 'genetically colonised' plant cell to synthesise and secrete opines. In Ti-plasmids the gene at the far right of the T-DNA specifies opine synthesis. This gene is called *ocs* in octopine Ti-plasmids because of its role in octopine synthesis (74). Similar genes encode nopaline synthase (*nos*) in nopaline Ti-plasmids (75) and succinamopine synthase in succinamopine Ti-plasmids (76). The TR region of the octopine Ti-plasmid also possesses genes which specify the synthesis of the opines agropine and manopine (77). Synthesised opines are secreted from the transformed plant cell via a secretory pathway encoded by the 6a gene in the TL-DNA region of the octopine Ti-plasmid (78). All other Ti-plasmids possess a similar gene, therefore all opines are thought to be secreted by a common pathway.

The mannopine Ri-plasmids contain only one T-DNA region compared with the agropine Ri-plasmids two (79). Transcriptional mapping and oncogenic analysis of the agropine Ri-plasmid TL-region revealed four loci involved in root induction, namely *rol A* , *rol B* , *rol C* and *rol D* (79). Although *rol A* , *rol B* and *rol C* individually can induce root proliferation, any two of these loci together act synergistically (80). If transgenic tobacco plants are produced containing *rol A* , *rol B* or *rol C* expressed under the cauliflower mosaic virus 35S promoter startlingly different phenotypes are produced (81). In *rol C* transgenes a phenotype is produced which inhibits the onset of senescence. In complete contrast *rol B* transgenes show premature senescence even prior to flowering. Transgenic *rol A* plants are normal except for wrinkled leaves (82). Neither *rol A* , *rol B* , *rol C* or *rol D* are involved in the synthesis of plant hormone like compounds. Therefore, it has been postulated that *rol B* may encode an auxin like receptor (Jeff Schell personal communication) rendering transformed plant cells more sensitive to auxin. *rol C* in contrast would counterbalance this affect, influencing the auxin signal transduction system in an as yet unkown manner.

1.7. T-DNA gene promoters

T-DNA genes are not dependent on proximal plant promoter units for expression. These genes already possess promoters which are active in the plant cell. The first functional map of a plant promoter was produced by Shaw and coworkers who showed that expression of the *nos* gene of the nopaline Ti-plasmid depended on typical eukaryotic promoter consensus sequences such as CAAT and TATA boxes (83). Expression of T-DNA genes has also been monitored in plants regenerated from transformed cells. Most T-DNA genes were, as expected, expressed constitutively. However, some demonstrated organ specific expression. The octopine TL-DNA gene 5 is activated by high auxin levels and reduced by high cytokinin levels (84). Expression from the TR1′2′ dual promoter of genes involved in agropine synthesis can also be increased several orders of magnitude by the use of auxins (85). Expression of the *rol A* , *rol B* , *rol C* and *rol D* genes from the TL-DNA of Ri-plasmids is also affected by organ specific factors (Jeff Schell personal communication).

Although, T-DNA genes have plant promoter like sequences the protein products appear to be of prokaryotic origin. For example the auxin biosynthetic pathway controlled by the *IaaH* and *IaaM* genes was first dissected in *Pseudomonas savastanoi* (86). Tumour proliferation induced by this organism is dependent on the continual secretion of IAA from the pathogenic bacteria itself.

1.8. Overdrive

Deletion analysis has shown that T-DNA transfer from the pathogenic *Agrobacterium* to the plant cell requires an intact right border repeat (87) , the left border repeat can be removed without any loss of tumourigenicity (84). Further investigation illuminated another sequence to the right of the right border repeat which was required for efficient T-DNA transfer, this sequence was coined overdrive (88). This sequence is located 13 and 14bp from the TL and TR-regions of the octopine Ti-plasmid respectively (88). Ream and coworkers have shown that overdrive may function in either orientation (89), and also if placed to the left of the right border (89). Experiments in which the distance of overdrive is increased from its normal position have shown that overdrive is functional up to a distance of 500bp to the right of the right border repeat but not functional at a distance of 3kb or greater (90).

1.9. The virulence region

The virulence (*vir*) region is approximately 35kb in lengh and lies to the left of the T-DNA on the Ti (Ri) plasmid. This region, unlike the T-DNA, is not transferred to the plant cell, instead the *vir* region encodes at least 20 proteins involved in T-DNA transfer and host range specificity (91). Heteroduplex analysis of octopine and nopaline Ti-plasmids revealed extensive DNA similarity between the two (92). This similarity was also found to extend to Ri-plasmids (93), indeed genetic complementation experiments demonstrated that the *vir* genes were interchangeable between Ti and Ri-plasmids (94).

The *vir* region specifies only *trans* -acting functions, the T-DNA can be present on another replicon, either plasmid or chromosome and efficient T-DNA transfer still occurs (95,96).

Transposon mutagenesis and complementation studies of the nopaline and octopine Ti-plasmids divided the *vir* region into 6 and 7 complementation groups respectively. These groups have been termed *vir A* , *vir B* , *vir C* , *vir D* , *vir E* for the nopaline Ti-plasmid, the octopine Ti-plasmid includes an extra group *vir F* (97) (Fig.1.9.). The nopaline plasmid does however contain a locus coined *tzs* which specifies the synthesis of the phytohormone transzeatin (98), this locus is absent in octopine Ti-plasmids. Some of the *vir* region complementation groups are large and consist of operons with more than one cistron, where this is the case each open reading frame is termed *vir B1* , *vir B2* , *vir B3* etc. The *vir* loci can be divided into one of two classes with respect to their function; namely, essential *vir* loci which include *vir A* , *vir G* , *vir D* and *vir B* and host range *vir* loci which include *vir C* , *vir E* and *vir F* .

Nucleotide sequence analysis of the octopine Ti-plasmid has revealed it encodes at least 20 *vir* proteins. *vir B* and *vir D* gene promoters showed strong similarity , while the other *vir* gene promoters exhibited weaker similarity to *E.coli* consensus sequences (99). Furthermore all promoters except *vir G* showed significant similarity to the *E.coli* Shine-Dalgarno ribosome binding consensus sequence (99). Analysis of codon usage revealed that all codons were utilized with uniform frequency, thereby suggesting the *vir* genes were not expressed at a high level (99).

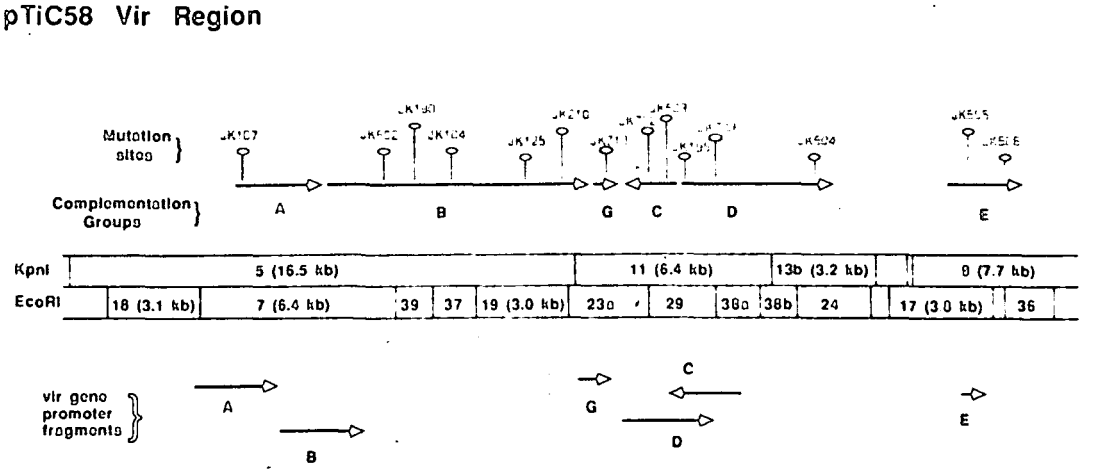


Fig.1.9. Physical map of a nopaline Ti plasmid *vir* region showing the positions of the *vir* complementation groups. (Courtesy of C.Kado).

1.10. *vir* gene expression

The transcriptional organisation of the virulence region has been dissected by transposon mutagenesis using the Tn3::*lacZ* transposon and assaying β-galactosidase production (100). Such experiments revealed all the *vir* genes were transcribed in a clockwise direction except *vir C* .

Unlike chromosomal virulence loci the genes of the Ti-plasmid *vir* region were found to be regulated. *vir A* and *vir G* were expressed constitutively on minimal medium, the other *vir* genes were only found to be expressed when virulent *Agrobacterium* were grown in conjunction with plant cells (101). Therefore a signal molecule from the plant cells was detected by *Agrobacterium* and the *vir* genes subsequently induced. Stachel and coworkers identified this signal molecule as the phenolic compound acetosyringone, present in plant exudates (102) (Fig.1.10.). Subsequently other phenolic compounds were demonstrated to act as signal molecules namely, sinapinic acid (102), syringic acid (102) , vanillin (103) and p-hydroxybenzoic acid (103). Structural analysis of these phenolics revealed that the presence of a 4′-hydroxyl group was essential for *vir* gene induction (103).

1.11. *vir A* and *vir G*

The *Agrobacterium* *vir A* and *vir G* loci were found to specify the signal transduction system through which these phenolic signal molecules act to bring about *vir* gene induction (104) (Fig.1.11.). Interestingly, the nucleotide sequence of *vir A* and *vir G* showed strong similarity with a number of prokaryotic two component positive regulatory systems eg. *EnvZ / OmpR* , *PhoR / PhoB* , *CpxA / Sfr* of *E.coli* and *NtrB / NtrC* of *Klebsiella pneumoniae* (105). Such systems possess a sensory protein, usually located in the cell membrane and an activator protein in the cytoplasm. *vir A* exhibits strong similarity to the sensory components of such systems ie to *Enz V* , *PhoR* , *CpxA* and *NtrB* and recently the 91.6 KDa *vir A* protein has been demonstrated to be located in the cell membrane (107). In addition *vir A* also possesses similarity with various chemoreceptor proteins (108). The *vir G* protein shows strong similarity with the activator proteins ie to *OmpR* , *PhoB* , *sfr* and *NtrC* (106). It has been postulated that *vir G* could be a DNA binding protein which is activated following binding to the signal molecule which has entered the cell via association with the *vir A* membrane protein which possibly acts as a transport protein. Activated *vir G* protein could then bind to *vir* promoter regions

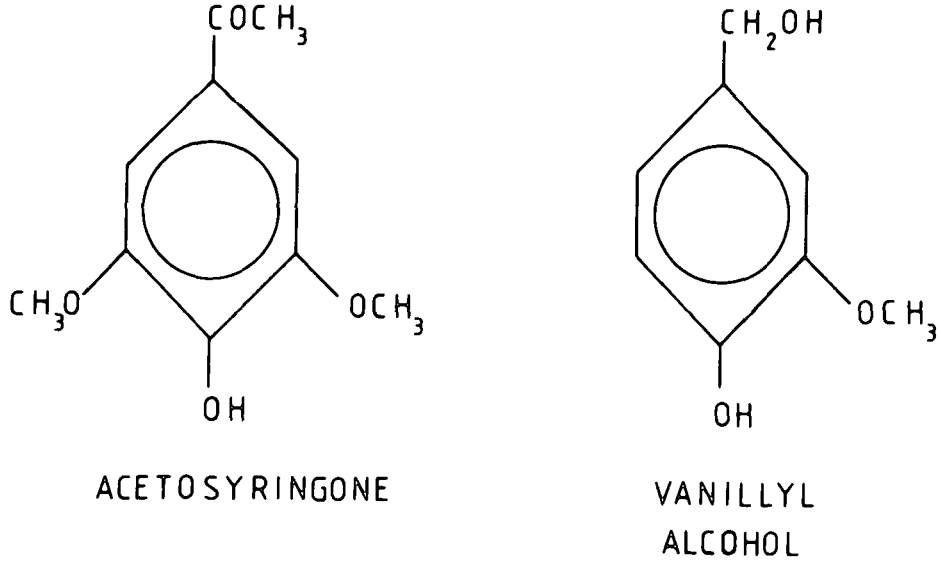


Fig.1.10. Structure of *vir* inducing molecules.

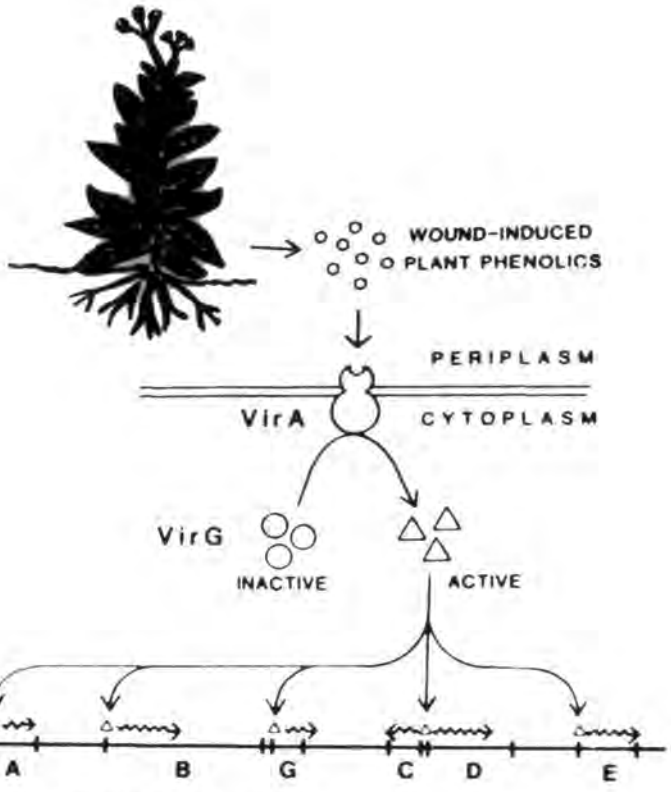


FIG 2. Model for *vir* gene induction

Fig.1.11. *vir* induction system of *A.tumefaciens* . The sensor protein vir A, detects the presence of phenolic compounds (like acetosyringone) from wound exudates, and tranduces a signal to the activator protein vir G, which subsequently provokes vir gene transcription. (Courtesy of E.W. Nester).

and induce *vir* gene expression (109).

vir gene expression is also controlled by the *ros* locus (110). In *ros* mutants the expression of *vir C* and *vir D* is increased in octopine and nopaline Ti-plasmids (111). Thus, the *ros* gene product acts as a negative regulator of *vir C* and *vir D*.

1.12. *vir B*

vir B is the largest *vir* gene operon and is thought to code for at least 11 transcripts. Using immunodetection methods *vir B* proteins have been located to the cell membrane (112). In support sequence data shows that the *vir B* proteins are very hydrophobic (113). It has thus been proposed that *vir B* proteins are involved in the formation of a transmembrane pore, through which the T-DNA could pass.

1.13. *vir C*

The *vir C* operon encodes two proteins *vir C1* (25KDa) and *vir C2* (22KDa) which are involved in host range specificity (114). The stop and start codons of *vir C1* and *vir C2* are separated by only two nucleotides, suggesting the two genes may be transcriptionaly coupled. There is also evidence to support a role for the *vir C* gene products in T-DNA processing (115).

1.14. *vir D* and *vir E* : T-DNA Intermediate formation

Activation of the *vir* genes leads to the nicking of the bottom strands of the 24bp border repeats on each side of the T-DNA (116). Border nicking is greatly increased by the presence of the enhancer element overdrive. Double stranded breaks at border repeats have also been reported (117).

The *vir D* operon encodes four proteins *vir D1* (16KDa), *vir D2* (47KDa), *vir D3* (21KDa) and *vir D4* (72KDa). Studies have revealed that *vir D1* and *vir D2* are essential for border cleavage (118). Therefore, *vir D1* and *vir D2* must possess specific endonucleolytic activity in cleaving border repeats. It has been proposed that the 3′ end of DNA at the nicked right border serves as a primer for DNA synthesis

and hence displacement of the nicked lower T-DNA strand (114). The genes encoding the helicase, polymerase(s) and ligation functions which would also be required are unknown. The end product, the T-strand is proposed to be the T-DNA intermediate (119). T-strands can be detected eight hours after *vir* gene induction by southern blot analysis (120). In octopine Ti-plasmid strains which contain a TL and TR-region six species of T-strand can be found corresponding to TL, TR, TC, TC TL, TC TR and TR TC TL although there seems to be a preference for the transfer and (or) integration of TL (120).

Circular T-DNA copies can also be isolated from *Agrobacterium* following *vir* gene induction (121). Such T-circles contain a hybrid border repeat consisting of a fusion between the TL and TR border repeats (121). However, strong selection methods must be employed and no T-circles can be detected by southern blot analysis. Therefore, the T-strand is more likely to represent the real T-DNA intermediate. Recent evidence suggests that the *vir D2* gene product remains tightly bound to the 5′ end of the T-strand (122). It has thus been suggested that *vir D2* could act as a 'pilot'protein involved in transferring the T-strand from the bacterium to the plant cell. 'Pilot' proteins have been proposed to play a crucial role in bacterial conjugative transfer (123). Further analogies with bacterial conjugation were illuminated when it was discovered that the origin of transfer (oriT) from a conjugative *E.coli* plasmid RSF1010 could substitute for the 24bp border repeats in directing T-DNA transfer to plant cells (124). This chimaeric transfer system also required an intacted *vir* region and the segment of RSF1010 encoding plasmid transfer functions. It is therefore likely that *Agrobacterium* has modified a pre-existing bacteria-to-bacteria DNA transfer process to satisfy its own peculiar vegetal bestiality (125).

The *vir E* operon encodes two proteins *vir E1* (7KDa) and *vir E2* (60KDa) (60). The *vir E2* gene product has been found to be a T-strand specific DNA binding protein (126). In this capacity the *vir E2* protein may form part of a protein-DNA complex with the T-strand protecting it from DNA endonucleases during the transfer process. In support the *vir E2* protein exhibits several classical properties of single-stranded DNA binding proteins, including tight stoichometric and cooperative binding to single-stranded DNA (127).

1.15. A model for T-DNA transfer

The first step in T-DNA transfer is assumed to be the binding of the virulent

bacterium to the plant cell. This binding is dependent on four different chromosomal loci *cel* (27), *pscA* (29), *chv A* and *chv B* (28). Phenolic compounds such as acetosyringone released from the plant wound site act as signal molecules for *Agrobacterium* (102), and *vir* gene induction occurs mediated by the *vir A* and *vir G* gene products (104). The 24bp border repeats flanking the T-DNA are then nicked on the bottom strand by *vir D* proteins acting as sequence specific DNA endonucleases (118). T-strands are subsequently generated following displacement of the bottom T-DNA strand by DNA synthesis using the 3′ end of the nicked right border as a primer (114). The *vir D2* protein remains attached to the 5′ end of the T-strand where it may act as a 'pilot' protein mediating DNA transfer (122). The released T-strand is converted into a protein-DNA complex following association with the *vir E2* gene product (127). This putative DNA intermediate could then pass through a membrane pore in the bacterium formed by associated *vir C* proteins (115). How the T-DNA intermediate could penetrate the plant cell membrane and integrate efficiently into the plant genome which is encased in a complex chromatin structure is still unknown.

1.16. The genus *Rhizobium*

The Rhizobia are symbiotic nitrogen fixing bacteria which are divided into three genera *Rhizobium* (including *Rhizobium leguminosarum* biovars *viciae* , *trifolii* and *phaseoli* and *Rhizobium meliloti*) , *Azorhizobium* and *Bradyrhizobium* .

Rhizobium and *Agrobacterium* are taxonomically closely related (32). Several loci that play crucial roles in symbiosis and pathogenicity are genetically interchangeable between the two genera. Such loci include the *ndvA* and *ndvB* genes of *Rhizobium* critical for nodule development, mutants of which can be complemented with the chromosomal virulence genes of *Agrobacterium* *chv A* and *chv B* respectively (32). The same holds true for the *exoC* gene of *Rhizobium* , involved in exopolysaccharide synthesis and the *pscA* gene of *A.tumefaciens* (33). In addition it has been shown that nitrogen fixing nodules could be induced in *A.tumefaciens* harbouring *R.phaseoli* plasmids (128), that expression of *R.meliloti* nodulation genes occured in *Agrobacterium* backgrounds (129) and that the *Agrobacterium* plasmid encoding agrocin 84 production could be expressed in *R.meliloti* (130).

More recently further similarities in the plant-microbe interaction of *Agrobacterium* and *Rhizobium* have been unearthed. Murphy and coworkers (131) have dis-

covered that a strain of *R.meliloti* possesses a large auxillary plasmid which carries genes designated *mos* , that specify the production of a unique carbon and nitrogen containing compound termed a rhizopine. *mos* genes are only expressed when *R.meliloti* is in close association with the plant. The free living bacteria however, express a set of genes (*moc genes*) responsible for the catabolism of this rhizopine. Thus bacteria of the same strain occupying the outside of the nodule benefit from the activity of their descendents inside.

1.17. Infection

Rhizobium infect plant roots, here they induce cellular differentiation of plant cells which subsequently evolve into nitrogen fixing nodule like structures. Rhizobia generally infect the legume family, *Leguminosae* ; different species being defined by the subset of host plants infected. At the plant surface Rhizobia interact with lectin components of the host cell wall, this interaction has recently been shown to be a determinant of host specificity (132). Following attachment, host defense polysaccharide-degrading enzymes may modify the *Rhizobium* cell surface polysaccharides which subsequently induce host responses which prepare for infection (133). The epidermal hairs on the root surface grow deformed, even curled (134). Simultaneously the cells of the root cortex, under the epidermis, begin dividing (135). Bacteria trapped in a curled root hair, or between a hair and another cell, proliferate and begin to infect the outer plant cells. The invaded plant cells are somehow stimulated to produce a cell wall sheath or 'infection thread ' (136). As cell divisions in the plant root establish the body of the nodule, infection threads penetrate and ramify individual target cells within the nodule. Bacteria are then released into the plant cytoplasm itself, they are enveloped in plant plasma membrane and subsequently differentiate to form bacteroids (137). Symbiotic nitrogen fixation and metabolic exchange then ensues (138).

1.18. Nodulation genes

Rhizobium nodulation (*nod*) genes have been defined by sequencing, transposon mutagenesis and in some cases protein analysis. *nod* genes are involved in the early stages of infection and host range specificity and reside on large symbiotic (*sym*) plasmids. In *R.leguminosarum* (139) and *R.trifoli* (140) the *nod* genes lie within a 14Kb region and in *R.meliloti* within two regions separated by about 12Kb (141). In contrast *Bradyrhizobium nod* genes are found on the chromosome (142). Mutants

in *nod A*, *nod B* or *nod C* are completely *nod*$^-$ (no nodules form). These genes are required by the bacteria to induce cell division (143) and deformation of root hairs (144). The *nod ABC* genes appear to be functionally interchangeable among all *Rhizobia* (111).

Downstream from the *nod C* gene two other *nod* loci have been defined, *nod I* and *nod J* , whose gene products are membrane associated and together with *nod C* may form a membrane transport system (143). The *nod D* gene is expressed constitutively and mediates the induction of the other *nod genes* in the presence of plant derived signal molecules. *nod D* has been shown to be a DNA binding protein, binding to the promoter sequences of the inducible *nod* genes (145). *nod D* may therefore be considered analogous to the *vir G* protein of *Agrobacterium* , in that they both mediate induction of genes involved in the plant microbe-interaction in the presence of plant derived signal molecules.

The *nod E* and *nod F* gene products inhibit nodulation (146), and together with *nod G* and *nod H* are thought to influence host range specificity. *nod I* and *nod J* share the same transcriptional unit as *nod ABC* and are thought to be involved in the uptake of plant derived metabolites (147). Other defined *nod* genes include *nod K* in *Bradyrhizobium* and *Azorhizobium* and *nod L* and *nod M* in *R.leguminosarum var viciae* , their functions are still unkown.

1.19. *nod* inducers

The plant signal molecules mediating *nod* gene induction via *nod D* have been purified from plant extracts. These signal molecules were found to be flavonoids, three-ring aromatic compounds derived from phenylpropanoid metabolism. In alfalfa and clover the major inducer was luteolin (148) and in soybean daidzein (149). This inducer specificity could play an important role in *Rhizobium* host range.

1.20. *nod* inhibitors

Compounds from plant extracts have been defined which antagonise the induction of *nod* genes by *nod* inducers (150). These molecules include certain flavonoids, isoflavonoids and monocyclic phenolics such as acetosyringone . Inducer and inhibitor molecules are structually similar, therefore the inhibitor compounds may act by competing for binding sites on the *nod D* protein.

Signal molecules therefore are the crux in both the *Agrobacterium*-plant and *Rhizobium*-plant interaction.

1.21. Plant hormones and nodulation

Recent experiments have begun to unearth a further anology between *Agrobacterium* and *Rhizobium* -plant interactions. Long and Cooper have reported that a nod^- (*nod ABC*) *R.meliloti* strain constitutively expressing the cytokinin zeatin is capable of forming primitive nodules on alfalfa (151). The concept that plant hormone balance pertubation may play a central role in nodulation has been further supported by Hirsch and coworkers (152). Their work has shown that anti-auxins such as NPA (naphthylpthalamic acid) produce nodules on the roots of alfalfa.

Therefore, growth controlling compounds may not only play an important role in tumour formation by *Agrobacterium* but may also be pivotal in plant nodule formation by *Rhizobium* .

1.2.0. Bacterial Chemotaxis

1.2.1. Historical Overview

Bacterial chemotaxis may be defined as the detection by motile bacteria of temporal changes in concentrations of specific chemicals, behaviourally responding to these changes, and then adapting to the new concentration of the chemical stimulus.

The phenomenon of chemotaxis was first described by Pfeffer almost a century ago (153). Over the last decade a renaissance has taken place in chemotaxis research. The relevance of the sensory-adaptation response in prokaryotic chemotaxis to neurobiology and the advent of recombinant DNA technology has done much to fuel such research.

Adler was the first to initiate the renaissance, he repeated Pfeffer's studies and designed a quantitative assay to study the phenomenon (154). The results of recent labours have now reached fruition and a molecular understanding of bacterial chemotaxis is almost at hand.

Most chemotaxis research to date has been carried out on the genetically and biochemically well defined *Enterobacteriaceae*, namely, *E.coli* and *Salmonella typhimurium*, although work has also been reported on *Caulobacter crescentus* (155), *Halobacteria halobium* (156), *Bacillus subtilus* (157), *Rhizobium meliloti* (158) and *Pseudomonas putida* (159).

The components of the chemotaxis system can be divided into three groups; the molecular device for sensing and adapting to enviromental stimuli, the signal transduction system carrying this information to the flagellar motor and finally the motor and flagellum itself.

1.2.2. Swimming and Tumbling

In the absence of chemical stimuli, the *Enterobacteriaceae* propel themselves

through their liquid enviroment in a series of smooth runs which terminate in a tumble (160). This tumble serves to randomly change the direction of travel. The smooth runs are a consequence of the flagellar motor turning counterclockwise (CCW), a tumble results when the motor reverses direction to a clockwise rotation (CW) (161). Therefore, the bacterium is said to undergo a 'random walk' in three dimensions (160).

In the presence of a chemical stimulus the frequency of tumbling is determined by the direction of movement of the bacterium with respect to the gradient. If the bacterium is moving in a favourable direction, the rate of tumbling is less than in the absence of a gradient, and if the bacterium is moving in an unfavourable direction, the rate of tumbling is approximately the same as that observed in the absence of the gradient (162). This results in the net movement of the bacterium in a favourable direction. To achieve this bacteria must maintain a record of their environment over the recent past and if the current environment is detected to be more favourable than the previous one, then the tumbles are suppressed. If the environment is detected to be less favourable than previously, then tumbling is enhanced (163). If the environmental and recorded levels do not differ, then the bacterium returns to the classic random walk.

1.2.3. Swarm plates

A chemotactic bacterial strain inoculated onto the centre of a semi-solid agar plate containing a chemoattractant begins to metabolise that chemoattractant, and thereby creates a concentration gradient of chemoattractant, which increases towards the outside of the plate. Bacteria are attracted up this concentration gradient and hence swarm outwards towards the periphery of the plate. This phenomenon may be utilised to assess bacterial motility, isolate motile strains and as a qualitative assay to investigate the positive or negative chemotactic potential of given chemicals.

1.2.4. Capillary assays

Chemotactic bacteria will preferentially enter one compartment from another if there is a suitable chemical gradient between the two compartments. An application of this phenomenon is the capillary assay (154); a glass capillary tube containing medium plus attractant is immersed in a cell suspension in medium without attractant, and

after suitable intervals the contents of the capillary tube is plated out and counted. The results provide a population-average to the imposed spatial gradient of attractant.

1.2.5. Microscopic observation of free living cells

Microscopic observation of motility can provide a lot of information, permitting rough estimates of speed, tumble frequency and the type of motility employed. For more quantitative information, it is necessary to track individual cells and record their position as a function of time. This can be done by the use of a video camera and playback, with either manual or automatic analysis. A more complex approach involves actual tracking in real time, using feedback circuitry to actually lock onto the cells image (165).

1.2.6. Microscopic observation of tethered cells

If a bacterium can be attached ('tethered') to glass by only a single flagellar filament, the operation of the corresponding flagellar motor can be monitored via the rotation of the cell body (166). Observations can then be made regarding the CCW versus CW rotation of the flagellar motor. Again, this can be monitored in real time or with the use of a video recorder.

1.2.7. Stimuli detected

Using the swarm plate method and the capillary assay or modifications of it, a whole range of stimuli have been identified in a number of different motile bacteria, namely; pH (167), temperature (168), divalent cations (169), specific amino acids (170), specific sugars (171), dipeptides (172) and carboxylic acids (173).

1.2.8. Behavioural Mutants

The application of microbial genetic techniques has provided the key to unlock the door on bacterial chemotaxis, especially so because of the advanced state of molecular genetic techniques currently available.

The isolation of mutants has played and continues to play a crucial role in elucidating the tactic behaviour of bacteria. Spontaneous chemotaxis mutants may be isolated by continually picking the bacteria from the centre of swarm plates following repeated rounds of swarming (174); or, by maintaining cells in stationary phase for several days, whereupon the culture for some unknown reason, becomes enriched for chemotaxis mutants (175). Alternatively, mutagenesis procedures may be employed, utilising chemical mutagens such as ethylmethane sulphonate (EMS) (176) or *Mu* phage in the case of *E.coli* (177).

Following culture enhancement or mutagenesis, behavioural mutants may be isolated on swarm plates by virtue of their visible phenotype (176).

1.2.9. Nonflagellate (*fla*) mutants

These mutants lack flagella, are nonmotile and form tight dense colonies on swarm plates (178).

1.2.10. Nonmotile (*mot*) mutants

Nonmotile mutants have morpholologically normal flagella, however, these flagella fail to rotate. Such mutants are thought to be defective in energy transduction and form tight dense colonies on swarm plates (178).

1.2.11. General nonchemotactic (*che*) mutants

General *che* mutants are fully motile but fail to respond to any or a wide variety of stimuli. These mutants are thought to possess a defect in the central sensory transduction mechanism that is shared by all stimuli. General *che* mutants form smaller swarming colonies than their respective wild-type strain on swarm agar containing any or a wide variety of chemoattractants. (176).

1.2.12. Specifically nonchemotactic (*che*) mutants

This class of *che* mutant fails to respond to a specific chemical and may also be found to be defective in response to a limited range of other stimuli (179). Such mutants are thought to be defective in a component shared only by those stimuli to which the mutant fails to respond. Specific *che* mutants form smaller swarming colonies compared to their respective wild-type strain on swarm plates which contain the subset of chemicals which fail to provoke a response. However, on swarm plates containing other chemoattractants normal sized swarms are produced.

1.2.13. Genes and genomic organization

The isolation of behavioural mutants has identified genetic loci which specify chemotaxis function. To date approximately 60 such genes have been defined in *E.coli* and *S.typhimurium* . Most of these genes (approximately 40) are thought to be involved in flagellar structure and function, the remainder specify functions involved in the communication of sensory information to the flagellar motor or in the energy transduction or switching process of the flagellar motor itself. Behavioural genes with only a few exceptions were mapped to two clusters in *E.coli* and *S.typhimurium* . The order of motility and chemotaxis genes and their organization into operons is indicative of their functional relationships.

In the *Enterobacteriaceae* many of these loci have been cloned and their protein products analysed. This work has allowed a detailed picture of the components of the chemotaxis system to be drawn.

1.2.14. Periplasmic binding proteins

Periplasmic binding proteins bind specific molecules with high affinity, chaperone them across the periplasmic space and deliver them to membrane associated transport systems. Some periplasmic binding proteins can interact with components of the chemotaxis system when associated with their transported ligand (180). Such proteins include the maltose binding protein (MBP), galactose binding protein (GBP), ribose binding protein (RBP) and the dipeptide binding protein (Dpp).

The true chemoreceptors however are located in the cell membrane and associate either directly with their ligand or with a ligand-periplasmic binding protein complex.

1.2.15. Transducer Proteins

Transducer proteins span the cytoplasmic membrane, unlike the periplasmic binding proteins their role extends beyond that of recognition, as these proteins initiate events within the cytoplasm itself (181).

From the analysis of specific *che* mutants in *E.coli* four such transducer proteins have been defined, namely, Tar, Tap, Tsr and Trg.

The *Tar* protein is the receptor transducer for the chemoattractants aspartate, α-methyl aspartate, glutamate, MBP and its ligand and the repellents cobalt and nickel (182,183,184).

The transducer Tap interacts with the dipeptide binding protein Dpp and its ligand, a variety of dipeptides (172). The dipeptides are thought to induce a conformational change upon binding Dpp that allows the attractant-receptor complex to bind Tap (185).

For most amino acid attractants the chemoreceptor is Tsr, which mediates taxis to serine, alanine, glycine, and the non-metabolisable amino acid anologue alpha-isobutyric acid (186). Tsr also acts as transducer for the repellent leucine (182).

The Trg protein is the receptor transducer for the ribose and galactose binding proteins when associated with their ligands ribose and galactose respectively (187,188).

1.2.16. Transducer protein structure

All four transducer proteins are of similar molecular weight (approx. 60KDa) and show extensive homology (189). There are distinct regions on Tsr and Tar (190)

and probably on Trg (191) and Tap (192) proteins that are responsible for signal transduction (Fig.1.2.16.). Most of the amino terminal portion of each transducer lies in the periplasmic membrane and is exposed to the periplasmic space. The carboxy portion however, is located extensively in the cytoplasm. Each transducer protein has two highly hydrophobic domains termed transmembrane 1 (TM1) and transmembrane 2 (TM2), these regions are thought to span the cytoplasmic membrane (191). The amino terminal domain has been shown to mediate the binding of the transducer to its specific ligands (193), while the carboxy terminal domain is concerned with intracellular signalling and adaptation.

1.2.17. Methyl-accepting chemotaxis proteins

Transducer proteins were first identified as methyl-accepting chemotaxis proteins (MCP's) by virtue of their ability to accept radiolabeled methyl groups from methionine or more precisely from S-adenosyl methionine (194) (Fig.1.2.17.). Five methylation sites have been identified for Trg (195) and Tsr (196), four for Tar (197) and a similar number for Tap (198). These sites fall into 2 clusters centred around residues 300 and 490 of the approximately 500 residue carboxy terminal domain (199). Methylation is mediated by the product of the *che R* gene (a methyl transferase) and occurs at specific glutamate residues, which are subsequently converted to gamma-glutamyl methyl esters (200). Conversely, methyl groups can be removed again by the product of the *che B* gene (a methyl esterase) which catalyses the hydrolysis of the gamma-glutamyl methyl ester bond forming methanol and regenerating the gamma-carboxylate bond of glutamic acid (201).

In *E.coli* , *S.typhimurium* (202,203) and *B.subtilus* (204) MCP methylation is thought to be the crux of the adaptive response.

Adler and coworkers were the first to show that following exposure to a positive chemical stimulus, the transducer protein specific for that stimulus exhibited enhanced methylation (194). This increase in transducer methylation was proposed to down-grade the transducer's intracellular signalling ability and hence restore behaviour to its prestimulus state, a process termed adaptation (205). Further support for this idea came from the discovery that a negative chemical stimulus resulted in a decrease in methylation of its specific transducer (203). Therefore, the relative methylation states of MCP's may be the molecular manifestation of adaption (205).

Fig.1.2.16. Model of transducer protein structure. Abbreviations are: TMI and TMII, membrane spanning domains I and II; P, periplasm; IM, inner membrane and C, cytosol. Asterisks represent the sites of methylation.

Chemoeffector
binding
TMI
NH2
Signal
TMII
P
C
IM
COOH

Following ligand binding the transducer proteins are thought to transmit an excitatory signal to the cytoplasmic sensory transduction system which, at least in molecular terms, links the transducer protein to the flagellar motor. It has been proposed that the occupancy of the transducer periplasmic binding domain induces a conformational change in the transducer, which results in the transfer of a molecular excitatory signal to the sensory transduction pathway (192).

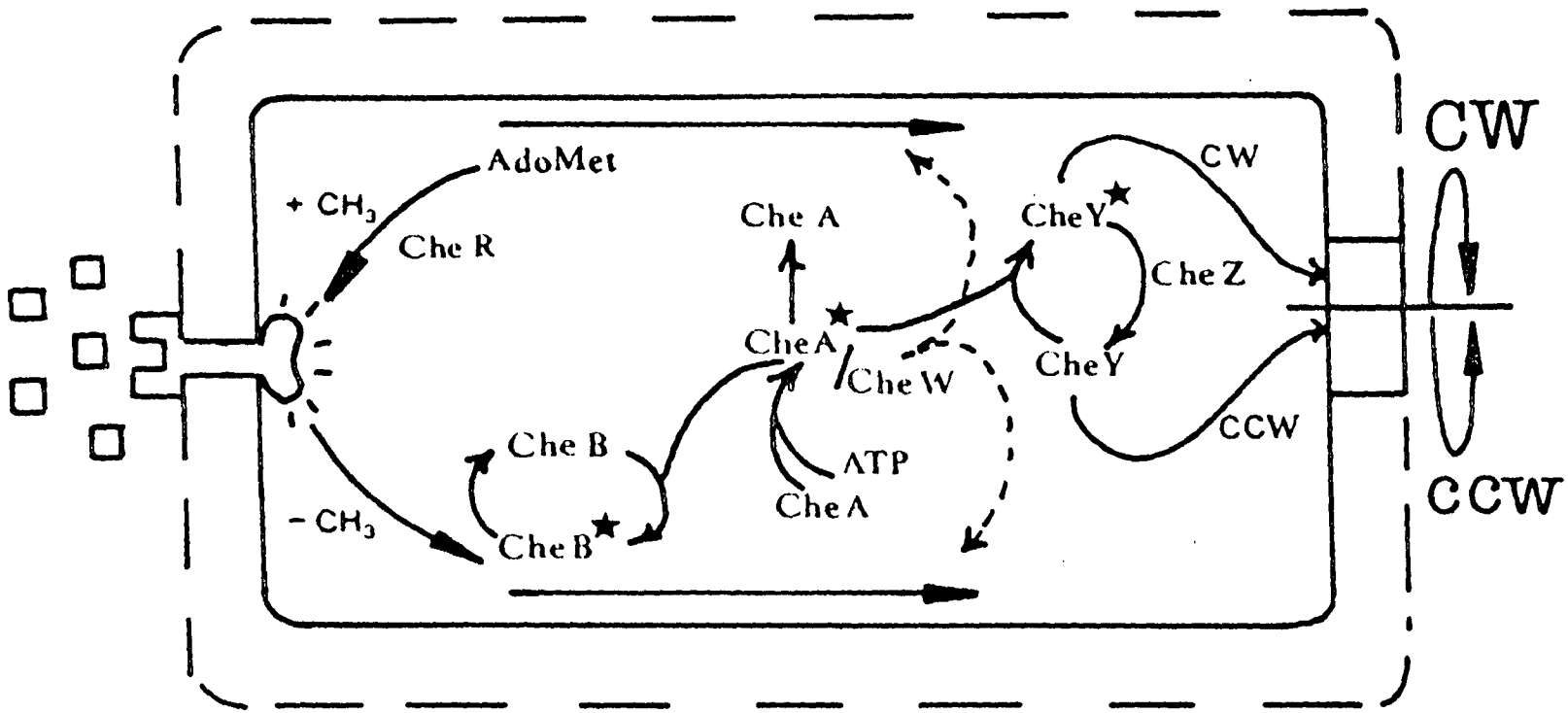
1.2.18. Cytoplasmic signal transduction

From the analysis of non-specific *che* mutants, four cytoplasmic proteins, namely, che A, che W, che Y and che Z have been defined that are involved in the transmission of sensory signals from the transducers to the flagellar motors (206) (Fig.1.2.18.).

che Y and che Z are thought to control the diretion of flagellar rotation, perhaps through interactions with the switching mechanism of the motor. che Y is needed for CW rotation (207) and che Z seems to somehow inhibit or antagonise che Y action, resulting in enhanced CCW bias (208). Therefore, flagellar motor responses may be carried out by regulating the activity of che Y and possibly che Z. The roles of che A and che W proteins are less well understood. *che A* and *che W* mutants are CCW biased and appear to lack any signalling activity, such as changes in MCP methylation states (209). Therefore, it has been proposed that che A and che W interact with the transducer proteins to generate the signal which is relayed to che Y and che Z (209).

ATP is not needed to energize the flagellar motors, however, a continuous supply is an essential requirement for chemotaxis (210). ATP-depleted cells rotate their motors only in the CCW direction, indicating ATP could be required for the production of a CW flagellar signal (211). This idea was substantiated by Hess and coworkers who discovered that che A undergoes ATP-dependent autophosphorylation (212). Further work showed that phosphorylated che A could transfer its phosphate group to either che Y or che B *in vitro* (213). che Y and che B were then found to undergo a slower, possibly self-catalysed loss of their phosphates. When che Z was added to the reactions, it accelerated the deposphorylation of che Y (but not che B), by stimulating hydrolysis of che Y phosphate rather than by acting as a phosphate acceptor itself (213). These results have allowed Hess and coworkers to design a working model for chemotactic sensory transduction.

Fig.1.2.18. Model of signal transduction from the sensory transducer to the flagellar motors via the cytoplasmic che proteins. Abbreviations: CH_3, methyl groups; AdoMet, S-adenosyl methionine; CW, clockwise; CCW, counterclockwise. Phosphorylated forms of the che proteins are indicated by asterisks.


AdoMet
+ CH3
Che R
Che A
CheY
CW
Che Z
CheA
Che W
CheY
CW
CCW
Che B
ATP
- CH3
Che B
Che A
CCW

The phosphate groups are first attached to che A, this che A autophosphorylation reaction is thought to be the likely site of chemoreceptor control. che A then rapidly transfers its phosphate groups to che Y and che B which then become activated. Activated che B may then remove methyl groups from the MCP transducers and che Y could be a CW signal that acts at the motor and controls flagellar rotation.

1.2.19. The Flagellum

Bacterial flagella can be divided into three substructures, the filament, hook and basal body (Fig.1.2.19.). The flagellar motors reside at the base of each flagellum (214) and are powered by proton motive forces (215).

1.2.20. The Flagellar Filament

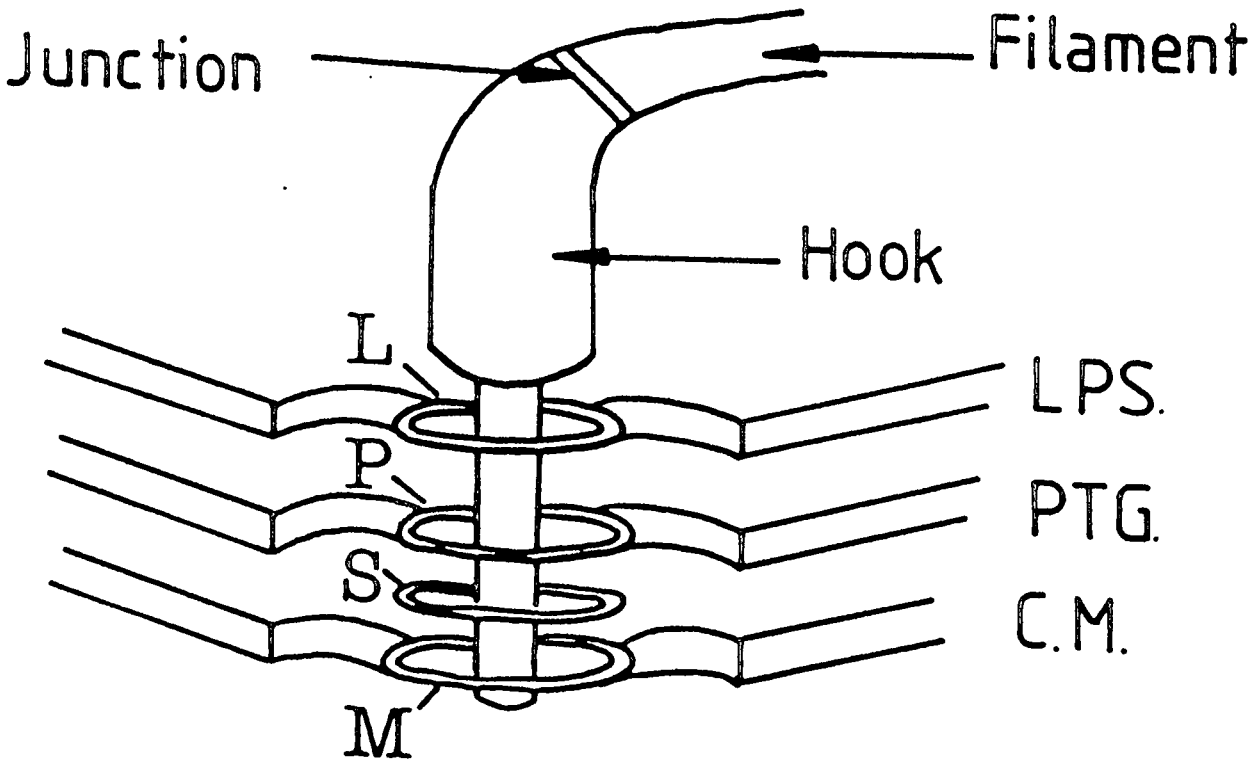
The filament is a long (10μm), thin (20nm), rigid structure and is composed of subunits of flagellin protein(s). In *E.coli* there is one flagellin protein of 54KDa encoded by the *hag* gene (216). *S.typhimurium* however has two flagellins H1 and H2 (217).

In peritrichously flagellated bacteria the individual flagellar filaments combine to produce a flagellar bundle that can propel the bacterium at speeds of up to 20μ m/sec.

1.2.21. The Flagellar Hook

The flagellar hook is thought to function as a flexible universal joint (218) transmitting the rotational movement of the motor to the filament resulting in propulsion of the bacterium. The hook is composed of a single 42KDa protein encoded by the *fla K* gene (219). Three hook associated proteins termed HAP1, HAP2 and HAP3 define the proximal and distal ends of the flagellar filament (220). HAP1 and HAP2 are located at the hook filament junction and are encoded by *fla W* (*fla S*) and *fla U* (*fla T*) for *S.typhimurium* and *E.coli* respectively. HAP3 encoded by *fla V* (*fla C*) is localized at the tip of the flagellar filament and is thought to function in the assembly of flagellin monomers.

Fig.1.2.19. Structure of the bacterial flagellum, showing hook, filament and L, M, P and S rings. The abbreviations LPS, PTG and C.M, represent the lipopolysaccharide and peptidoglycan layer of the outer membrane and the cytoplasmic membrane respectively.


Junction
Filament
Hook
L
P
S
M
LPS.
PTG.
C.M.

1.2.22. The Basal Body

The basal body is the most complex of the flagellar components and is comprised of about nine proteins. Four ring like structures termed lipopolysaccharide (L), peptidoglycan (P), stator (S) and motor (M) surround a central rod that terminates at the flagellar hook. *fla A* and *fla M* are thought to encode the M-ring, *fla FV* the hook, *fla FV1* and *fla F2*, the rod, *fla FV3* the L-ring and *fla F9* the P-ring (220,221,222). Two other genes *mot A* and *mot B* encode integral membrane proteins that may function as oxido-reductases, required for movement of the S/M ring (223).

1.2.23. Chemotaxis in the rhizosphere

Many Rhizobacteria are actively motile and chemotactic (224,225,226,227,228). Plant extracts have been shown to be strong positive stimuli for *Rhizobium* (229), *Pseudomonas* (227), *Azospirillium* (230) and recently *A.tumefaciens* (231). More specifically, organic compounds can act as chemoattractants for Rhizobacteria. Harwood and coworkers have demonstrated that *Pseudomonas putida* is chemotactically attracted to aromatic acids (232). In addition the *nod* gene inducing flavonoids luteolin and naringenin have been shown to be chemoattractants for *R.meliloti* (233) and *R.leguminosarum* (234). Indeed chemotaxis to these phenolic compounds has been proposed to play an important role in host range specificity (234). Bauer and co-workers analysed the chemotactic responses of various *R.meliloti* nodulation mutants to the flavone luteolin, and concluded that chemotaxis required functional *nod A* , *nod C* and *nod D* genes (233). Exactly how these loci could mediate *nod* gene induction and chemotaxis to luteolin is unknown.

In *R.leguminosarum* an enteric bacteria like transducer protein has been reported (235). Following exposure to L-serine but not naringenin a *R.leguminosarum* protein of approximately 60KDa becomes increasingly methylated. *R.leguminosarum* may therefore, mediate chemotaxis to amino acids but not flavonoids through an MCP based transducer system analogous to that of *E.coli* and *S. typhimurium* .

Many Rhizobacteria have been shown to be actively motile in the soil. *Pseudomonas aeruginosa* can move through moist soil a distance of 2cm in 24 hours (236) and *P.putida* a distance of 2cm in 48 hours to imbibing soybean seeds in raw soil (238); and *Azospirillum brasilense* has been shown to be both horizontally and

vertically motile in a variety of soil types (230). The only Rhizobacterial propulsion system to have been extensively studied however is that of *R.meliloti* . Schmitt and coworkers have observed the presence of 5-10 peritrichously inserted flagella on *R. meliloti* (237). The flagella, unlike the plane flagella of *Enterobacteria* , have a helical pattern of alternating ridges and grooves, which may reflect environmental selection (237). These complex flagella are encoded by two *fla* genes designated *fla A* and *fla B* in *R.meliloti* which specify gene products of 40.4 and 41KDa respectively (238). Interestingly, *R.meliloti* displays a totally different motillity pattern compared to *E.coli* and *S.typhimurium* (237). Unlike plane flagella which may rotate CW and CCW, complex flagella only show CCW rotation. It has been proposed that whenever the majority of the flagellar motors stop, a reorientation of the bacterium can occur, which is equivalent to a tumble in bacteria possessing plane flagella. Therefore, *R.melilioti* motility consists of a series of runs and stops.

Spontaneous *R.meliloti* chemotaxis mutants have been isolated by Bergman and coworkers (239). These behaviourial mutants like those reported for *E.coli* and *S.typhimurium* could be classified as either *fla* , *mot* or *che* on semi-solid agar. The corresponding loci to these mutants all mapped in a cluster near the *his 39* and *met 56* markers (240). Complementation of these mutants with a *R.meliloti* cosmid gene library provided by Ausubel and coworkers revealed that all the mutants could be complemented to wild-type by one of two cosmids designated pRZ2 or pRZ4 (240).

The pertinence of bacterial motility and chemotaxis in the rhizosphere has been open to question. However, the designation of a substantial portion of the bacteria genome to motility and chemotaxis, suggests the organism must derive some substantial benifit. This supposition has been substantiated by De Weger who illuminated the importance of flagella in the effective colonization of potato roots (241). Moreover, motility has been shown to play a significant role in root nodulation of alfalfa by *R.meliloti* (242). Therefore, the importance of chemotaxis and motility in plant-microbe interactions cannot be over-estimated.

Aims and objectives of the thesis

The aim of this thesis was to substantiatee primary discovery of chemotaxis in *A.tumefaciens* (224).

The initial objective was to identify and classify compounds that may act as chemoattractants for *A.tumefaciens* .

Following on from this research base, chemical and transposon mutagenesis was to be used in the production of a battery of *A.tumefaciens* behavioural mutants. It was envisaged that the characterization of these mutants would shed light on the genetics and biochemistry of chemotaxis in *A.tumefaciens* , and also the significance of motility in the rhizosphere.

In the final stage of the project it was planned to isolate and characterize genes, mutations in which produced the most interesting behavioural phenotypes. This task being facilitated by the insertion inactivation nature of transposons and their encoded drug resistance.

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, Dorset.

Restriction endonucleases, T4 DNA ligase, 5-bromo-4-chloro-3-indoyl-β - galactoside (X-gal), Tris (hydroxy methyl) aminomethane, Agarose, LMP Agarose and the M13 sequencing kit were from Bethesda Research Laboratories (U.K.) Ltd. BRL, Cambridge, U.K., The Boeringer Corporation (London) Ltd, Lewes, East Sussex or New England Biolabs, CP Labs Ltd, Bishops Stortford, Herts, U.K.

Yeast extract was from Sterilin Ltd, Teddington, U.K.

BBL trypticase peptone was from Becton Dickinson and Co, Cockeysville, M.D., USA.

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

Caesium chloride and Sodium chloride were from Koch-light Ltd, Haverhill, Suffolk, U.K.

Sephadex G-10 and Ficoll 400 were from Pharmacia Fine Chemicals, Uppsala, Sweden.

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

3MM paper was from Whatmann Ltd., Maidstone, Kent, U.K.

Acrodisc filters were from Gelman Sciences Inc., Ann Arbor, Michigan, USA.

Minisart filters were from Sartorius GmbH, Postfach 3243, D-300 Gottingen.

High vacuum grease was from Dow Corning S.A., Seneffe, Belgium.

Polaroid 667 film was from Polaroid (U.K.) Ltd., St. Albans, Hertfordshire.

Radiochemicals were from Amersham International plc., Amersham, Bucks, U.K.

2.2 Glassware and other equipment

All glassware, plasticware and other equipment used in the manipulation of DNA and in the growth and maintenance of bacterial cultures, was autoclaved before use.

2.3. Bacterial strains and plasmids

All bacterial strains and plasmids used during the course of this study are outlined in Table 2.4.

Table 2.4. Bacterial strains

Strains	Remarks	References
A.tumefaciens		
C58*C1*(pTiB6S3)	*vir*$^+$, *ocs*$^+$, Ery, Cm,	243
C58*C1*	Rif, *vir*$^-$	244
GM19023	cured of cryptic plasmid	308
C58*C1-met*-81	Rif, *met*-81	245
C58*C1-ile*	Rif, *ileu*	245
C58*C1-trp*	Rif, *trp*	245
C58*C1-ura*	Rif, *ura*	245

C58*CI-mot-1*	non-motile	this study
C58*CI-mot-2*	"	"
C58*CI-mot-2*	"	"
C58*CI-mot-3*	"	"
C58*CI-mot-4*	"	"
C58*CI-mot-5*	"	"
C58*CI-mot-6*	"	"
C58*CI-mot-7*	"	"
C58*CI-fla-1*	no flagellar	"
C58*CI-fla-2*	"	"
C58*CI-fla-3*	"	"
C58*CI-fla-4*	"	"
C58*CI-fla-5*	"	"
C58*CI-fla-6*	"	"
C58*CI-fla-7*	"	"
C58*CI-che-1*	tumbles incessantly	"
C58*CI-che-2*	small swarms	"
C58*CI-che-3*	"	"
C58*CI-che-4*	"	"
C58*CI-che-5*	"	"
C58*CI-pc-1*	paired cells	"
E.coli		
TG2	$\triangle$ *(lac-pro) supE thi*	
	hsdM hsdR recA	309
	FI(tra D36 proAB lacZ$\triangle$M15I^q)	
ED8767	*recA met*	283
1830	*pro met*	283
HB101	*recA13 rpsL proA2*	
	leuB6 thi-1	310
DH5α		311

Table 2.5 Bacterial Plasmids

Plasmid	Remarks	References
pSUP101	Tra$^-$ Mob$^+$ Cm^r Tn5	282
pSUP2011	Tra$^-$ Mob$^+$ Ap^r Cm^r Tn5	282
pJB4JI	Tra$^+$ Mob$^+$ *Mu* Tn5	281
pRK2073::Tn5	Tn5 Tn7	283
pUC18	Amp^r mcs	312
pLAFR3	Tc^r mcs	306
Ti pGV2201	*vir*$^+$ Sm^r Sp^r	313
pRZ2	Tc^r	239
pRZ4	Tc^r	239

Table 2.6. Growth Media

L-Broth

10g tryptone
5g yeast extract
5g NaCl
(1.5% agar per litre)

Min A

76ml distilled water
20ml Min A salts
1ml 20% glucose
0.1ml $MgSO_4$

Chemotaxis Medium

10^{-4}M EDTA pH7.0
10^{-2}M phosphate bufer pH7.0
phosphate buffer
1M KH_2PO_4 pH with 1M KOH

5X Min A Salts

52.5g K_2HPO_4
22.5g KH_2PO_4
1g $(NH_4)_2SO_4$
2.5g Na Citrate.$2H_2O$ per litre

Table 2.7. Antibiotics

Antibiotic	Stock solution ($mgml^{-1}$)	Final conc. (μgml^{-1})
Rifampicin (Rif)	50	100
Kanamycin (Km)	12.5	25
Chloramphenicol (Cm)	12.5	25
Ampicillin (Amp)	25	50
Tetracycline (Tc)	12.5	10
Streptomycin (Sm)	150	300
Spectinomycin (Sp)	50	200

Methods

2.8. Preparation of motile bacteria

Motile bacteria were periodically obtained by inoculating a loopful of bacteria onto the centre of an L-swarm plate which was subsequently incubated at 28°C for two days. Motile bacteria will spread to the periphery of the plate as metabolites are expended, therefore providing a suitable method for enrichment of motile populations. Bacteria from the edge of the swarm were picked and reinoculated onto a fresh L-swarm plate, this procedure was repeated at least three times. Bacteria from the edge of the final swarm plate were then picked and streaked to single colonies on a master plate containing selective medium. Single colonies of motile bacteria were taken from this plate for chemotaxis assays.

2.9. The capillary assay

The method used was a modification of that described by Adler (154).

Exponential phase bacteria were prepared by inoculating 500μl of an overnight culture of motile Agrobacteria into 10ml of L-broth and growing the culture at 28°C with shaking to an $O.D._{590}$ of 0.2-0.4. The culture was then centrifuged at 3600g for

10 minutes and washed twice in chemotaxis medium. The final pellet was resuspended in up to 10ml of chemotaxis medium.

A bacterial pool was formed by placing 0.2ml of the prepared bacterial suspension within a U-tube (bent from a melting point capillary tube and sealed at both ends) between a microscope slide and a coverslip. For large numbers of assays the slides were replaced by a glass plate.

All chemoattractants were filter sterilised through 0.22μm disposable filters prior to use, diluted with chemotaxis medium and assayed within the range 10^{-3} to 10^{-8}M.

Chemoattractant solutions were drawn up into a plastic capillary tube and the tube sealed at one end using high vacuum grease. Capillary tubes were handled with sterile forceps throughout the assay and were inserted open end first into the bacteria pools. The assay was left for 60 minutes at room temperature. After incubation the capillary was removed and its exterior washed with a thin stream of sterile distilled water from a wash bottle. The sealed end was broken and the contentents diluted serially in sterile distilled water down to 10^{-5}. 0.1ml aliquots of each dilution were spread onto L-agar plates containing the appropriate antibiotic. The resulting plates were incubated at 28°C for 48 hours and the colonies counted using a colony counter (Gallenkamp).

2.10. Microscopic observation of motile bacteria

The motility of *Agrobacterium* strains were assessed using a Nikon Optiphot microscope utilising phase contrast optics. Bacteria were observed following resuspension of overnight cultures in chemotaxis media.

2.11. Chemical mutagenesis of *A.tumefaciens*

The method used was as decribed by Parkinson (176).

Agrobacterium strain C58C*I* was grown in tryptrone broth at 28°C to about 1x10^9 cells per ml. The culture was then washed and resuspended at 2x10^9 cells per

ml in min A salts containing 3% ethyl methane sulphonate (EMS). After one hour of incubation at room temperature, the treated cells were washed twice with 5 volumes of min A salts and resuspended in min A salts plus glucose medium at $1x10^6$ viable cells per ml. They were then grown overnight growth at 28°C to allow for segregation and expression of new mutations.

2.12. Transposon Mutagenesis of C58C*1*

The method used for transposon mutagenesis of *A.tumefaciens* was based on that of Beringer *et al.*, (243). 0.1ml of overnight L-broth cultures of donor strain HB101(pRK2073, pJB4JI or pSUP2011), helper strain HB101 (pRK2013) and recipient strain C58*C1* were added to a 0.22μm millipore filter (Nuform) placed on the centre of a dry L-agar plate, which was subsequently incubated at 28°C overnight. The filter was removed using sterile forceps and placed in a universal bottle containing 10ml of sterile distilled water, which was then vortexed vigorously to release the bacteria. The number of transposition events were calculated by plating dilutions of the bacterial suspension onto L-agar plates containing the appropriate antibiotics. The plates were incubated at 28°C for 48 hours and the resulting colonies counted using an electronic counter (Gallenkamp).

2.13. Mini swarm technique

Approximately 50 mutagenized bacteria were mixed with 10 ml of L-swarm agar containing the appropriate antibiotics in a capped test tube which had just been removed from a 42°C water bath. The resulting suspension was poured into an empty petri dish. The plates were left 30 minutes to dry, were sealed with Nesco film to prevent drying and incubated 48 hours at 28°C. Putative chemotaxis mutants were picked with a sterile toothpick, cloned twice and their swarm phenotype retested on L-swarm plates.

2.14. Keto-lactose assay

The method was that of Bernaerts and De Ley (244). The test identifies 3-keto-lactose production which produces a characteristic yellow colour when reacted with Benedicts reagent (173g Trisodium Citrate, 100g of Sodium Carbonate and

173g of Copper Sulphate per litre). Putative *Agrobacterium* strains were streaked onto $CaCO_3$ medium (2% $CaCO_3$, 1.5% agar, 2% glucose and 1% yeast extract) and incubated overnight at 28°C. A loopful of bacteria were then plated onto lactose agar (1% lactose, 0.1% yeast extract, 2% agar) and plates incubated for 48 hours at 28°C. After this period, plates were swamped with Benedicts reagent and left at room temperature for up to 2 hours. Yellow rings appear only around *Agrobacterium* colonies.

2.15. Isolation of rifampicin resistant *A.tumefaciens met-81* auxotrophs

0.1 ml aliquots of an overnight *A.tumefaciens* C58C*I* culture were plated out onto L-agar plates containing 500μg/ml rifampicin. The plates were then incubated at 28°C for 48 hours. Putative rifampicin resistant colonies were picked and then retested for rifampicin resistance. Those colonies which again grew were tested for *met-81* auxotrophy by streaking them out on min A media plus glucose and minA media plus glucose and methionine to 20μg/ml.

2.16. Ti-plasmid transfer

This was based on the method of Shaw *et al* (245).

0.1 ml of an overnight culture of C58C*I* (pTi GV2201) and C58C*I* (*mot*, *che* or *fla*) grown in min A glucose medium were added to a 0.22μm millipore filter placed on the centre of a min A glucose plate. The plate was then incubated overnight at 28°C. The filter was then removed using sterile forceps, placed in a universal bottle containing 10 ml of distilled water and vortexed vigorously to wash the bacteria from the filter. Dilutions of the resulting suspension were plated out onto min A glucose plus spectinomycin and streptomycin plates to select for transconjugants.

2.17. *A.tumefaciens* virulence assay

This method has previously been described by Hooykaas *et al* (246).

5 ml of an overnight L-broth culture of the *A.tumefaciens* strains to be assayed

were poured into a 5ml syringe fitted with a fine bore needle. Two week old sunflower seedlings growing in a 1:1 mixture of peat:vermiculite were inoculated with a few drops of the bacterial cultures under investigation just below the first node. The seedlings were then allowed to grow in a plant growth cabinet and monitored for tumour formation for 2-3 weeks. Control seedlings were also inoculated with virulent C58C*I* (pTi B6S3) and avirulent C58C*I*.

2.18. Root colonization assay

The assay was based on that decribed by De Weger *et al.*, (241).

Potato tubers were kept in a warm damp place for three weeks until they began to sprout. Developing potato plantlets were then gently picked from the tubers. The *A.tumefaciens* C58C*I* behavioural mutant strains to be assayed were grown overnight in 10 ml of L-broth. Ten potato plantlets per strain were immersed in the overnight bacterial culture. Excess *Agrobacterium* were then gently shaken off and the plantlets planted in 12cm x 3cm PVC tubes containing a clay soil. The PVC tubes containing the plantlets rested on a layer of vermiculite. The soil moisture content was regulated by the addition of water to the vermiculite layer only, the water could subsequently enter the soil via capillary action. The plantlets were grown for 3 weeks in a plant growth cabinet and then removed carefully from the soil. 1 cm root sections of the plantlets were cut corresponding to 0-1cm, 3-4cm and 6-7cm along the length of the root from the base of the stem. Ten 1cm root sections were cut per plant for each given length. Root sections of the same lengh from different plants were combined in an eppendorf containing fine glass beads and 1% bacto tryptone. The eppendorf was vortexed thoroughly and dilutions plated out onto selective L-agar plates containing rifampicin and kanamycin. The resulting colonies were counted using an electronic (Gallenkamp) counter.

2.19. Triparental matings

Triparental matings were routinely used for the mobilisation of behavioural genes cloned in pLAFR3 from *E.coli* (HB101) to given *A.tumefaciens* behavioural mutants to test for genetic complementation.

0.1 ml of an overnight L-broth culture of *E.coli* donor HB101 (pLAFR3), helper HB101 (pRK2013) and recipient an *A.tumefaciens che, mot* or *fla* mutant were added to a 0.22μm millipore filter placed in the centre of a dry L-agar plate. The plate was then incubated at 28^{o}C overnight. The filter was removed using sterile forceps and placed in a universal bottle containing sterile distilled water. The universal was vortexed thoroughly to wash the bacteria from the filter and dilutions plated onto L-agar plates containing tetracycline, rifampicin and kanamycin.

2.20. Radiolabelling of methyl-accepting chemotaxis proteins

The method used was a modified version of that of Springer *et. al.*, (169).

20ml of L-broth was inoculated with a single colony of *A. tumefaciens* and incubated at 28^{o}C overnight. Stationary phase cells were harvested in a sterile McCartney bottle by centrifugation at 3500rpm for 5 minutes in a bench-top centrifuge (Wifug). The bacterial pellet was washed 3 times in chemotaxis medium and finally resuspended in 20 ml of chemotaxis medium. 0.1 ml aliquots were added to a number of Bijou bottles and glycerol and chloramphenicol were added to give a final concentration of 0.5% vol:vol and 200μg/ml respectively and the bottles placed in a water bath at 28^{o}C for 10 minutes. L-[^{3}H-methyl]-methionine (Amersham) of specific activity of 15ci/mM was added to a final concentration of 1μM and the bottles incubated a further 30 minutes at 28^{o}C. The selected chemoattractant was then added to the desired concentration and the bottles incubated one hour at 28^{o}C. Each 1ml sample was then removed and added to a 1.5 ml eppendorf tube. 62.5μl of 40% formalin and 0.2ml of 20% trichloroacetic acid were then added and the eppendorfs placed immediately on ice for 15 minutes. The proteins were then pelleted by 2 minutes centrifugation in a microfuge (MSE) and the supernatant discarded. The pellets were resuspended in 0.1ml of SDS PAGE gel buffer and neutralised by the addition of 5μl of 1M Tris.Cl (pH 6.9). The samples were then boiled for 90 seconds, left to cool and run on 15% polyacrylamide gels.

2.21. SDS polyacrylamide gel electrophoresis

The method used was that of Laemmli (248).

Two glass polyacrylamide gel plates were cleaned with 100% ethanol and with spacers placed between them clamped firmly together using bulldog clips. Stock solutions of 30% acrylamide and 2% bisacrylamide had been made up previously and stored at 4°C. A solution of the following was made up in a buchner flask :-

30% acrylamide	25ml
2% bisacrylamide	3ml
1M Tris.Cl	22.4ml
distilled water	9.3ml

The resulting acrylamide solution was then degassed and 0.3ml of 20% SDS, 50μl of TEMED (N,N,*N'N'*-tetramethyl-ethylene-diamine) and 200μl of ammonium persulphate added. The gel was then cast to a depth of 5cm below the smallest glass plate. A small amount of isobutanol was added to ensure an even gel surface. After the gel had set the isobutanol was poured off and a solution of the following prepared in a buchner flask.

30% acrylamide	3.4ml
2% bisacrylamide	1.4ml
1M Tris.Cl(pH 6.9)	2.5ml
distilled water	12.7ml

The acrylamide solution was then degassed and 100μl of 20% SDS, 50μl of TEMED and 100μl of 10% ammonium persulphate added. The stacking gel was then cast. A well comb was inserted and the gel left to set. The comb and the bottom spacer were then removed and the gel fitted to the electrophoresis equipment (Bio Rad) using bulldog clips. The top and bottom tanks were filled with PAGE buffer (Tris 30.2g, Glycine 144g and SDS 10g per litre) and the wells and bottom spacer sparged out with buffer. Protein samples were then loaded and the gel run at 8mA overnight.

2.22. Fluorography: Bonner and Laskey method

The method used was that of Bonner and Laskey (249).

Following PAGE the gel was placed in a sandwich box containing a solution of 10% trichloroacetic acid and 30% methanol for one hour.

The gel was then transferred to a second sandwich box and washed with dimethylsulphoxide (DMSO) for one hour, fresh DMSO was added after 30 minutes.

The gel was transferred to a third sandwich box containing a PPO DMSO solution (66g of PPO in 300ml of DMSO) and soaked for 3 hours. Finally the gel was placed in another sandwich box containing 7% acetic acid for one hour, then removed, placed between two pieces of cellophane and dryed overnight at room temperature on a vacuum line.

2.23. Fluorography: Amplify Method

Following PAGE the gel was placed in a tray and fixed in 20% methanol for 20 minutes. The methanol solution was then poured off and the gel washed in Amplify (Amersham). Fresh Amplify solution was then added and the gel soaked for at least 30 minutes. The gel was then removed, placed between two pieces of cellophane and dryed overnight at room temperature on a vacuum line.

2.24. Autoradiography

Dry polyacrylamide gels were taped inside a photo-cassette covered by a layer of cling film and exposed to a light sensitized film (Fuji RX saftety) for at least 6 weeks at a temperature of -80^{o}C. Films were developed in phenisol developer (Kodak) and fixed in Kodafix (Kodak).

General DNA manipulation techniques

2.25. Phenol:Chloroform extraction of DNA

DNA samples were deproteinised by the addition of 1 volume of redistilled phe-

nol equilibriated with TE buffer (10mM Tris, 1mM ethylenediaminetetracetic acid (EDTA))pH 8.0.). The phases were mixed by vortexing for 10 seconds and then separated by centrifugation at 13000g in a microfuge (MSE Microcentaur) for 2 minutes, or for larger samples, in a bench-top centrifuge (Wifug) at 3500rpm for 2 minutes. The aqueous phase was re-extracted with 1 volume of phenol:chloroform:isoamylalcohol (25:24:1) followed by 1 volume of chlorofom:isoamylalcohol (24:1) to remove excess phenol.

2.26. Ethanol precipitation of DNA

0.1 volume of 3M sodium acetate and 2 volumes of ethanol were added to the DNA solution, mixed by vortexing and stored at -80°C for 20 minutes. In mini-DNA preparations where sodium or potassium acetate was already present in the DNA solution, the sodium acetate was excluded in the ethanol precipitation step. The DNA was collected by centrifugation at 13000g for 10 minutes in a microfuge, or for larger samples at 12000g for 20 minutes at 4°C in a Sorvall RC-5B centrifuge. Pellets were washed in 80% ethanol, dried under vacuum and resuspended in sterile distilled water or TE buffer.

2.27. Dialysis of DNA solutions

Dialysis tubing (Medicell International Ltd., London) was prepared by boiling for 10 minutes in 2% (w/v) sodium carbonate solution, followed by 5 washes in distilled water. The tubing was stored for several weeks at 4°C and washed well with distilled water before use. The DNA solution was placed inside the tubing each end being secured with a clip. The samples were dialysed against 2 litres of TE buffer at 4°C with stirring for 24 hours and the buffer changed three times.

2.28. Spectrophometric quantitation of DNA solutions

1:100 dilutions of sample DNA were read at 260nm and 280nm on a UV spectrophotometer (LKB). A pure DNA sample has an $A_{260/280}$ ratio of 1.8. An A_{260} of 1.0 is equivalent to a DNA concentration of 50μg/ml, therefore sample DNA concentrations could be calculated.

2.29. Pretreatment of RNase A

Ribonuclease A was dissolved in water to a final concentration of 10mg/ml and boiled at 90°C for 10 minutes to inactivate contaminating DNAase. Aliquots were stored at 80°C.

Isolation of DNA

2.30. Mini-preparation of plasmid DNA

A modified method of Birnboim and Doly (250) was used. An overnight culture of *E.coli* was grown using antibiotic selection and 1.5ml aliquots put into sterile eppendorf tubes. The tubes were centrifuged for 1-2 minutes in a microfuge, the supernatants removed and the tubes inverted onto absorbant paper. 100μl of ice cold solution 1 (1% glucose, 10mM EDTA pH 8.0, 25mM Tris.HCl pH 8.0 and 0.1M NaCl) was added, the solution vortexed for 30 seconds and allowed to stand at room temperature for 5 minutes. 200μl of freshly made solution 2 (0.2N sodium hydroxide, 1% sodium dodecyl sulphate) was added, mixed by inversion and stored on ice for 5 minutes. 150μl of a solution which was 5M with respect to acetate and 3M with respect to potassium (which was made up as follows; to 60ml of 5M potassium acetate was added 11.5ml of glacial acetic acid and 28.5ml of distilled water) was added, the tubes vortexed briefly and stored on ice for a further 5 minutes. Cell debris was pelleted by centrifugation at 13000g for 5 minutes at 4°C. Supernatants were removed to fresh tubes and phenol:chloroform extracted. The aqueous phases were transferred to new tubes and the plasmid DNA obtained by ethanol precipitation. The DNA pellets were washed in 70% ethanol, dried under vacuum and resuspended in TE buffer plus RNAase at a concentration of 20μg/ml.

2.31. Large scale plasmid DNA preparation

10ml of L-broth was inoculated with a single bacterial colony and grown up overnight. The resulting culture was added to 500ml of L-broth containing the appropriate antibiotic selection and incubated at 37°C with shaking overnight. The bacteria were harvested by centrifugation at 4000rpm for 10 minutes in a MSE High

Speed 18 centrifuge. The resulting pellets were washed in 30ml of STE (10mM Tris, 1mM EDTA and 100mM NaCl) and pelleted again by centrifugation at 4000g for 10 minutes and the supernatent discarded. 10ml of solution 1 containing 5mg/ml lysozyme was added, the pellets resuspended, transferred to 2 oakridge tubes and left at room temperature 10 minutes. 20ml of solution 2 was then added, the tubes inverted gently and stored on ice 10 minutes. 15ml of an ice cold solution of 3M potassium 5M acetate was added, the tubes vortexed and left on ice for 10 minutes. Cell debris was removed by centrifugation at 18000rpm for 20 minutes in a MSE 18 High Speed 18 centrifuge. The resulting supernatant was poured into 4 clean 30ml corex tubes, 0.6 of a volume isopropanol added and the DNA left to precipitate at room temperature for 15 minutes. The DNA was pelleted by centrifugation at 8000rpm for 20 minutes. The pellets were washed in 70% ethanol, dried under vacuum and resuspended in 5ml of TE buffer.

2.32. Mini-preparation of chromosomal DNA

This method was as described by Dhaese *et al* ., (251).

A single bacterial colony was used to inoculate a 5ml L-broth culture which was grown at the appropriate temperature overnight. 1.5ml of the resulting culture was added to a 1.5ml eppendorf tube, and the bacteria pelleted by centrifugation for 2 minutes in a microfuge. The supernatant was discarded and the pellet resuspended in a solution of 1% SDS, 20mM EDTA, 50mM Tris.Cl and 1mg/ml pronase and incubated 60 minutes at 37°C. Pronase stock solution contained 20mg/ml of pronase disolved in sterile distilled water which was self digested for 60 minutes at 37°C and then stored at -20°C.

Following pronase digestion the cell debris was removed with 5 consequetive phenol extractions. The DNA was then ethanol precipitated, pelleted by centrifugation at 13000g in a microfuge, vacuum dryed and resuspended in 300μl of TE buffer.

2.33. Large scale preparation of chromosomal DNA

This was essentially a scaled up version of the mini-preparation. A single bacterial colony was inoculated into 5ml of L-broth and grown up overnight. 100μl of

the resulting culture was used to inoculate 40ml of L-broth containing the relevant antibiotics where appropriate and grown overnight. The bacteria were harvested by centrifugation in 2 McCartney bottles at 3500rpm in a bench-top centrifuge (Wifug). The supernatent was discarded and the pellets resuspended in a solution of 1% SDS, 20mM EDTA, 50mM Tris.Cl and 1 mg/ml pronase. and incubated 60 minutes at 37°C. The cell debris was removed with 5 successive phenol extractions, the DNA precipitated by centrifugation at 3500rpm for 5 minutes in a bench-top centrifuge, the pellets vacuum dryed and resuspended in 1ml of TE buffer.

2.34. Caesium chloride/Ethidium bromide density gradient centrifugation

22.4g of caesium chloride and 0.33ml of a 10 mg/ml ethidium bromide solution (giving a final concentration of 120μg/ml) was added to the DNA solution contained in a measuring cylinder and the volume made up to 30ml with TE. The resulting solution was added to a quickseal ultracentrifuge tube via a needle and syringe. The tube was balanced, heat sealed and centrifuged at 55000rpm for 17-20 hours at 15°C in a Sorvall OTD 65B ultracentrifuge. Following centrifugation the tube was carefully removed from the rotor and observed under ultra violet light. Usually 2 bands were clearly visible, the upper band corresponding to open circular plasmid DNA and the lower to supercoiled plasmid DNA. A hole was then made in the top of the quickseal tube and the lower plasmid band collected using a wide bore needle and a 5ml syringe. The needle was then removed to prevent shearing and the solution extruded into a sterile plastic tube. 1 volume of isopropanol saturated with caesium chloride was added and the tube shaken vigorously. The upper pink phase was then removed and the procedure repeated until no ethidium bromide could be seen in the lower phase. The sample was then dialysed against TE buffer, ethanol precipitated and the DNA concentration estimated spectrometrically.

Enzymatic reactions used in DNA manipulation

2.35. Restriction endonuclease digests

10X stock solutions of restriction endonuclease buffers were made up as described in Maniatis (252).

Table 2.36. Restriction enzyme buffers (10X stock solutions)

	Low μl	Medium μl	High μl
5M NaCl	0	100	200
1M Tris pH7.4	100	100	500
1M $MgSO_4$	100	100	100
1M DTT	10	10	0
Distilled water	790	690	200

Plasmid DNA was digested with the desired restriction enzyme (1-2 units), 1/10 volume of the appropriate restriction enzyme buffer added, the volume made up with distilled water and the DNA solution incubated for 60 minutes at 37°C. Chromosomal digests were carried out in a total volume of 240 μl with 25 units of enzyme and incubated overnight at 37°C. Digestion reactions were terminated by the addition of 1/10 volume of stop-dye made-up as follows:-

1ml	10% SDS
2ml	250mM EDTA pH 8.0
0.2ml	1M Tris. pH 8.0
5ml	Glycerol
10mg	Bromophenol blue
1.8ml	Distilled water

2.37. Phosphorylation of DNA

Calf intestinal phosphatase removes the 5′ phosphates from the termini of linear DNA and thus prevents the religation of termini which possess cohesive ends.

4μl of 10X phosphatase buffer (0.5M Tris.Cl(pH 9.0), 10mM magnesium chloride, 1mM zinc chloride and 10mM spermidine) and 1μl of calf intestinal phosphatase were added to the DNA solution and the volume made up with sterile distilled water. The reaction was incubated for 30 minutes at 37°C. 0.1 volume of 0.1M nitriloacetic acid (NTA) was then added and the reaction incubated at 70°C for 15 minutes to denature the phosphatase. The solution was then phenol:chloroform extracted, ethanol

precipitated and the DNA resuspended in TE buffer.

2.38. Ligation of DNA

For subcloning steps DNA fragments were mixed in the ratio 3:1 insert to vector. 0.1 volume of ligase buffer (0.66M Tris.HCl (pH 7.5), 50mM magnesium chloride, 50mM DDT and 10mM ATP) and 2 units of T4 ligase were added to the DNA solution. The sample was then incubated at either 4°C overnight or for 2 hours at room temperature. The ligation mix was then used directly to transform competent *E.coli* cells.

2.39. Agarose gel electrophoresis

The desired concentration of agarose was dissolved in either 200ml of TBE buffer (Tris 216g, Boric acid 110g and EDTA 18.6g for 2 litres) for large gels or 80ml of TBE for mini-gels. The agarose was then heated in a microwave for 3 minutes, left to cool and 3μg/ml of ethidium bromide added. Large gels were cast in a horizontal 180 X 150mm perspex mould sealed to a glass plate with high vacuum grease. Mini-gels were cast in the mould provided by the manufacturer. The gel was then placed in a tank containing TBE buffer, DNA samples loaded into the wells and electrophoresis carried out for the desired time. The gel was then viewed using a UV transilluminator (UVP, Inc.) and photographed using a red filter and Polaroid 667 film.

2.40. DNA fragment isolation using the low melting point (LMP) method

DNA samples were loaded onto a LMP agraose gel and electrophoresis carried out as appropriate. The gel was then observed under UV light and the required fragments cut from the gel using a clean scalpel. The fragments were placed in a sterile eppendorf tube and incubated at 60°C for 15 minutes. 2 volumes of 50mM Tris.HCl, 0.5mM EDTA pH 8.0 was then added and the tube incubated at 37°C for a few minutes. The DNA was then recovered by phenol extraction and ethanol precipitation and resuspended in sterile distilled water.

2.41. DNA fragment isolation using the freeze-squeeze method

The gel slices containing the desired DNA fragments were excised from a standard agrose gel and placed in a sterile 15ml corex tube containg 0.9ml of distilled water, 0.1ml of 3M sodium acetate, 40μl of EDTA and left in the dark 15 minutes. The gel slices were placed onto clean absorbant paper and dried. A small hole was made in a 0.5ml eppendorf tube using a needle and a small amount of glass wool added to the bottom of the tube. The fragments were then placed in the eppendorf tube and incubated at -80^{o}C for 20 minutes. The tube was then placed inside a 1.5ml eppendorf tube and microfuged 10 minutes at 13000g. The 0.5ml eppendorf was then discarded and the solution present in the large eppendorf tube ethanol precipitated following the addition of 5μl of a 1M MgCl, 10% acetic acid solution. The resulting DNA pellet was resuspended in sterile distilled water.

2.42. [α-^{32}P]-dCTP Random primer labelling of DNA

The method was that of Feinberg and Vogelstein (253).

Random primer labelling was carried out using a Sratagene kit. 5μl of buffer, 10μl of a solution containing dATP, dGTP and dTTP nucleotide triphosphates and 2μl of klenow fragment were added from the kit to a 100-200ng DNA sample contained in 28μl of distilled water. 5μl of [α-^{32}P]-dCTP (equivalent to 50μCi) (Amersham) was then added and the reaction incubated at 37^{o}C for 2 hours. The labelled DNA was then either boiled for 5 minutes and used immediately or stored at -20^{o}C.

2.43. [γ-^{32}P]-dCTP End-labelling of synthetic oligonucleotide probes

The method used was as described by Maniatis (252).

4μl of [γ-^{32}P]-dCTP (equivalent to 40μCi)(Amersham), 5μl of klenow buffer and 5μl of klenow enzyme was added to 1μg of DNA sample contained in 36μl of distilled water. The reaction was incubated at 37^{o}C for 2 hours and the labelled DNA either used immediately or stored at -20^{o}C.

2.44. Transfer of DNA to nitrocellulose

The method used was a modification of that of Southern (254).

The agarose gel was photographed and then soaked in dilute HCl (15ml 1M HCl in 2L of water) for 15 minutes. The gel was then washed twice with distilled water and soaked in denaturation buffer (1.5M sodium chloride, 0.5M sodium hydroxide) for 1 hour with occasional shaking. Again the gel was washed twice in distilled water and then soaked in neutralisation buffer (3M sodium chloride, 0.5M Tris pH7.0) for 1 hour. The neutralisation buffer was then discarded and the gel washed twice in 20X SSC (1.5M sodium chloride, 0.15M sodium citrate) and placed on top of Whatman 3MM paper previously soaked in 10X SSC and positioned on a glass plate such that the paper dipped into a reservoir of 10X SSc. A piece of nitrocellulose the same size as the gel was soaked in distilled water, immersed in 10X SSC and placed on top of the gel. Any air bubbles were removed by rolling a glass pipette along the surface of the nitrocellulose. 3 sheets of Whatman 3MM paper the same size as the gel were immersed in 10X SSC and then placed on top of the nitrocellulose, again any air bubbles were removed. 3 layers of absorbant nappies were placed on top of the Whatman 3MM paper followed by a glass plate. The apparatus was compressed by the addition of a lead weight to the surface of the glass plate. DNA transfer was then allowed to proceed overnight.

After transfer the filter was placed between 2 pieces of Whatman 3MM paper and dryed at 80^oC under vacuum for 2 hours.

2.45. Hybridisation of labelled probes to Southern blots

The dry Southern filter was placed inside a plastic bag which was heat sealed along 3 sides of the filter. Prehybridisation fluid (6xSSC, 0.5% SDS, 1x Denhardts solution (0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin) was added to the inside of the bag and the top of the bag heat sealed. The filter was prehybridised for 2 hours at temperatures ranging from 42-65^oC depending on the required stringency with the bag placed in a sandwich box. A corner of the bag was then cut off and the prehybridisation fluid discarded. Hybridisation fluid $50\mu l/cm^2$ of filter (6xSSc, 0.01M EDTA, 1x denhardts solution, 0.5100μ/ml denatured salmon sperm DNA) and the boiled labelled DNA probe were then added to the bag which

was heat sealed. Hybridisation was carried out overnight at temperatures ranging from 42-65°C depending on stringency.

The bag was then cut in one corner and the hybridisation fluid containing the probe poured into a capped beaker and stored for future use at -20°C. The plastic bag was then cut along 3 sides, the filter removed and placed in a sandwich box.

2.46. Washing probed Southern blots

For high stringency washes 500ml of 0.1X SSc and 1% SDS was added to the sandwich box containing the filter and the filter washed at 65°C for 2 hours, with fresh washing solution being added after 1 hour.

For low stringency washes 500ml of 3X SSC and 0.1% SDS was used and the washes carried out at a temperature of 42°C.

2.47. *In situ* hybridisation of bacterial colonies

Colony hybridisation was carried out using the method of Grunstein and Hogness (255). This procedure allows large numbers of bacterial colonies to be screened for the desired recombinant clone.

A 22 X 22cm sheet of nitrocellulose was cut, divided into 1200 squares using permanent ink, placed between two pieces of whatman 3MM paper, wrapped in tin foil and autoclaved. A 22 X 22cm perspex plate was soaked in 70% ethanol and dryed in a lamina flow hood to achieve some degree of sterility. 400ml of L-agar containing the appropriate antibiotic was then poured into the perspex plate and left to set. The nitrocellulose filter was then picked up using sterile forceps and gently placed down on the surface of the L-agar. The bacterial colonies to be screened were picked with sterile toothpicks and patched onto the squares marked on the filter. The plate was then incubated overnight at 37°C.

Four 28 X 28cm pieces of Whatman 3MM paper were cut. One piece was placed in a large tray, saturated with 10% SDS and any excess liquid poured off. The ni-

trocellulose filter containing the bacterial colonies was removed from the plate using sterile forceps and placed on the SDS saturated Whatman 3MM paper colony side up for 3 minutes. Any air bubles between the filter and the Whatman paper were removed. The nitrocellulose sheet was then transferred to a second piece of Whatman 3MM paper that had been saturated with denaturation buffer (0.5M sodium hydroxide, 1.5M sodium chloride) and left for 5 minutes. When the nitocellulose filter was transferred from one tray to another the edge of the tray was used as a scraper to remove as much fluid as possible from the underside of the filter. The filter was transferred to a third sheet of Whatman 3MM that had been saturated with neutralization buffer (1.5M sodium chloride, 0.5M Tris.Cl (pH 8.0)) and left for 5 minutes. The nitrocellulose sheet was then placed colony side up on a dry piece of Whatman 3MM paper, left to dry 60 minutes, then sandwiched between two dry sheets of Whatman 3MM paper and baked for 2 hours under vacuum.

2.48. Hybridisation to nitrocellulose filters containing bacterial colonies

The filter was floated on the surface of a tray containing 6XSSC for 2 minutes and then submerged for 5 minutes. The filter was then transferred to a large plastic bag which was heat sealed around the filter and 300ml of prewashing solution (50mM Tris.Cl, 1mM sodium chloride, 1mM EDTA, 0.1% SDS) was added, the bag was placed in a large plastic box and the filter incubated 1-2 hours at 42°C with shaking. The prewashing solution was then poured off, 150ml of prehybridization solution added and the filter incubated at 65°C for 4 hours. The prehybridization solution was then discarded, hybridization solution and the given labelled probe added and the filter incubated overnight at 65°C.

2.49. Washing probed nitrocellulose filters following colony hybridisation

Filters were washed twice in 500ml of 1XSSC, 0.1% SDS for 2 hours. Following baking at 80°C under vacuum for 1 hour the filters were set up for autoradiography.

2.50. Partial digestion of high molecular weight DNA

A reaction mixture was prepared with 10μg of chromosomal DNA and restriction enzyme buffer in a final volume of 150μl and mixed well. 30μl was dispensed into an eppendorf tube (tube 1). 15μl aliquots were then dispensed into eppendorf tubes 1-8. The remainder was added to tube 9 and all tubes were chilled on ice. 4 units of restriction enzyme was then added to tube 1 and mixed well. The concentration of restriction enzyme was thus 2 units/μg of DNA. 15μl of the reaction mixture was then transferred to tube 2. The enzyme concentration was now 1 unit/μg of DNA. The twofold serial dilution was continued through to tube 8, nothing was added to tube 9. Tubes 1-8 were incubated at 37^{o}C for 1 hour. The reaction was stoped by chilling the tubes to 0^{o}C and adding EDTA to a final concentration of 20mM. 3μl of stop dye was added and the samples analysed by electrophoresis through 0.4% agarose gels at 1-2V/cm. Accurate size markers in the 10-30-kb ran were also run. The gel was analysed under UV light and the conditions giving the greatest amount of high molecular weight DNA in the 20-23-kb range were selected for the large scale preparation of high molecular weight DNA.

2.51. Large scale preparation of high molecular weight DNA

Using the optimum conditions determined from the pilot experiment, 500μg of chromosomal DNA was digested. 1μg of the digested DNA was analysed on an agarose gel to confirm a successful digestion while the remainder was phenol:chloroform extracted, ethanol precipitated and resuspended in 500μl of TE.

2.52. Sucrose density size fractionation of DNA

The prepared DNA fragments were size fractionated on a 38ml 10-40% sucrose gradient prepared in a Beckman SW27 polyallomer tube. The 10 and 40% sucrose solutions were made up in a buffer containing 1M NaCl, 20mM Tris.Cl (pH 8.0), and 5mM EDTA. The two sucrose solutions were added to each vessel of a gradient former. The clip separating the two solutions was released and a magnetic stirrer mixed the incoming 40% with the resident 10% sucrose solution. The developing mixture was slowly transferred to a polyallomer tube through plastic piping driven by a peristaltic pump (Gilson).The piping was attached to the bottom of the mixing vessel and terminated in a fine needle placed at the bottom of the polyallomer tube.

The pump was set at the its lowest speed and the gradient left to form.

The DNA sample was heated for 10 minutes at 68°C, cooled to 20°C and gently loaded onto the top of the gradient. The gradient was then centrifuged at 26000rpm for 24 hours at 20°C.

0.5ml fractions were then collected in eppendorf tubes using a Gilson fraction collector.

10μl of every third fraction was mixed with 10μl of water, 5μl of gel loading dye and analysed through a 0.4% agarose gel. The sucrose and sal concentrations of the DNA markers were adjusted to those of the samples. The gradient fractions containing DNA in the 20-23-kb size range were pooled and dialysed against TE buffer. The dialysed DNA was extracted several times with an equal volume of 2-butanol until the volume was reduced to about 5ml. The DNA was then ethanol precipitated and resuspended in TE at a concentration of 300μg/ml. 0.5μg was then analysed on a 0.4% agarose gel to check the size distribution.

2.53. Preparation of cosmid arms

5μg of the cosmid vector pLAFR3 was digested with either *Hind* III or *Eco* RI. The resulting fragments were treated with bacterial alkaline phosphatase and finally digested with *Bam* HI.

2.54. Ligation and packaging

Each ligation reaction contained 4μg of Bam H1 digested pLAFR3 and 0.5μg of Bam H1 digested *A.tumefaciens* high molecular weight chromosomal DNA in a final volume of 10μl. Post ligation the reaction mixture was packaged using packaging extract (Giga Pack) following the instructions provided by the manufacturer.

2.55. Preparation of competent cells

This was based on the method of Mandel and Higa (256). 100ml of L-broth was inoculated with 1ml of an overnight L-broth culture of the appropriate *E.coli* strain and incubated at 37°C with shaking until the $O.D._{600}$ was between 0.2-0.4 (usually reached after about 2 hours). The culture was then stored on ice for 10 minutes and centrifuged at 4000g for 5 minutes at 4°C. The supernatant was discarded and the pellet resuspended in half a volume of ice cold 100mM calcium chloride pH 7.0. The cell suspension was kept on ice for 15 minutes and centrifuged as previously. The pellet was resuspended in 1/15 volume of 100mM calcium chloride and dispensed into 200μl aliquots in eppendorf tubes. 80μl of 40% glycerol was then added and the competent cells stored at -80°C until required.

2.56. Transformation of *E.coli*

Competent cells were thawed on ice for 5 minutes. Either pure DNA or a ligation mixture was added in a volume of not more than 100μl, the tube was gently shaken and left on ice 20 minutes. The cells were then heat shocked at 42°C for 2 minutes and placed back on ice for 5 minutes. 1ml of L-broth was then added and the cells incubated at 37°C for 1 hour. 100μl of the transformation mixture was then plated per L-agar plate containing the appropriate antibiotic selection.

CHAPTER 3

3.0 Chemotaxis in *Agrobacterium tumefaciens*

3.1 Introduction

Motile bacteria are able to detect, respond and then adapt to temporal and spatial gradients of specific chemicals. This phenomenon is called chemotaxis. Bacteria move-up a concentration gradient of chemoattractants and down a concentration gradient of chemorepellents.

Chemotaxis in the rhizosphere is well documented (225,226,227,228). However, chemotaxis in *A.tumefaciens* has only recently been reported (224). It was shown that specific phenolic compounds, characteristic of plant wound extracts, could act as chemoattractants for *A.tumefaciens* . This attraction was Ti-plasmid dependent unlike chemotaxis per se. Moreover, at low conentrations (10^{-7}M) plant phenolics acted as chemoattractants for *A.tumeaciens* and at high concentrations (10^{-5}M) functioned as *vir* gene inducers (231). Based on these observations the authors proposed a biological scenario; in which, low concentrations of phenolics attract *A.tumefaciens* to the plant wound site, where higher concentrations induce *vir* gene expression and thereby T-DNA transfer (260).

Although the chemotactic response towards phenolic compounds is very sensitive, the intensity of the response is low. In addition, the response is predominatly dependent on the Ti-plasmid, which is possessed by less than one percent of soil *Agrobacterium* . Therefore, chemotactic responses towards phenolic compounds are likely to be important to the infection process only around the immediate area of the wound site. Other compounds may act as more powerful chemoattractants for *A.tumefaciens* explaining their abundance in the rhizosphere (2,261).

This chapter describes the intense and highly sensitive chemotactic response of *A.tumefaciens* to a variety of sugars and amino acids, many of which are characteristic of plant tissues.

3.2. Isolation of motile bacteria

Motile bacteria were obtained periodically by inoculating a loopful of stock cul-

ture into the centre of a L-swarm plate. A concentration gradient is formed as metabolites are expended, and bacteria migrate outwards towards the edge of the plate (Fig.3.2). This provides a rapid method for the enrichment of motile populations. Motile bacteria for capillary assays are picked from the edge of a swarm plate, the bacteria having undergone two previous cycles of swarming, and streaked out onto selective media. Single colonies were picked for chemotaxis assays.

3.3. Motility in *Agrobacterium tumefaciens*

In collaboration with Dr C.H. Shaw the pattern of motility in *A.tumefaciens* was assessed.

Under microscopic observation, *A.tumefaciens* C58C' was an active swimmer. Other genotypes of *A.tumefaciens* , such as LBA4301 (262) or A136 (263) were observed to be much less vigorously motile.

Motility was characterized by continuous straight or curved runs (Fig.3.3.) with few of the tumbling motions and abrupt changes of direction observed in *Escherichia coli* . Average speeds of $60\mu ms^{-1}$ over runs of $200\mu m$ were recorded. On longer continuous runs of over $500\mu m$, mean speeds of $50\mu ms^{-1}$ were maintained. Cells close to the underside of the coverslip turned consistently clockwise, while those adjacent to the surface of the glass slide moved counter-clockwise. This bias was shown by cells in rich and minimal media, and also those washed and resuspended in chemotaxis medium. Thus, *A.tumefaciens* appears to differ from *E.coli* and more closely resembles *Rhizobium* in its motility behaviour (237,264).

3.4. Capillary assay time course experiment

A time course for the chemotactic response of *A.tumefaciens* was monitored for the capillary assay, using L-broth as the chemoattractant. The result would allow the calculation of suitable times for the lengh of *A.tumefaciens* chemotaxis assays.

After a period of 60-70 minutes the number of bacteria in the capillary tubes did not increase significantly (data not shown). Therefore, all subsequent chemotaxis assays were carried out for a period of 60 minutes.

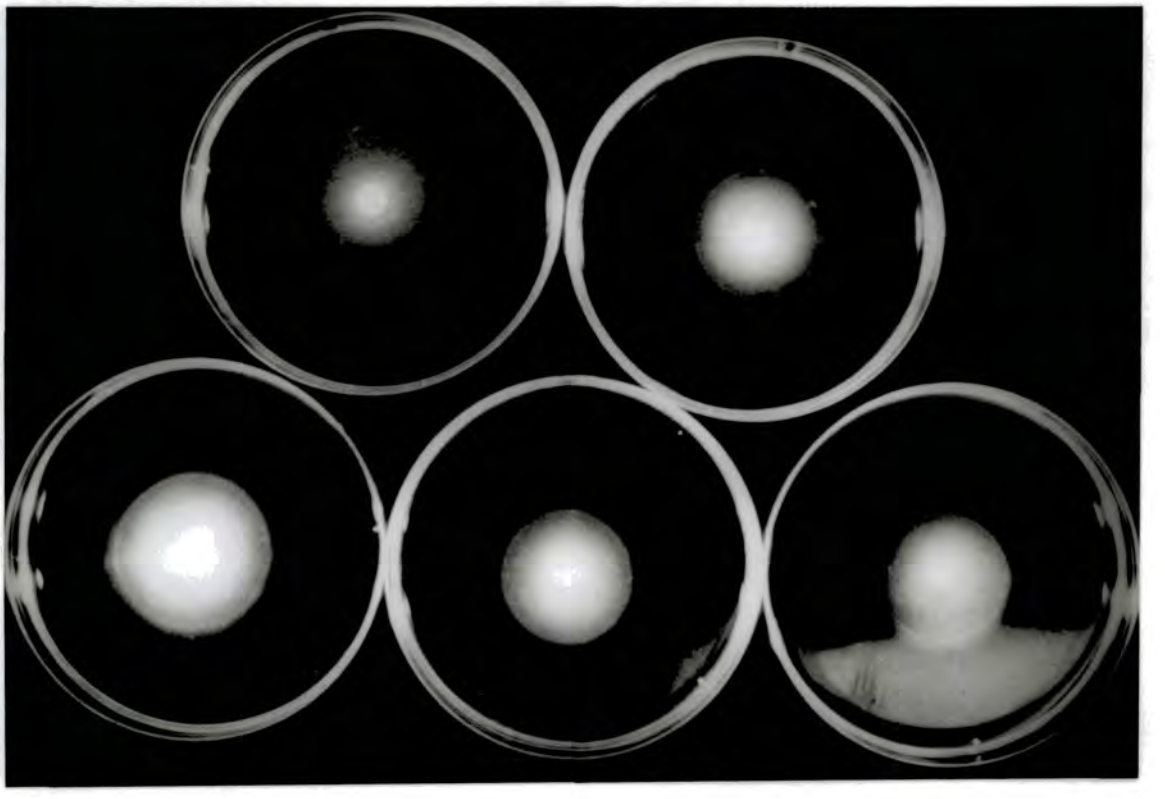
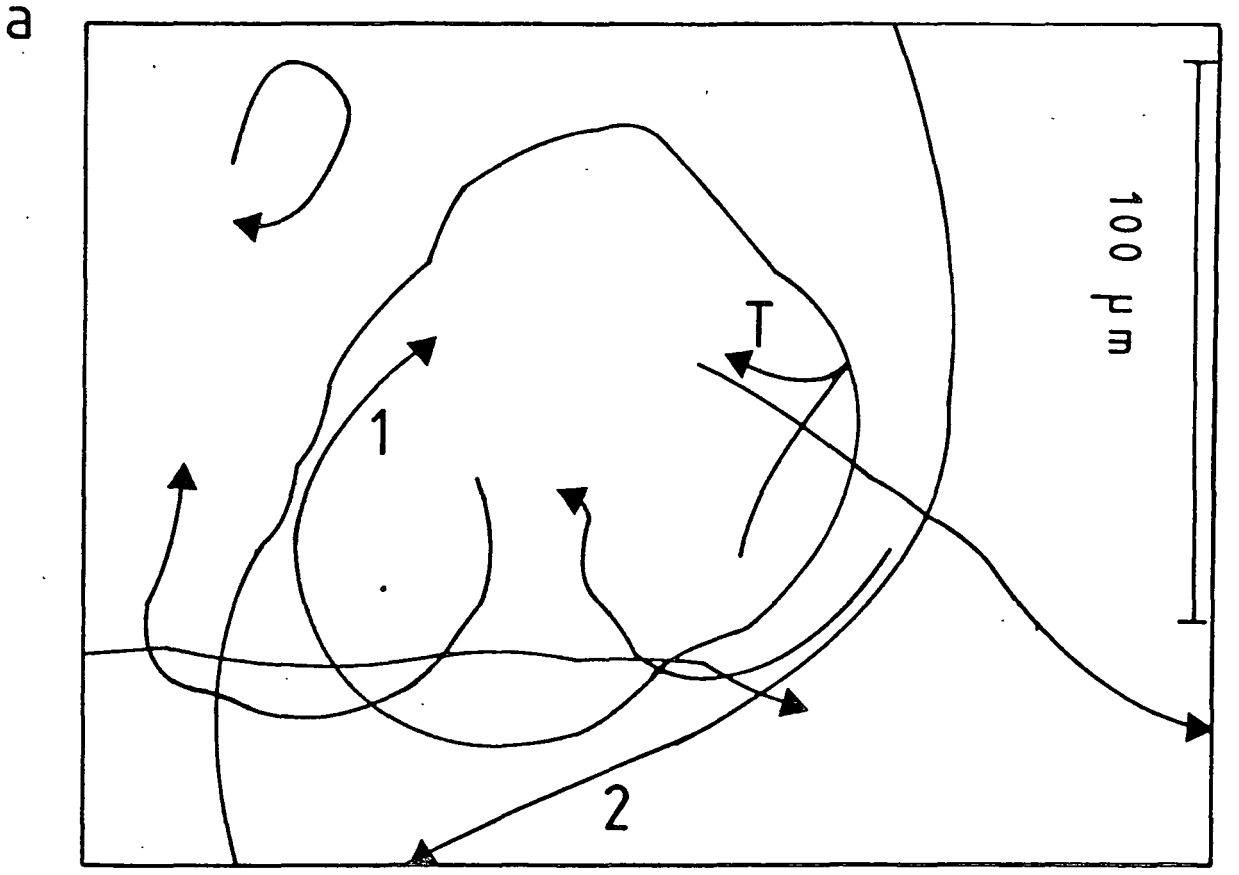
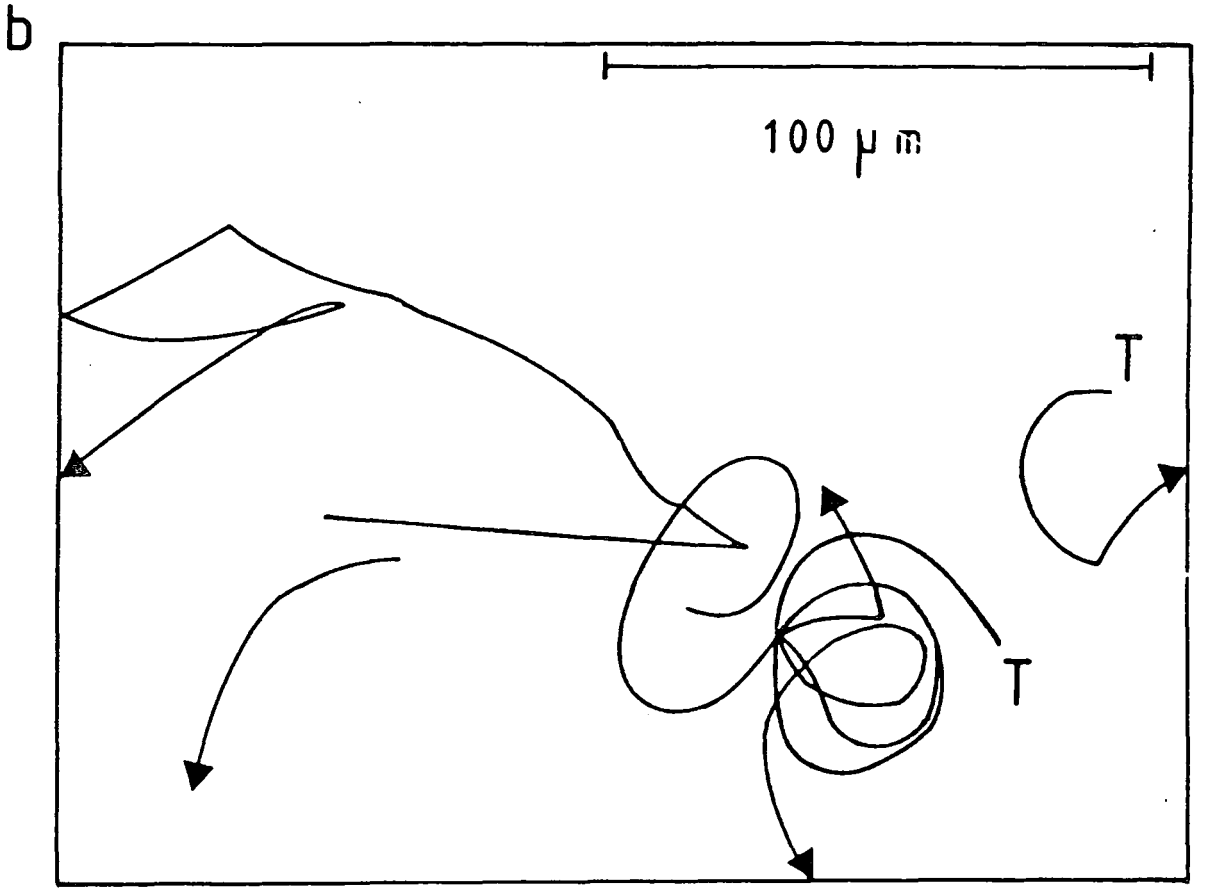


Fig.3.2. Bacterial swarms of *A.tumefaciens.*

Fig.3.3. Trajectories of *A.tumefaciens* C58C/ observed under 400X magnification, using phase-contrast optics. Tracks of individual bacteria were traced onto transparent film superimposed onto a video monitor during slow motion playback of recorded motility. All runs are delinated by the edge of the field of view or by the plane of focus, except tracts marked T, which denote runs commencing or ending in apparent tumbles. Arrowheads indicate direction of motion. (a) Cells adjacent to the inside of the coverslip; (b) cells near the glass slide surface. Track 1 was visible for 10 s during which time the cell covered 514μm. Track 2 covered 218μm in 3.6 s. Bars represent 100μm.


a
100 µm
T
1
2


b
100 µm
T
T

3.5. Capillary assay control experiment

In order to assess the background 'noise' level of the capillary assay, a series of simple control experiments were undertaken. Capillary tubes were filled with an identical concentration of a given attractant, and a capillary assay carried out to determine the variation in the number of bacteria accumullated in each tube.

A typical experiment showed a difference of 6.5 times between the capillary tubes which had acculmulated the greatest and the least number of bacteria within them.

Therefore, in the following set of assays, only values which exceeded the control value by more than seven times were considered significant.

3.6 Chemotaxis of *A.tumefaciens* towards sugars and amino acids

3.7. Introduction

Sugars are a common component of plant tissues. The largest component of the phloem is sucrose, which is typically present at 0.2-0.7M. Reducing sugars such as glucose and fructose are less abundant. However, oligosaccharides for example raffinose (trisaccharide), stachyose (tetrasaccharide) and verbascose (pentasaccharide) are frequently found as major translocation sugars. Most oligosaccharides are based on sucrose with additional galactose units attached by glycosidic linkage. Some sugars are even species specific in a number of plants. Sucrose is thought to be the major translocated sugar rather than monosaccharides because the non-reducing sugar is more easily transported and is less prone to absorption. The reason why larger oligosaccharides are used in some plants is unkown.

Amino acids constitute the main translocatable source of nitrogen and may be present in phloem up to a value of 0.5%. Other translocatable sources of nitrogen include the amides glutamine and asparagine.

3.8. Attraction towards sugars

A range of monosaccharides and oligosaccharides were tested as chemoattractants for *A.tumefaciens* C58*C'*, a Ti-plasmid cured strain. Therefore, any tactic responses detected would be mediated by a chromosome or mega-plasmid encoded system. The sugars chosen were mainly abundant components of plant extracts (265). Capillary assays defined three groups of attractants, with optimum chemotactic concentrations at 10^{-6}M (sucrose, glucose and fructose), 10^{-5}M (maltose, lactulose and galactose), 10^{-4}M (raffinose, stachyose and arabinose) and a group to which no appreciable response was detected (palatinose, lactose, cellobiose and xylose).

3.9. 10^{-6}M optima sugars

In this class the most potent response was observed with the disaccharide sucrose, with a threshold at 10^{-7}M and a peak at 10^{-6}M (Fig.3.9.). The peak response reached a value of 220 times that of the control, in chemotaxis terms this is a massive response.

Interestingly, the monosaccharides glucose and fructose both produced similar curves to sucrose with peaks at 10^{-6}M (Fig.3.9.). However, the intensity of the glucose and fructose response at 34 and 25 times that of the control value is significantly less than that recorded for sucrose. To investigate this further capillary assays were done using 10^{-6}M solutions of glucose, fructose, the two monosaccharides combined and sucrose (Table 3.9.). Glucose and fructose separately evoked similar responses, approximately 4-5 times lower than those towards sucrose. However, the mixture of the two monosaccharides produced a much greater response, of a similar magnitude to that of sucrose. This result suggests that the two monosaccharides of sucrose may act synergistically in producing a chemotactic response from *A.tumefaciens* C58*C'*.

Fig.3.9. Dose response curves of motility of *A.tumefaciens* towards sugars with 10^{-6}M chemotactic optima in capillary assays: Closed boxes, sucrose (α-D-glucopyranosyl-β-D-fructofuranoside); closed circles, glucose; open circles, fructose.

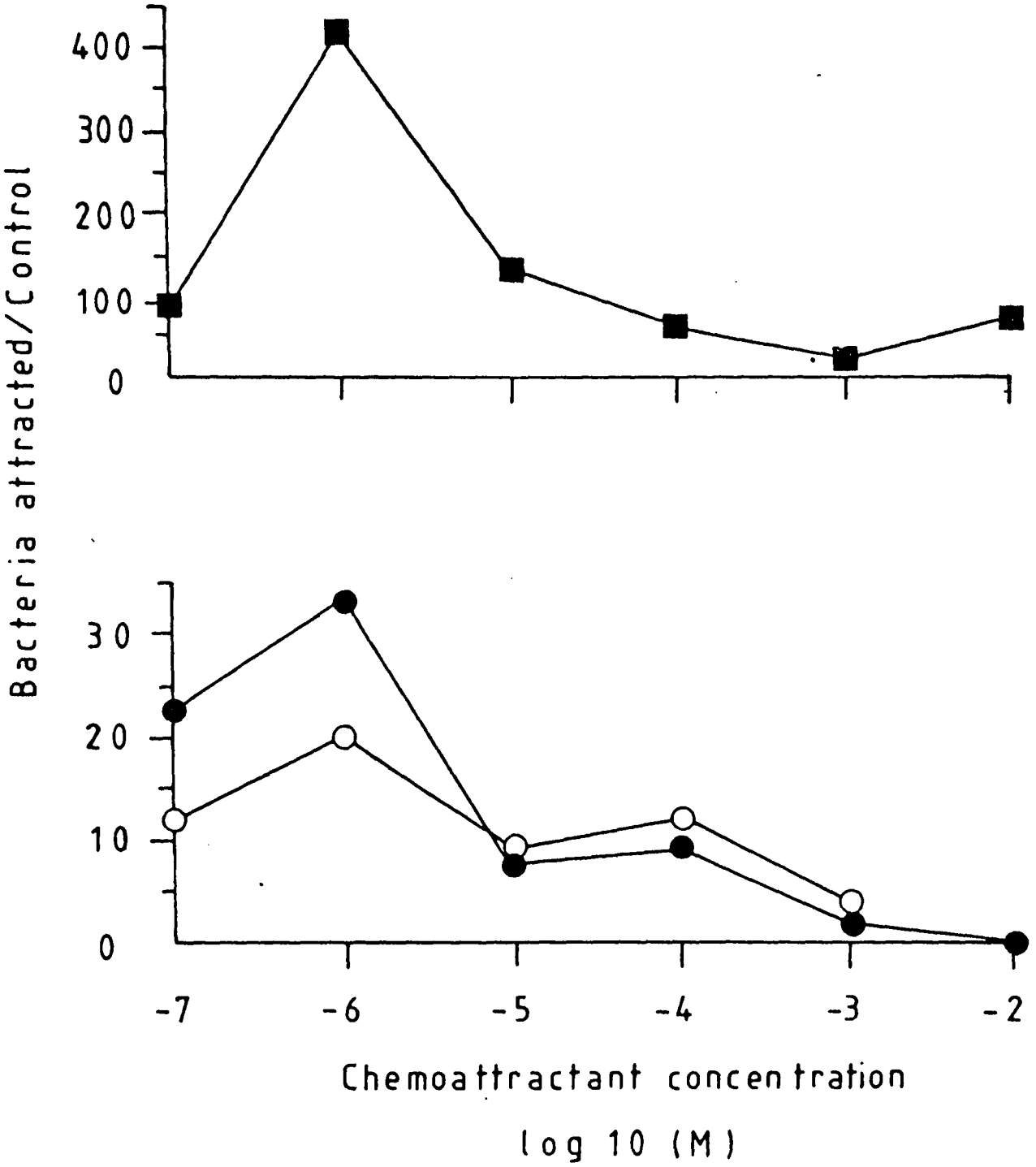

400
300
200
100
0
Bacteria attracted/Control
30
20
10
0
-7
-6
-5
-4
-3
-2
Chemoattractant concentration
log 10 (M)

Table 3.9. Glucose and fructose provoke a synergistic chemotactic response

Attractant	No. bacteria in capillary
Control	1
Fructose (10^{-6}M)	33
Glucose (10^{-6}M)	28
Fructose and Glucose (10^{-6}M)	141
Sucrose (10^{-6}M)	165

3.10. 10^{-5}M optimum sugars

Sugars which provoked a maximum chemotaxis response at 10^{-5}M include the disaccharides maltose and lactulose and the monosaccharide galactose. Again, the disaccharides produced the most intense response with similar peak values of 62 and 58 times that of the control for maltose and lactulose respectively. Galactose incited a peak response 2-3 times lower, at 22 times that of the control value (Fig. 3.10.).

3.11. 10^{-4}M optimum sugars

In this class were the oligosaccharides raffinose and stachyose and the monosaccharide arabinose. All three sugars produced a similar magnitude of response, 15,13 and 14 times that of the control for raffinose, stachyose and arabinose respectively (Fig. 3.11.).

3.12. Sugars which produce no detectable chemotaxis response

The group of sugars which provoked little or no detectable response from *A.tumefaciens* C58C' included xylose, cellobiose and lactose. Palatinose evoked a weak response at 10^{-2}M and 10^{-7}M (data not shown). These weak or non-attracting oligosaccharides are all compounded from sugars which as separate monosaccharides are good chemoattractants.

In summary, the diferent sugars evoked varying responses, both in terms of the

Fig.3.10. Dose response curves of motility of *A.tumefaciens* towards sugars with 10^{-5}M chemotactic optima in capillary assays: Closed circles, maltose (O-α-D-glucopyranosyl-(1-4)-D-glucopyranose); open circles, lactulose (4-O-β-D-galactopyranosyl-D-fructofuranose); closed boxes, galactose.

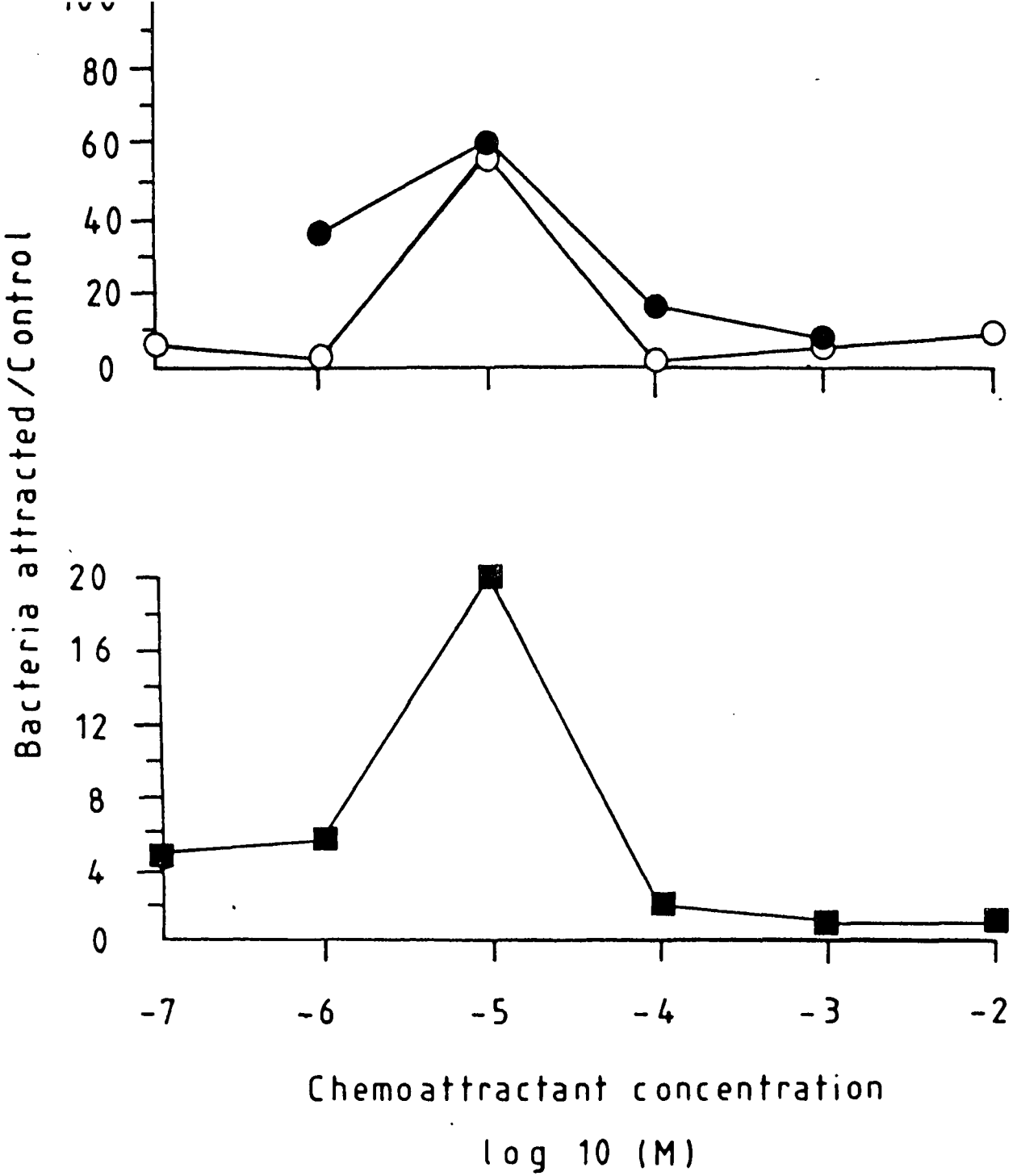
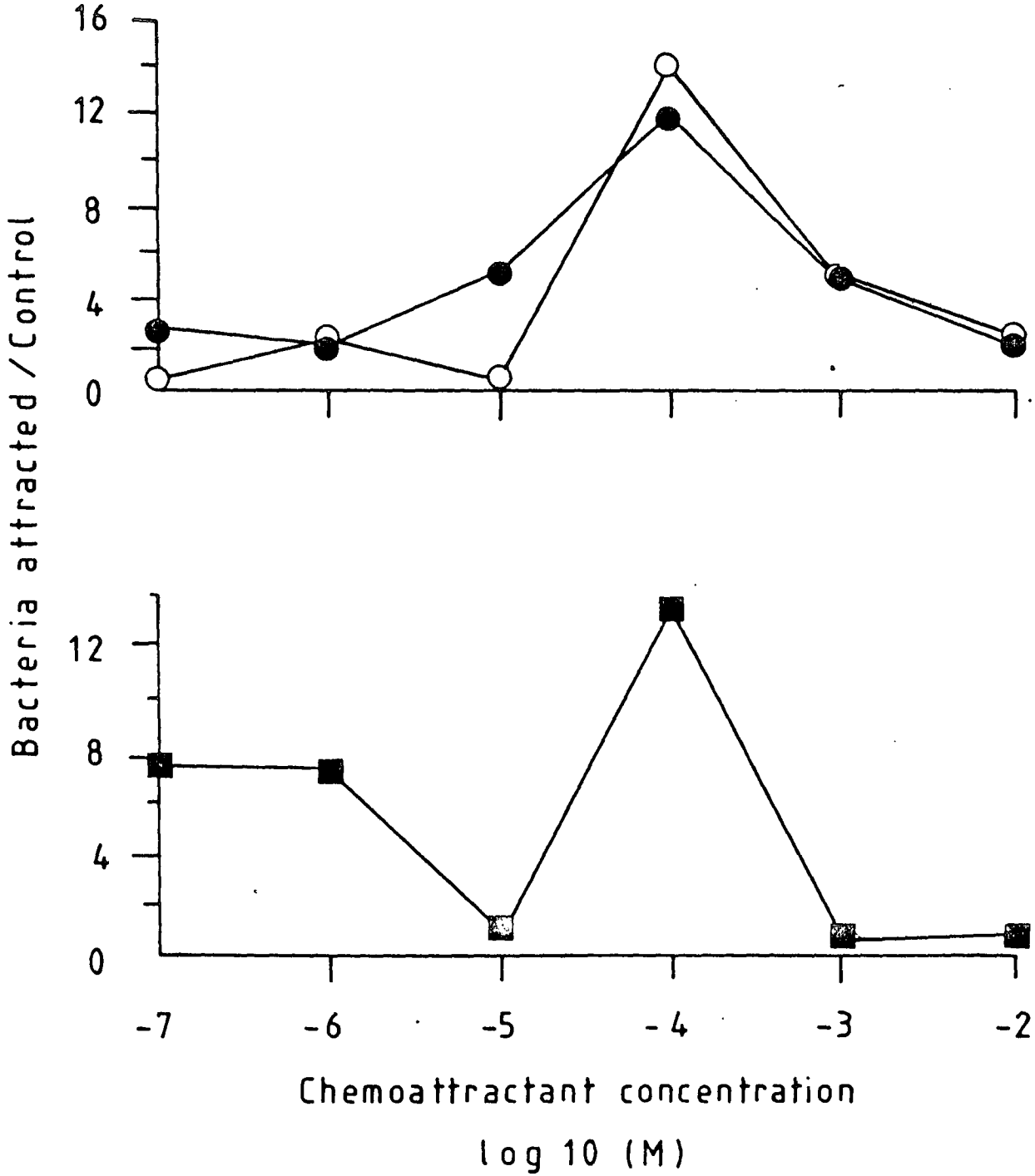

80
60
40
20
0
20
16
12
8
4
0
Bacteria attracted/Control
-7
-6
-5
-4
-3
-2
Chemoattractant concentration
log 10 (M)

Fig.3.11. Dose response curves of motility of *A.tumefaciens* towards sugars with 10^{-4} chemotactic optima in capillary assays: open circles, raffinose (O-α-D-galactopyranosyl-(1-6)-O-α-D-glucopyrano syl-(1-2)-β-D-fructofuranoside); closed circles, stachyose, (O-α-D-galactopyranosyl-(1-6)-O-α-D-galactopyranosyl-(1-6)-O- α-D-glucopyranosyl-(1-2)-β-D-fructofuranoside); closed boxes, arabinose.


16
12
8
4
0
Bacteria attracted / Control
12
8
4
0
-7
-6
-5
-4
-3
-2
Chemoattractant concentration
log 10 (M)

molarity producing the peak response, and its magnitude. The order of response magnitudes was sucrose >> maltose > lactulose > glucose > galactose/fructose > stachyose/arabinose/raffinose. There was a trend for the least sensitive response to have the lowest magnitude.

3.13. Attraction towards amino acids

A.tumefaciens C58C' was tested against a restricted range of amino acids as potential chemoattractants. Valine and arginine evoked a detectable response with both peaks at 10^{-3}M (Fig.3.13.). The intensity of the response was 20 and 13 times above the control value for arginine and valine respectivly.

The amino acids alanine, glycine, cysteine and methionine failed to produce a significant positive response (data not shown).

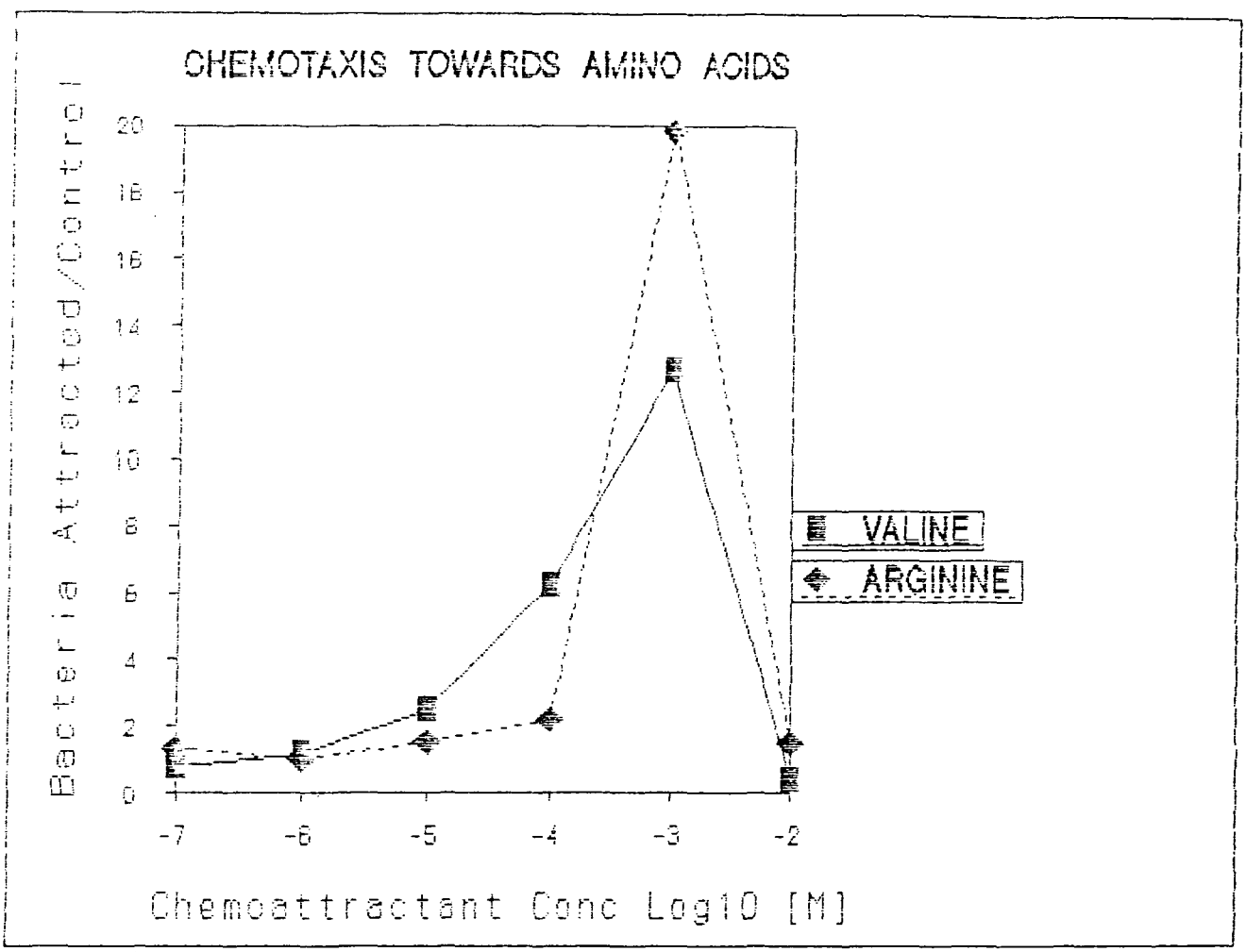
3.14. Discussion

Bacterial chemotaxis has been extensively studied in members of the *Enterobacteriaceae*, but in other bacteria it is not well understood. Of the rhizosphere bacteria, only in *Rhizobium* has chemotaxis been examined in any detail (237,240,239,264). However, it has been recorded in other rhizosphere bacteria prevalent in this habitat (266,267).

Motility in *A.tumefaciens* C58C' seems to more closely resemble that of *Rhizobium meliloti* MVII-1 (237,264) rather than that of *E.coli*. Like *Rhizobium* , *Agrobacterium* exhibits long straight runs, with brief reorientations of direction. This contrasts with shorter runs and longer tumbles in *E.coli*. The switch in direction of flagella rotation from counter-clockwise (CCW) to clockwise (CW) in *E.coli* is responsible for the induction of tumbling. Conversly, *Rhizobium* swim and change direction by an alteration between CW rotation and brief flagella stops, which reorientate the bacterium.

Analysis of *A.tumefaciens* C58C' bacteria, either adhered to the underside of the glass coverslip or to the slide below, suggests that like *R.meliloti* , *A.tumefaciens* motility is mediated only by CW flagella rotation. Therefore, this motility system

Fig.3.13. Dose response curves of motility of *A.tumefaciens* towards amino acids in capillary assays: closed circles, arginine; closed squares, valine.


CHEMOTAXIS TOWARDS AMINO ACIDS
Bacteria Attracted/Control
20
18
16
14
12
10
8
6
4
2
0
-7
-6
-5
-4
-3
-2
Chemoattractant Conc Log10 [M]
VALINE
ARGININE

seems peculiar to rhizobacteria.

Two types of flagella filaments, termed plain and complex have been observed. Plain filaments are found in *E.coli* and *S.typhimurium* , complex in *Pseudomonas rhodos* (268), *Rhizobium lupini* (269) and *R.meliloti* (270). The motility pattern observed for *A.tumefaciens* suggests that it too may possess complex flagella. However, conformation will require the analysis of highly resolved electron micrographs.

The sugars assayed were selected due to their abundance in plant extracts, or to their similarity to sugars already tested. Sucrose, the most abundant translocated plant sugar (265) produced the most intense response and one of the most sensitive responses. The disaccharides were more powerful attractants than the monosaccharides present in the same group; sucrose was greater than glucose or fructose and maltose and lactulose greater than arabinose. This could be explained by the nature of the receptors involved. Alternatively, released monosaccharides following periplasmic degradation of the sugar could act synergistically. Data from Table 3.9. suggests that for glucose and fructose, such synergistic responses may occur. It is unkown at present if *A.tumefaciens* possesses the necessary enzymes for carbohydrate degradation. However, determining the fate of radiolabelled oligosaccharides in the periplasm may resolve the problem. Failure of *A.tumefaciens* to metabolise some oligosaccharides may account for their behaviour as non-attractants.

The tactic potential of the tri- and tetra-saccharide may be influenced by the permeability of the *A.tumefaciens* outer-membrane to these carbohydrates. In *E.coli* raffinose does permeate through (271), but nothing is known of the permeability of the outer-membrane of *A.tumefaciens* .

Arginine and valine were the only amino acids tested to produce a detectable tactic response. The sensitivity of the amino acid responses were an order of magnitude lower than any sugar tested, and the intensity equivalent to the least powerful carbohydrates. The responses (where positive) were however similar to those reported for *Rhizobium* (264). However, all of the amino acids tested in this study were good attractants for *Rhizobium* (264) but alanine, cysteine, glycine and methionine do not attract *A.tumefaciens* C58C'.

Chemotaxis towards phenolic wound exudates has been suggested to play a role in

attracting virulent *A.tumefaciens* towards susceptible plant cells (224). The response to amino acids and certain phenolics are similar in sensitivity to those of *E.coli* and *R.meliloti* . However, the response evoked by *vir* -inducing phenolics and sugars are indicative of a highly sensitive chemotaxis system in *A.tumefaciens* C58*C'*. As many of these carbohydrates are characteristic of plant extracts, this suggests that chemotaxis towards sugars is involved in attracting *Agrobacterium* to the vicinity of plants, which may in part explain its abundance in the rhizosphere (2,261).

CHAPTER 4

4.0. Isolation and characterisation of *A.tumefaciens* behavioural mutants

4.1. Introduction

Genetics provides a powerful tool for the study of processes characteristic of microorganisms, this is especially so because of the current advanced state of molecular biological techniques.

E.coli and *S.typhimurium* represent the model systems for bacterial chemotaxis. In each case a range of behavioural mutants has been isolated which define some 40 genes. These mutants fall into three classes termed *fla* , *mot* and *che* , each producing characteristic colonies on swarm plates (178,179,176). The characterisation of *fla* , *mot* and *che* mutant phenotypes and the analysis of the genes which they define has enabled a detailed molecular model of chemotaxis in enteric bacteria to be constructed (272).

A number of mutagenic strategies for bacteria have been described, chemicals (176), UV light (273) and insertion elements (274). Chemicals and UV light produce rapid results, however, mutagenesis is not specific and may occur at many sites in the genome. Insertion elements, while possibly more difficult to use, have the advantage of producing a single mutation per mutagenized cell (272).

The transposon Tn5 is 5.4Kb in size and possesses 1.4Kb inverted repeats at its termini (275). Its original host is the R-factor JR67 (276). Tn5 has the ability to integrate into non-homologous DNA in an almost random fashion (274). This property makes it ideal for use as an insertional mutagen. In addition to disrupting the physical integrety of a gene, Tn5 may have polar affects on genes within the same operon (277). The insertion of Tn5 into a gene provides a 'molecular tag ' for the mutation it has induced. Moreover, the kanamycin resistance determinate which Tn5 encodes, provides a positive selective marker facilitating the rapid isolation of the mutated gene and its chromosome mapping (274).

In order to introduce the insertion element into the bacterial chromosome, a vehicle (temperate phage or plasmid) is required which carries the element. By selecting against the maintenance of the vehicle in the recipient bacteria, the element must

become stably inserted into the bacterial genome to be maintained. A variety of plasmid vehicles have been constructed using temperature sensitivity, incompatability, *Mu* -induced instability and narrow host-range to select against vehicle maintenance (278,279,280).

The vehicle pJB4JI was constructed from the P1 plasmid pPH1JI and contains Tn5 and *Mu* (281). The lysogenic phage *Mu* renders the vehicle unstable and reduces its ability to become established in a new host. P1 plasmids can infect most Gram-negative bacteria, therefore following infection Tn5 must integrate into the host chromosome to be maintained. This vehicle has proved particularly useful in the mutagenesis of *Rhizobium* (281).

The common *E.coli* vectors pACYC184 and pBR325, which are neither self-transmissible nor mobilizable have been modified into the suicide vehicles, pSUP101 and pSUP201 respectively (282). In addition to Tn5, the mobilization site from RP4 has been added to both vectors. Therefore, the resulting vehicles may be mobilized *in trans* by transfer functions present on a RP4 replicon sharing the same host. Following transfer, mutagenesis is facilitated by virtue of the narrow host-range ColE1 OriR possessed by both vehicles.

An alternative strategy to the pSUP vehicles is provided by pRK2073::Tn5 (283). This plasmid contains both a ColE1 OriR and its own transfer functions provided by RK2. Therefore, pRK2073::Tn5 requires no helper plasmid.

All four of these 'suicide vehicles ' have been instrumental in the low level mutagenesis of a wide variety of Gram-negative bacteria.

This chapter describes the isolation and characterisation of a battery of *A.tumefaciens* behavioural mutants using the technique of transposon mutagenesis.

4.2. Chemical mutagenesis of *A.tumefaciens*

Although a range of chemoattractants has been defined for *A.tumefaciens* (224,284), nothing is known of the genetics of chemotaxis in this organism. Therefore, the ease of isolation of behavioural mutants and the variety of swarm phenotypes they may

produce is unknown.

Chemical mutagenesis provides an easy and rapid method for the isolation of bacterial mutants (176). Therefore, to quickly assess the variety and ease of isolation of *A.tumefaciens* behavioural mutants, chemical mutagenesis using ethyl methane sulphonate (EMS) was carried out.

The bacteria were mutagenized in tryptone broth at 28°C with 3 or 5% EMS fo one hour. Treated cells were then washed and grown overnight to allow for segregation of new mutations.

The bacterial survival rates using 3 and 5% EMS were 96.9% and 29% respectively, therefore only the latter mutagenized bacteria were studied further. Following mutagenesis approximately 100 bacteria were plated per plate from 10 independently mutagenized cultures using the mini-swarm technique (176). Mutant candidates were identified by virtue of their swarm morphology.

Six *A.tumefaciens* behavioural mutants were isolated, yielding a behavioural mutant frequency of 6 x 10^4. Five mutants produced tight dense colonies and were therefore probably *fla* or *mot* mutants. One mutant gave a significantly smaller colony than wild-type but was still fully motile. By analogy with the chemotactic system of enteric bacteria this was probably a *che* mutant.

Therefore, *A.tumefaciens* behavioural mutants can be rapidly isolated and easily identified by virtue of their swarm morphology.

4.3. Mutagenic efficency of 'suicide vehicles' for *A.tumefaciens*

A number of plasmid vehicles have been constructed which carry the insertion mutagen Tn5 (281,282,283). The mutagenic efficency of these vectors varies from host-to-host. Therefore, the 'suicide vehicles' pSUP101, pSUP2011, pRK2073::Tn5 and pJB4JI were tested for their mutagenic efficency in *A.tumefaciens* C58*C1*.

pSUP101 and pSUP2011 were transferred to *A.tumefaciens* in a triparental

mating between pSUP101 or pSUP2011/ED8767, pRK2013/ED8767 and C58*C1*. Rif selected for the recipient and Km for stable Tn5 integration.

Transfer of pRK2073::Tn5 and pJB4JI required no helper plasmid and was accomplished by mating pRK::Tn5/ED8767 or pJB4JI/1830 directly with C58*C1*. Again, Rif selected for the C58*C1* recipient and Km for stable Tn5 integration.

Table 4.3. Tn5 transfer frequency to *A.tumefaciens*

Donar Strain	**Suicide Vehicle**	**Transconjugant Frequency** (Km^r)
ED8767	pSUP1011	5×10^{-6}
ED8767	pSUP2011	3×10^{-6}
ED8767	pRK2013::Tn5	7×10^{-7}
1830	pJB4JI	10^{-6}

Table 4.3. shows the Tn5 transfer frequencies produced by the 'suicide' vehicles. The frequencies are presented per final number of recipient cells.

The transconjugants could not transfer Km^r at high frequency and were sensitive to antibiotics, the resistance to which was specified by the vehicle. These results suggested that the Km^r determinant resided in the chromosome.

From Table 4.3. pJB4JI shows the highest Tn5 transfer frequency at 10^{-6}. pSUP1011 and pSUP2011 gave a similar level of Tn5 transfer, which was significantly higher than that observed for pRK2073::Tn5. Therefore, pJB4JI is the most efficent vehicle for transposon mutagenesis of C58*C1*.

4.4. Transposon mutagenesis of *A.tumefaciens* C58*C1*

pJB4JI/1830 was mated directly with *A.tumefaciens* C58*C1*. The mating mixture was resuspended in distilled water and a dilution series plated onto LA Rif Km plates to calculate the number of transconjugants present. Approximately 50 transconjugants per plate were then screened for behavioural mutant phenotypes on

LA Rif Km mini-swarm agar. Mutant candidates were picked, cloned twice and retested.

22 mutant candidates were isolated at a frequency of 1.8 x 10^{-2} per mutagenized bacteria. Initial classification gave 14 non-motile (*mot* or *fla*) and 8 putative *che* mutants.

To investigate if the reduced swarm size of putative behavioural mutants could be attributed to metabolic defects, their growth in culture was compared to C58*C1*. All but two candidates grew at a rate similar to that of wild-type C58*C1*, those two mutants were therefore discarded.

4.5. Probing putative behavioural mutants for Tn5

A southern blot was used to assess if Tn5 had integrated into the chromosome of behavioural mutant candidates and also that each candidate was unique.

Chromosomal DNA from each candidate was isolated, purified on a caesium chloride ethidium bromide density gradient, digested with *Eco*R1, run on a 0.8% agrose gel and southern blotted. The resulting blot was probed with a *Bgl* II fragment of Tn5 isolated from pAG60 (285). There is no *Eco* R1 site in Tn5, therefore each Tn5 chromosomal insertion should produce a discrete band, the size of which depending on the location of *Eco* R1 sites either side of Tn5.

Fig.4.5. shows a single Tn5 insertion in each mutant candidate. The size of the bands in kb, differs in each case except perhaps for 9 and 10. A second blot (not shown) showed all the 10 remaining mutant candidates to be unique. Therefore, it is likely that at least 18 of the 20 mutant candidates may possess a different mutagenized behavioural gene. Further genetic or phenotypic analysis will be required to discriminate between 9 and 10.

4.6. Phenotypic analysis of C58*C1* behavioural mutants

The detailed behavioural phenotype of the 20 mutant candidates was analyzed

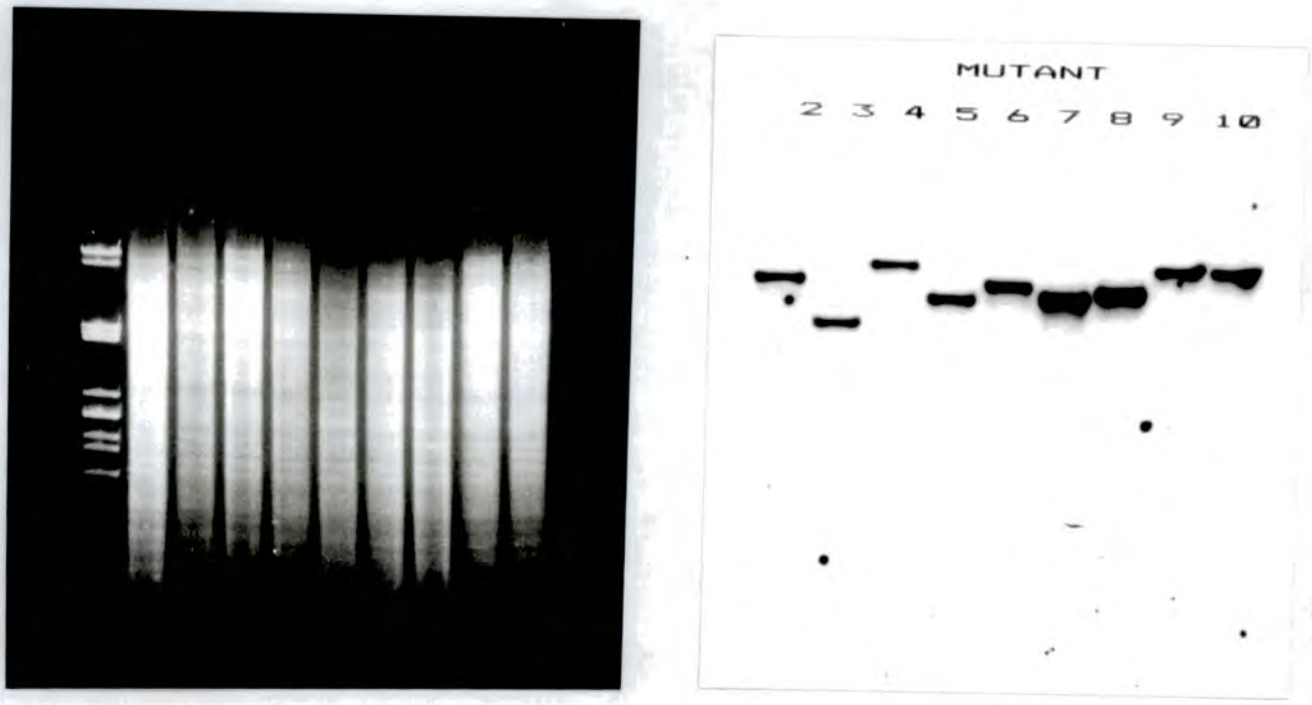


Fig.4.5. 0.8% agarose gel of *Eco* RI digested genomic DNA from 9 putative behavioural mutants (left). The DNA was Southern blotted onto nitrocellulose and probed with a 1.1-kb *Bgl* II fragment of Tn5 from pAG60 (right).

by: light microscopy, using a Nikon Optiphot microscope with phase contrast optics and a Hitachi HV-720K CCTV camera; flagella staining (286) and high powered electron microscopy in collaboration with Dr C.H. Shaw.

All 20 candidates showed aberrant motility and chemotaxis compared to wild-type C58*C1*. The behavioural mutants were classified as either non-motile(*mot*), non-flagellated (*fla*) or non-chemotactic (*che*). 7 *mot* , 7 *fla* and 5 *che* mutants were observed (Table 4.6. and Fig. 4.6.). One putative *che* mutant existed continually as paired cells and was therefore a cell separation rather than a behavioural mutant.

Table 4.6. Behavioural mutants of *A.tumefaciens* **C58***C1*

Behavioural Mutant	**Phenotype**
C58*C1* *mot-1*	non-motile
C58*C1* *mot-2*	"
C58*C1* *mot-3*	"
C58*C1* *mot-4*	"
C58*C1* *mot-5*	"
C58*C1* *mot-6*	"
C58*C1* *mot-7*	"
C58*C1* *fla-1*	non-flagellated
C58*C1* *fla-2*	"
C58*C1* *fla-3*	"
C58*C1* *fla-4*	"
C58*C1* *fla-5*	"
C58*C1* *fla-6*	"
C58*C1* *fla-7*	"
C58*C1* *che-1*	non-chemotactic
C58*C1* *che-2*	"
C58*C1* *che-3*	"
C58*C1* *che-4*	"
C58*C1* *che-5*	"
C58*C1* *pc-1*	paired cells

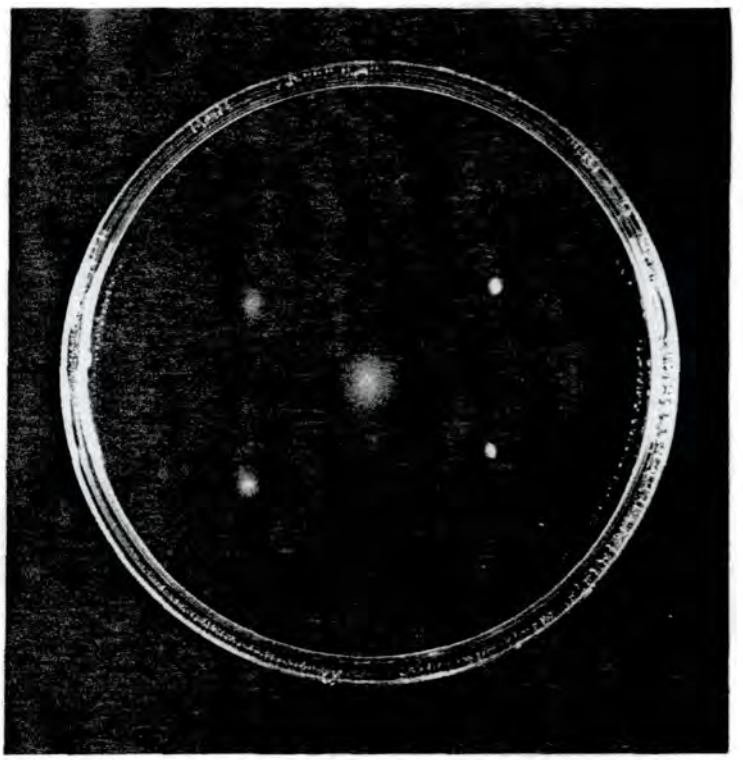


Fig.4.6. Behavioural mutants of *A.tumefaciens*. Wild-type C58C1 centre, *che* mutants small swarms and *mot* mutants tight dense colonies.

4.7. Virulence of *A.tumefaciens* behavioural mutants

To unearth a possible role for motility and chemotaxis in *Agrobacterium* virulence, the 20 behavioural mutants were assessed for virulence or attenuated virulence on sunflower seedlings.

C58*C/* is cured of its Ti-plasmid, therefore Ti pGV2201 was mobilized to all behavioural mutants via a direct mating with pGV2201/*C/*. Km^r Sp^r Sm^r transconjugants were inoculated at the first and second nodes of sunflower plantlets (287). pGV2201 C58*C/* and C58*C/* were used as positive and negative controls. After 3 weeks tumour induction was assessed. All 20 behavioural mutants produced large rough tumours characteristic of pGV2201. Therefore, non of the behavioural mutants were either avirulent or attenuated in virulence in conventional assays.

4.8. Root colonization efficiency of *A.tumefaciens* behavioural mutants

In order to evaluate the importance of motility in the rhizosphere, the ability of C58*C/* behavioural mutants to efficiently colonize plant roots was assessed.

Two week-old potato plantlets were washed with an overnight culture of either *mot-1* , *che-5* or C58*C/*, gently shaken and planted in a PVC tube containing a light clay soil, kept moist by capillary action from a bed of wet vermiculite below. After 14 days incubation the plantlets were removed and their roots cut to give 1cm samples at given distances along their lengh. These samples were placed in an eppendorf tube of peptone broth and vortexed thoroughly with a small quantity of fine glass beads. Dilutions of the resulting suspension were plated onto Rif Km plates to select for behavioural mutants. The number of bacterial colonies reflected the population size of *A.tumefaciens* present at given distances along the lengh of the root (241).

From Table 4.8. no difference in population size along the lengh of the root could be detected between wild-type C58*C/* and *che-5* . However, at a distance of 6cm along the root from the point of inoculation the population size of *mot-1* was significantly lower than that of either wild-type C58*C/* or *che-5* . Therefore, *mot-1* shows reduced colonizing efficency compared to wild-type C58*C/*.

Table 4.8. Root colonization efficency of C58C/ behavioural mutants

	Root Depth (cm)		
	0-1	3	6
Strain			
C58C/	1.2 x 10^3	1 x 10^3	5.1 x 10^2
che-5	9 x 10^2	8.9 x 10^2	7.1 x 10^2
mot-1	1.4 x 10^3	9.1 x 10^2	19

Figures represent the number of Km^r and Rif^r CFU of a given *A.tumefaciens* strain at a stated depth of root. The values given are mean values of 10 replicates.

4.9. Discussion

Mutagenesis by EMS was used to rapidly assess both the nature and ease of isolation of any possible behavioral mutants induciable in *A.tumefaciens* . It was discovered that *A.tumefaciens* produced, at a relatively high frequency, a similar range of behavioural mutants to enteric bacteria.

Although chemical mutagenesis is easily accomplished, it produces a high level of background mutations. Therefore, in order to facilitate an in depth genetic study of chemotaxis in *A.tumefaciens* it was decided to use low level mutagenesis by transposable elements.

A number of suicide vehicles using the transposon Tn5 as an insertional mutagen have been described (281,282,283). The mutagenic efficiency of these vehicles in a C58C/ genetic background was therefore assessed. pSUP1011, pSUP2011, pRK2073::Tn5 and pJB4JI all proved efficient mutagens for *A.tumefaciens* .

Transposon mutagenesis with pJB4JI, followed by selection using the mini-swarm technique (176), expedited the isolation of 20 *A.tumefaciens* behavioural mutants. After detailed analysis using light and electron microscopy and flagella staining the mutants were classified into three groups. 7 *fla* , 7 *mot* and 5 *che* behavioural mutants were defined (Fig.8.4.). One mutant (*pc-1*) existed continually as paired

cells and was therefore classified as a cell separation mutant. Behavioural mutants were produced at a relatively high frequency, 1.8 x 10^2 per mutagenized bacteria. Therefore, if Tn5 integration is assumed to be almost random, behavioural genes represent a significant proportion of the genome.

The *che-1* mutant was especially interesting, this mutant was motile but tumbled incessantly. A similar mutant phenotype has been reported for *E.coli* and *S.typhimurium* (176,198). Such mutants were found to be deficient in *che B* function, a MCP specific methyl esterase. Not surprisingly *che B* mutants showed enhanced MCP methylation. C58*C1 che-1* could therefore be a *che B* mutant and possess enhanced MCP methylation (288). *In vivo* MCP labelling studies with L-[methyl-^{3}H]-methionine may confirm this hypothesis.

The other *che* mutants are motile but produce an attenuated or uncoordinated response. These mutants are likely to be defective in the functioning of the cytoplasmic signal transduction pathway, which relays information from the primary chemoreceptors to the flagellar motor (288). The use of mini-swarm plates containing a complex rather than a defined media would have biased the selection in favour of this type of *che* mutant.

Mutations in the chromosomal virulence genes *chv A* and *chv B* (28), are pleiotropic. In addition to being avirulent, these mutants also lack flagella and are consequently non-motile. Therefore, a link may exist between virulence and motility. Moreover, isolated behavioural mutants classified as *fla* may really be *chv A* or *chv B* mutants. Virulence assays on all 20 mutants were therefore carried out to investigate these possibilities. Every behavioural mutant tested was found to be virulent, at least on sunflower. Therefore, none of the behavioural mutants expressing a *fla*$^-$ phenotype contained mutations in *chv A* or *chv B* . Moreover, this assay detected no correlation between motility and chemotaxis and virulence. However, because *Agrobacterium* were inoculated directly into plant tissues, it is possible the assay superceded the steps where motility and chemotaxis may have been critical. A more pertinent assay might entail only the wounding of plantlets, whose seeds had been inoculated prior to planting. This may allow the role (if any) of motility and chemotaxis in virulence to be more accurately assessed.

Root extracts have been shown to be a powerful chemoattractant for *A.tumefaciens* (23). Furthermore, rhizobacteria have been shown to be motile in moist soil (11).

Pseudomonas aeruginosa moves a distance of 2cm in 24 hours to imbibing seeds (289). Therefore, chemotaxis in the rhizosphere does occur and may play a role in the efficient colonization of plant roots.

The root colonization efficiencies of *A.tumefaciens mot-1* , *che-5* and C58*C1* wild-type were assessed using the assay decribed by De Weger *et al* (241). No significant difference between the root colonizing ability of *che-5* and C58*C1* wild-type could be detected, even at the most extreme point from inoculation. However, a significant difference was apparent in the population of *mot-1* and wild-type C58*C1* at a distance of 6cm along the root. This result suggests that motility but not chemotaxis is important for efficient root colonization.

There is currently a major world-wide research effort to develop effect micro-inoculants for crop plants. The construction of bacterial delivery systems to protect the root against deleterious organisms requires the bacterial vector to efficiently colonize the whole root. Therefore, for effective biocontrol, this study suggests highly motile bacterial vectors would be desirable.

Because of the insertion-inactivation nature of transposon mutagenesis, each defined behavioural gene possesses effectively a 'molecular tag' in the form of Tn5. The drug resistance encoded by this element can therefore serve as a positive selectable marker for the rapid recovery of the given inactivated gene (275). Gene sequences flanking Tn5 can then be used as molecular probes to isolate the wild-type behavioural gene from an *A.tumefaciens* gene library. The application of this technology will permit the isolation of *A.tumefaciens* genes involved in flagella sructure and biosynthesis, intracellular signalling and chemoreception.

This chapter has described the isolation and characterization of a battery of *A.tumefaciens* behavioural mutants. The role of motility and chemotaxis in virulence and root colonization has also been addressed. The behavioural mutants should now facilitate a study of *Agrobacterium* chemotaxis at the molecular genetic and biochemical level.

CHAPTER 5

5.0. Evidence for a methionine-dependent methyl-accepting chemotaxis protein system in *A.tumefaciens*

5.1 Introduction

The majority of motile bacteria swim by rotation of helical flagella. This phenomenon has been extensively studied in *E.coli* and *S.typhimurium* (289). Motility consists of straight runs punctuated by brief tumbles. In the prescence of a spatial gradient of attractant or repellent, tumbling frequency decreases in the favourable and increases in the unfavourable direction. This results in the net movement of bacteria towards attractants and away from repellents.

In enteric bacteria many chemotactic responses are mediated by a class of inner membrane proteins termed methyl-accepting chemotaxis proteins (MCPs) (203). These proteins function as sensory transducers, communicating with cytoplasmic proteins which transmit signals to the flagellar motor. In *E.coli* four distinct MCPs have so far been defined: Tar, which mediates the chemotactic response to aspartate, maltose and repellents; Tap towards dipeptides ; Tsr towards serine and repellents; and, Trg towards ribose and galactose (182,172,186,187). Following exposure to tactic stimuli, methyl groups are thought to be transferred from S-adenosyl methionine to specific sites in the highly conserved MCP terminal domains (196,289,290). This reaction is thought to be crucial in the adaptation process. The change in methylation state is determined by the modulation in activity of a specific methyl transferase and methylesterase, the protein products of the *che R* and *che B* genes respectively (198,291).

A.tumefaciens has been shown to possess a highly sensitive chemotaxis system with threshold responses below 10^{-8}M (224,284). Chemotaxis functions seem to be encoded by two distinct genetic systems; the Ti-plasmid and the bacterial chromosome. Chromosomally encoded gene products mediate taxis towards sugars and amino acids. Highly specific and sensitive tactic responses towards wound exuded phenolics such as acetosyringone, are thought to be mediated via a Ti-plasmid specified system involving *vir A* and *vir G* (292).

It has been suggested that many, if not all, motile bacteria utilise a MCP like system (293). This has been shown to be the case for *Halobacterium halobium* (294),

Caulobacter crescentus (295), *Rhodospirillum rubrum* (296) and *Bacillus subtilis* (297) among others.

Therefore, as a first step in the biochemical characterisation of chemotaxis in *A.tumefaciens* , it was important to ascertain if this bacterium, like others, mediated its tactic responses through a MCP-like system.

5.2. Methionine auxotrophs of *A.tumefaciens* are non-chemotactic

Sucrose is an extremly potent chemoattractant for *A.tumefaciens* , with a peak response at 10^{-6}M (284), while methionine is chemotactically neutral for *Agrobacterium* (284). *A.tumefaciens* strains with auxotrophic mutations affecting either methionine, isoleucine or uracil biosynthesis (kindly provided by G.R.K. Sastry) were fully motile under microscopic analysis. However, when grown under starvation conditions for their respective auxotrophic amino acid, only *ile* and *ura* auxotrophs gave a tactic response to sucrose in capillary assays. No response could be detected from methionine starved C58*C1 met-81* . However, a detectable tactic response could be restored by the supplementation of 10^{-5}M methionine. No further increase in chemotaxis was recorded when the methionine supplement concentration was increased to 10^{-2}M (Fig.5.2.).

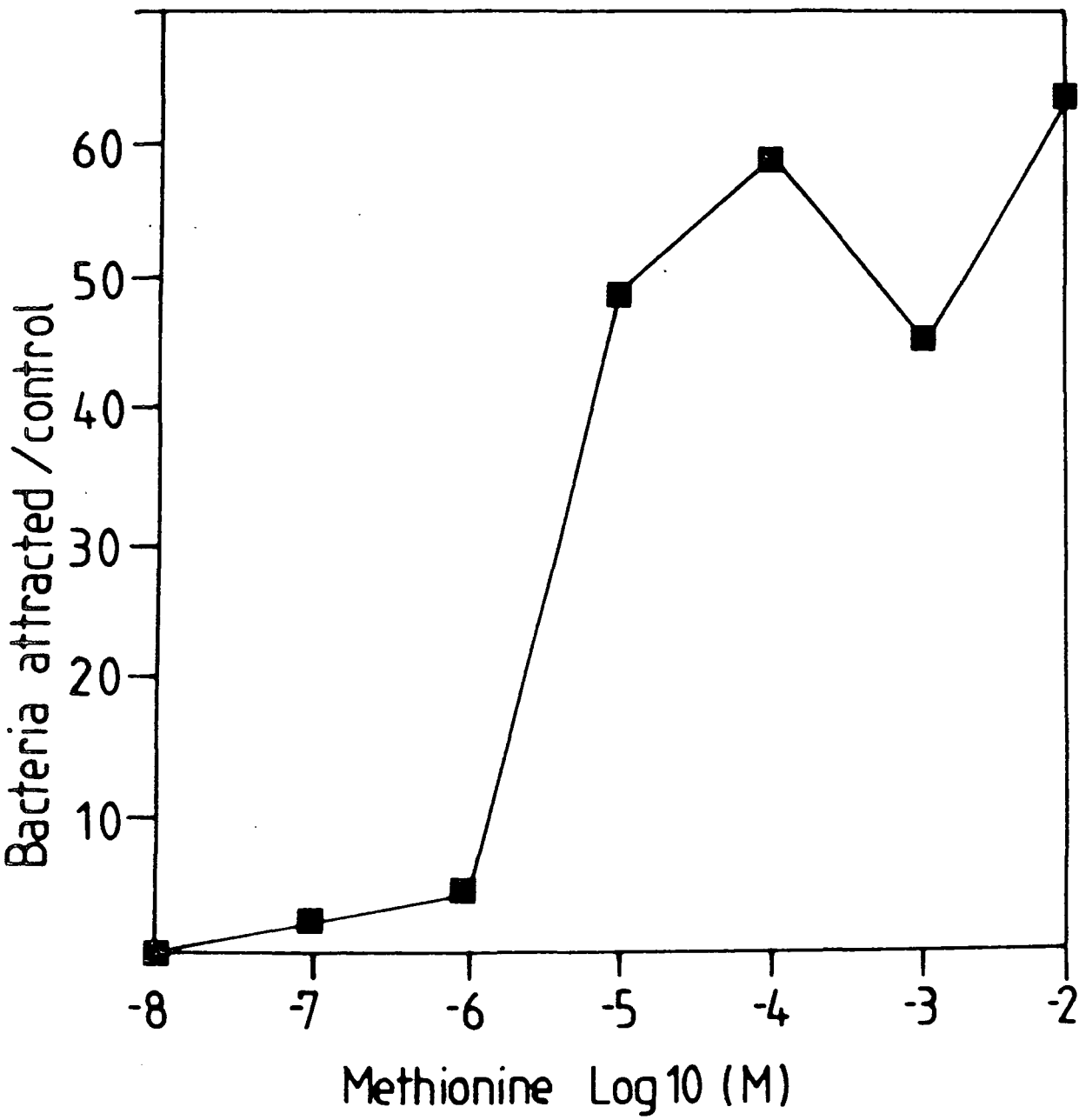
Experiments performed in collaboration with an undergraduate project student suggeseted that acetosyringone chemotaxis in *met-81* (pTiC58) is also methionine dependent (data not shown).

These results suggest there is an absolute requirement for methionine in the tactic response of *A.tumefaciens* towards sucrose and acetosyringone.

5.3. Reduced protein synthesis has no effect on taxis

It is possible that the reduced tactic response observed with methionine starved C58*C1 met-81* could be due to an effect on protein synthesis rather than methionine starvation per se. This is unlikely in view of the tactic response shown by the *ile* and *ura* auxotrophs and the fully motile character of the *met* auxotroph. However, to confirm this capillary assays were performed with methionine starved C58*C1 met-81*

Fig.5.2. Capillary chemotaxis assays on *A.tumefaciens met-81* , using 10^{-6}M sucrose as attractant, with varying levels of methionine supplementation in the bacterial pool.


Bacteria attracted / control
60
50
40
30
20
10
-8
-7
-6
-5
-4
-3
-2
Methionine Log 10 (M)

in the presence of 10^{-2}M methionine and/or 25μg/ml chloramphenicol (Table 5.3.).

Table 5.3. Requirement for methionine but not protein synthesis in taxis

Methionine in pool ($10^{-2}M$)	−	+	+	−
Chloramphenicol in pool ($25mug.ml^{-1}$)	−	−	+	+
No. bacteria in capillary (x10^{-5})	4	57	38	8

C58*CI-met-81* exhibited no chemotactic response towards sucrose unless supplemented with 10^{-2}M methionine. An appreciable tactic response was detected even in the presence of chloramphenicol, although it seemed slightly attenuated. Residual cell lysis could be an explanation for this reduced response.

This result supports the contention that the non-chemotactic phenotype displayed by C58*cI met-81* upon methionine starvation, is not due to an effect on protein synthesis, but for an absolute requirement for methionine during the tactic response.

5.4. Methionine analogues suggest a specific requirement for S-adenosyl methionine

In bacteria possessing MCPs the requirement for methionine has been shown more specifically to be a requirement for S-adenosyl methionine, which directly supplies the methyl groups essential for adaptation.

Previous studies have correlated the ability of methionine analogues to act as substrates for S-adenosyl methionine synthetase with their capacity to restore taxis to methionine starved *met* auxotrophs. Good substrates (seleno-DL-methionine) restored taxis, while poor substrates (ethionine) and non-substrates (α-methyl-DL-methionine) could not.

The ability of methionine analogues to restore taxis of methionine starved C58*CI-*

met-81 towards sucrose was tested (Table 5.4.). Neither α-methyl-DL-methionine nor ethionine restored taxis. However, supplementation with 10^{-2}M seleno-DL-methionine did give a consistently small peak and seemed to partially restore the tactic response.

Table 5.4. Restoration of taxis by methionine analogs

Analogue	Concentration in pool (Log_{10}M)							
	0	-8	-7	-6	-5	-4	-3	-2
Methionine	24	-	-	-	-	-	-	473
α-methyl-DL-methionine	-	25	27	31	17	22	24	42
Ethionine	-	19	8	43	20	3	31	22
Seleno-D-L-methionine	-	39	14	28	41	91	71	103

Figures represent the number of bacteria per ml^{-1} attracted into a capillary tube containing 10^{-6}M sucrose.

Therefore, like enteric bacteria, the absolute requirement for methionine in *A.tumefaciens* reflects the need for S-adenosyl-methionine, presumably to satisfy the demand for methyl groups during adaptation.

5.5. Radiolabelling of MCPs in *A.tumefaciens* .

In enteric bacteria methyl groups are transferred from S-adenosyl-methionine to the C-terminal domains of a class of inner membrane proteins termed MCPs with a molecular weight of 55-65,000 (196,290,291). MCPs can be detected by labelling cells with L-[methyl-^{3}H]-methionine in the presence of an attractant and protein synthesis inhibitor followed by SDS page.

In *E.coli* , *S.typhimurium* and *B.subtilus* mutants have been isolated which show either enhanced or reduced MCP methylation (289,290). Such mutants display exaggerated tumbly or smooth swimming phenotypes. An *A.tumefaciens* chemotaxis mutant (*che-1*) has been isolated which tumbles incessantly. Conceivable, this mutant may possess an aberrant methylation pattern. Experiments were therefore undertaken to assess this possibilty.

Using the method of Terwilliger (194), MCPs were labelled with L-[methyl-^{3}H]-methionine in the presence of acetosyringone and a protein synthesis inhibitor. Under such conditions only MCP proteins of 55-65KDa and the 22KDa elongation factor EFT-U may become labelled.

MCP labelling was clearly visible in the lane corresponding to wild-type C58*C1* but was significantly reduced in the C58*C1* *che-1* lane (Fig.5.5.). Furthermore, MCP labelleing was also detected with C58*C1* using sucrose as an attractant (data not shown).

These results show that MCP-like proteins exist in *Agrobacterium* . Moreover, an *A.tumefaciens* chemotaxis mutant *che-1* , which has an incessantly tumbly phenotype, displays reduced levels of MCP methylation.

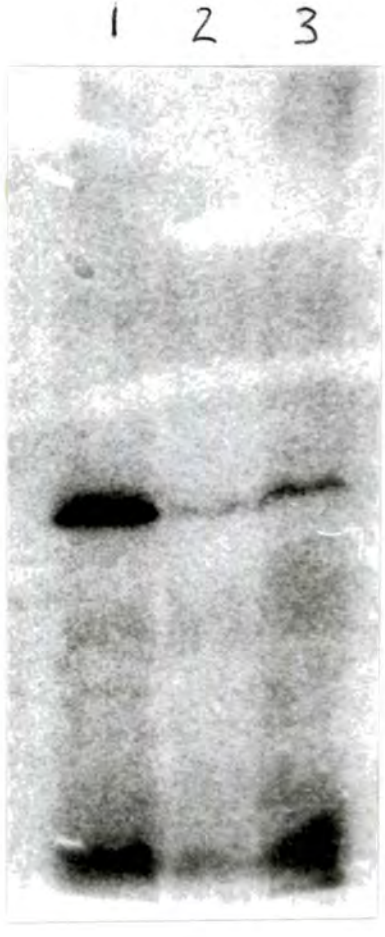


Fig.5.5. Radiolabelling of *A.tumefaciens* MCPs *in vivo*. Fluorograph of SDS-PAGE. Lane 1: C58C*1*. Lanes 2 and 3: C58C*1 che-1*.

5.6. Discussion

This chapter presents evidence for a methionine dependent MCP system in *A.tumefaciens* .

MCP systems have been relatively well characterized in enteric bacteria (289,290), but little is known of the biochemistry of chemoreception in the *Rhizobiaceae* . This study suggests that chemotaxis in *A.tumefaciens* is mediated by an MCP system similar, but subtly different from that of *E.coli* and *S.typhimurium* .

Like enteric bacteria the *A.tumefaciens* MCP system is methionine, or more specifically S-adenosyl methionine dependent and involves the transfer of methyl groups to proteins of approximately 60KDa. However, the analysis of *A.tumefaciens* chemotaxis mutants reveals differences between the two systems.

C58*C'* *che-1* possesses an incessantly tumbly phenotype and has reduced MCP methylation. In enteric bacteria reduced MCP methylation is associated with smooth swimming biased mutants (289). Such mutants are usually defective in the activity of the che-R protein, a MCP specific methyl transferase. However, reduced MCP methylation could occur for a number of other reasons: slow methyl group turnover, lack of available methylation sites and rapid hydrolysis. Therefore, the chemotactic systems of *A.tumefaciens* and enteric bacteria may differ in one or more of these processes.

Moreover, smooth swimming in *A.tumefaciens* is produced by CW and not CCW flagella rotation as in the enteric bacteria. It is therefore possible that some component of the chemotactic mechanism in *A.tumefaciens* is inverted with respect to *E.coli* and *S.typhimurium* .

Chemotaxis to acetosyringone in *A.tumefaciens* is Ti-plasmid dependent (242) and mediated by the vir A and vir G proteins (292). vir A has recently been localized to the cytoplasmic membrane (298), thus the possibility arose that it may function as an MCP like protein. However, methylation of MCPs occurred in the absence of the Ti-plasmid. Therefore, the acetosyringone specific MCP cannot be Ti-plasmid encoded. Moreover, Ti-plasmid specified chemotaxis must be mediated at a level

other than that of the MCP receptor.

In enteric bacteria chemotactic responses to a small number of related compounds are mediated through a single MCP. In *A.tumefaciens* taxis to many phenolic compounds is not Ti-plasmid dependent. Therefore, acetosyringone may share its MCP with one or more of such compounds.

This brief study has provided the basis from which a more detailed biochemical analysis of chemotaxis in *A.tumefaciens* may follow.

CHAPTER 6

6.0 Molecular cloning of genes that specify chemotaxis in *A.tumefaciens*

6.1. Introduction

The cloning of genes from Gram-negative bacteria other than *E.coli* was dependent on the development of a broad host-range cloning system. Such a system required a cloning vehicle that is both transmissable at a high frequency and stably maintained in a wide variety of Gram-negative bacteria.

The Inc P-1 plasmid RK2 confers restistance to ampicilin, tetracycline and kanamycin and is present in *E.coli* at 5-8 copies per cell (299). Inc P-1 plasmids are capable of conjugal self-transfer to a wide variety of Gram-negative bacteria (300). This unique property was the basis in the development of a cloning system in *E.coli* with widespread applicability (301). Due to the large size of RK2, the transfer and replication functions were separated to produce a binary vector system. The 20-kb vector plasmid pRK290, encodes tetracycline resistance and can be mobilized at high frequency by a helper plasmid pRK2013 that encodes kanamycin resistance and is non-self transmissable. pRK2013 contains a colE1 replicon and RK2 transfer functions (302). Foreign DNA can be cloned into the pRK290 vector at unique *Eco* RI and *Bgl* II sites.

Bacteriophage lambda *cos* sequences define the unit lengh of lambda DNA molecules to be packaged within the viral capsid (303). For efficient packaging to occur *cos* sites must be 37-52-kb apart (75-105% the size of lambda DNA) along the lambda replicative concatemer.

Plasmid vectors have been constructed which possess a fragment of DNA containing the *cos* site (304). Such plasmids, termed cosmids, may be packaged in an analogous fashion to lambda DNA, provided the sum of the vector plus insert DNA is 37-52-kb.

Ausubel and coworkers employed this technology to develop further the broad host-range vector pRK290 (305).

A *Bgl* II fragment purified from the cosmid pHC79 (304), containing the lambda

cos site was ligated into the unique *Bgl* II site of pRK290. The resulting plasmid pLAFR1, was an *Eco* RI cloning vector. When ligated to foreign DNA fragments in the size range 15-31-kb pLAFR1 could be packaged into lambda phage capsids which could subsequently infect *E.coli* .

The size selection cosmids impose on foreign DNA inserts results in all the library clones being of a similar size. The average DNA insert of 23-kb would both increase the probability of preserving functional gene clusters intact and minimise the number of clones comprising the gene library. In addition transfection increases the cloning efficiency (measured as the number of clones generated per μg of insert DNA) to a value ten times that obtained for transformation (305).

Thererfore, cosmids possess a number of advantages over their plasmid vector counterparts.

The cloning vector potential of pLAFR1 was further optimized by the addition of a multiple cloning site to form the vector pLAFR3 (306).

The pLAFR1 cloning system has recently been exploited to clone a number of *R.meliloti* chemotaxis genes (239).

By continually picking bacteria from the centre of L-swarm plates Bergman and coworkers isolated a range of spontaneous *R.meliloti* chemotaxis mutants (239). Using cotransduction with bacteriophage ϕM12 the chemotaxis mutations were genetically mapped. All the mutations except *fla-101* mapped to a single *fla-che* region on the chromosome (239). Therefore, like enteric bacteria chemotaxis genes in *R.meliloti* are clustered.

A genomic library from a partial *Eco* RI digest of wild-type *R.meliloti* DNA in pLAFR1 (supplied by Ausubel (305)), was conjugated (en masse) from *E.coli* HB101 into each behavioural mutant. Two cosmid clones, pRZ2 and pRZ4 (Fig.6.1.), complemented most of the mutations in the *fla-che* region.

This chapter concentrates on the construction of an *A.tumefaciens* gene library using the cosmid pLAFR3. The isolation of Tn5 DNA flanking sequences from

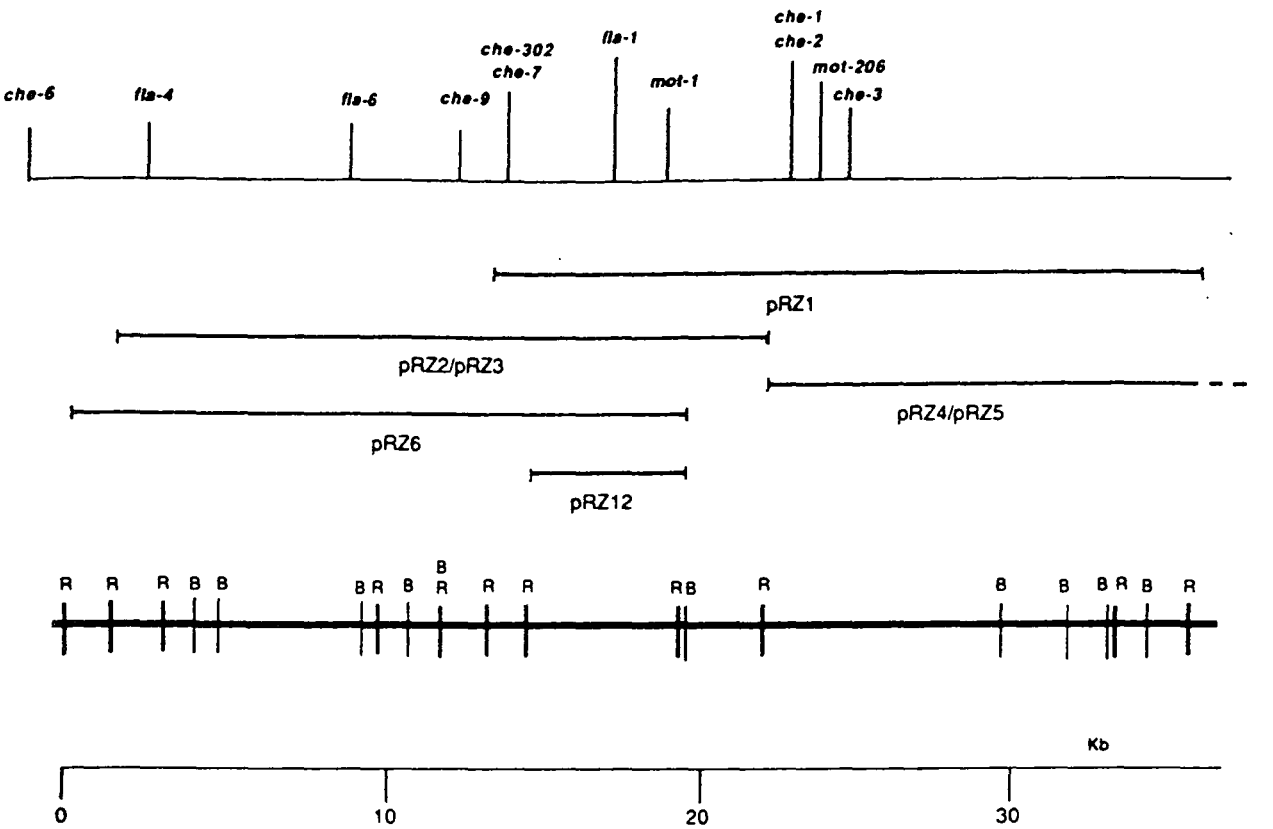


Fig.6.1. Map of the *R.meliloti fla-che* region. Cosmid clones drawn as thick lines underneath the mutations they complement. *Eco* RI (R) and *Bam* HI (B) endonuclease sites within the cloned DNA are indicated. (Courtesy of K.Bergman).

behavioural mutants and their employment as molecular probes to screen the constructed *A.tumefaciens* gene library for wild-type behavioural genes is discussed. And finally, the relationship of *A.tumefaciens* chemotaxis genes to the chemotaxis genes of other rhizobacteria is outlined.

6.2. Production of 23-kb *Bam* HI fragments of *A.tumefaciens* DNA

A.tumefaciens genomic DNA was isolated and purified on a caesium chloride/ ethidium bromide density gradient.

23-kb is the insert size which is packaged with maximum efficency into the lambda capsid in the pLAFR3 cosmid system. Therefore, a series of small scale partial *Bam* HI digests were carried out on purified *A.tumefaciens* genomic DNA to optimize the yield of 23-kb *Bam* HI fragments.

After optimizing the digestion conditions three large scale partial *Bam* HI digestions were carried out using 0.5, 0.25 and 0.12 *Bam* HI units per μg of DNA respectively for one hour at 37^{o}C. Each digest contained a total of 65μg of *A.tumefaciens* genomic DNA.

The digested DNA was fractionated by size on a 10-40% sucrose gradient. Every third fraction was electrophoresed on a 0.4% agarose gel overnight. Fractions containing 23-kb DNA fragments were pooled and concentrated.

6.3. Preparation of pLAFR3 vector arms

5μg of pLAFR3 was digested with either *Hind* III or *Eco* RI and the resulting linear pLAFR3 treated with bacterial alkaline phosphatase to prevent vector arm religation. Finally, both *Hind* III and *Eco* RI digested phosphatased pLAFR3 was digested with *Bam* HI.

6.4. Ligation and packaging

400μg/ml of *A.tumefaciens* 23-kb *Bam* H1 fragments was mixed with 100μg/ml of each vector arm and the resulting mixture ligated at 4°C overnight.

2μg of the ligation mixture was then packaged using a Gigapack packaging extract. The resulting phage were titrated by transfecting *E.coli* HB101. Transfectants were plated onto LA tetracycline X-gal. White colonies among a background of blue suggested the presence of recombinant pLAFR3. Plasmid mini-preparations carried out on 20 randomly selected white colonies showed this to be the case.

6.5. Library maintenance

The number of recombinant pLAFR3 clones required to constitute a library of 99% probability is 820. This estimate was based on the equation P=1-$(1-f)^N$: where P is the probability of a gene sequence being present in the library, f is the fraction of the genome represented by the average insert and N is the number of clones in the library (307).

1200 clones were picked and each inoculated into the well of a microtitre dish containing 65μl of LB tetracycline. The bacteria were grown overnight at 37°C with gentle shaking. 50μl of 80% glycerol was then added to each of the 1200 wells and mixed by gentle pipeting. The 13 microtitre dishes containing the gene library were stored at -80°C.

6.6. Isolation of Tn5 DNA flanking sequences

A battery of 20 Tn5 induced behavioural mutants has been generated using the suicide vector pJB4JI. The kanamycin resistance determinant encoded by Tn5 has been employed as a positive selective marker for the recovery of mutated behavioural genes.

Genomic DNA was isolated from each of the given behavioural mutants and purified on caesium chloride/ethidium bromide density gradients. 28μg of purified

DNA was digested to completion with *Eco* RI. Tn5 does not contain an *Eco* RI site, therefore its kanamycin resistance determinant remains intact.

400μg/ml of *Eco* RI digested chromosomal DNA from each mutant was added to 50μl/ml of *Eco* RI digested pUC18 in a volume of 80μl and the mixture ligated overnight at 4°C. The high insert:vector ratio decreased the probability of vector religation.

The amount of recombinant pUC18 was titred by transforming competent DH5α cells with 1μl of the ligation mixture. Transformants were plated onto LA ampicillin X-gal plates. White colonies indicated the numbers of recombinant pUC18 clones.

If the average insert size generated by the hexanucleotide cutter *Eco* RI is assumed to be 4-kb, 4715 recombinant pUC18 clones would be required to guarantee the presence of the kanamycin resistance determinant in the library. This estimate is again based on the equation P=1-$(1 - f)^N$ (307).

If a sufficient number of recombinant pUC18 were present equal volumes of the ligation mix were added to 12 eppendorfs of competent DH5α cells in a large scale transformation. Transformants were selected on 60 kanamycin ampicilin plates. Plasmid mini-preparations were carried out on putative kanamycin resistant colonies. The isolated plasmids were digested with *Eco* RI and *Hpa* I. Tn5 has no *Eco* RI site but *Hpa* I sites almost define the unit lengh of the transposon. An *Eco* RI *Hpa* I digest would therefore release the 2.7-kb pUC18 vector, Tn5 and the *Eco* RI *Hpa* I flanking sequences (Table 6.6. and Fig.6.6).

Table 6.6. Sizes of behavioural mutant Tn5 DNA flanking sequences

Mutant	*mot-1*	*mot-2*	*mot-3*	*mot-4*	*che-1*	*che-2*
Size of flanking sequences (kb)	0.95, 0.35	2.8, 2.6 2.4	2.6, 1.0	3.0, 1.1 0.9	2.2, 2.8	2.5, 1.8

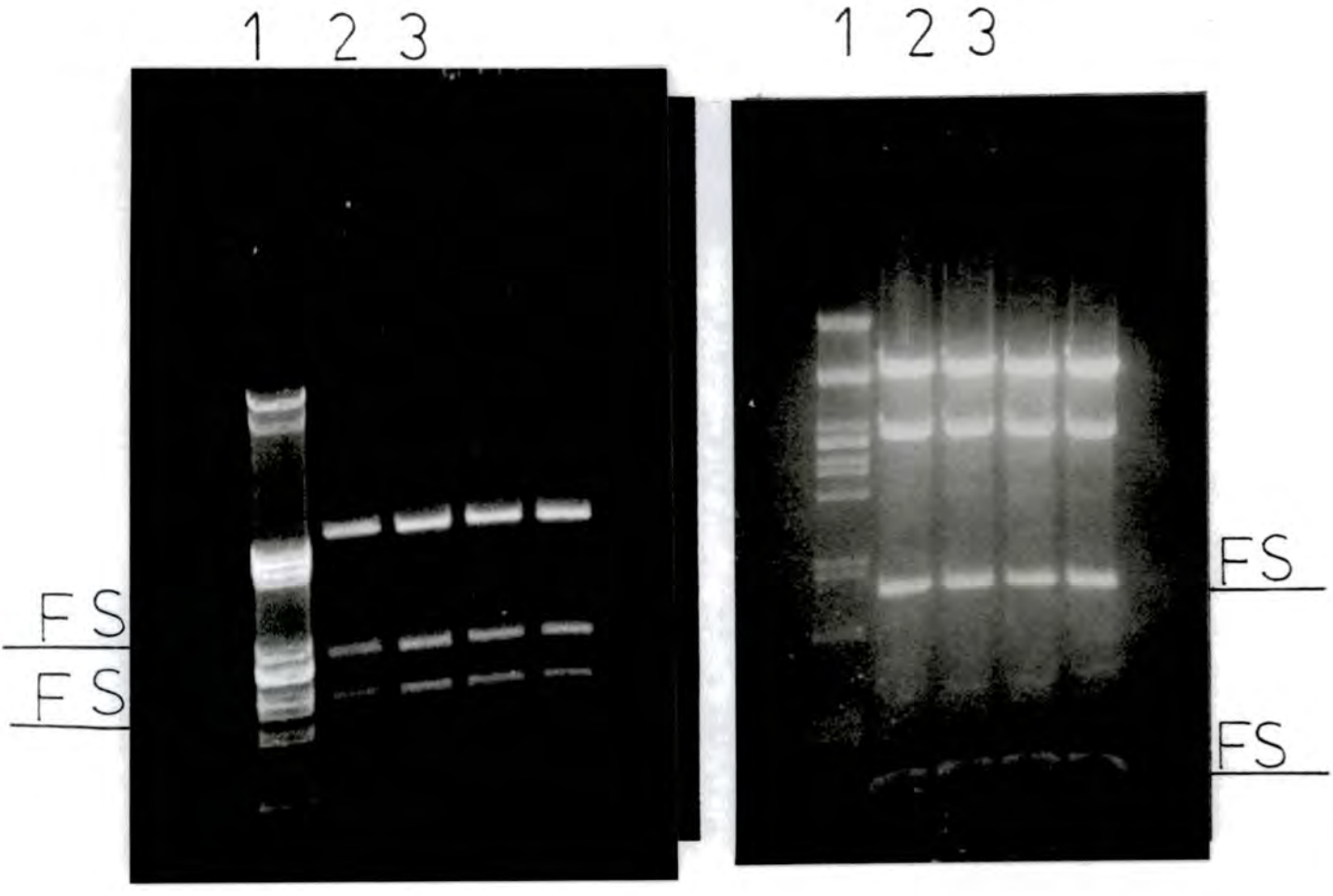


Fig.6.6. 0.8% agarose gel of the cloned *A.tumefaciens* behavioural genes *mot-1* (right) and *che-1* (left) mutated with Tn5. Lanes 1: *Pst* I digested λ DNA. Lanes 2 and 3: *Eco* RI, *Hpa* I digested *mot-1* (left) and *Eco* RI, *Hpa* I digested *che-1* (right).

The abbreviation FS denotes Tn5 flanking sequence.

6.7. C58C/ *mot-1* Tn5 flanking sequences show similarity to *R.meliloti* genomic DNA

Gradient purified genomic DNA from *A.tumefaciens* , *R.meliloti* and *E.coli* was digested with *Bam* H1 or *Hind* III and electrophoresed through a 0.8% agarose gel. The resulting DNA fragments were southern blotted onto nitrocellulose. The resulting blot was subsequently probed at high stringency (6XSSC, 0.5% SDS and 65^{o}C) with Tn5 *mot-1* DNA flanking sequences random primer labelled with [α-^{32}P]-dCTP (Fig.6.7.).

Not surprisingly the *mot-1* Tn5 flanking sequence hybridised strongly to two *Bam* HI and two *Hind* III *A.tumefaciens* DNA fragments. Intriguingly, the flanking sequence also hybridized to three *Bam* HI and three *Hind* III *R.meliloti* DNA fragments. No hybridization could be detected with *E.coli* genomic DNA. Because of the high stringency hybridization conditions employed, *R.meliloti* genomic DNA must possess a nucleotide sequence with at least 99% similarity to the *mot* -1 Tn5 flanking sequences.

6.8. *A.tumefaciens chemotaxis* genes are not encoded by the cryptic plasmid

Although C58C/ was cured of its Ti-plasmid the possibility still existed that behavioural genes resided on the large cryptic plasmid. To resolve this problem genomic DNA from a cryptic plasmid cured strain GM19023 (kindly provided by Dr J.Denarie) was probed with the *mot-1* flanking sequence.

In view of the detected similarity between sequences in the *R.meliloti* genome and the *mot-1* Tn5 DNA flanking sequences, other rhizobacteria were also tested for similarity.

Gradient purified DNA from GM19023, C58C/, *R.leguminosarum* , *R.leguminosarum* biovar *viciae* , *Pseudomonas reactans* and *Pseudomonas talassii* was digested to completion with *Hind* III and electrophoresed through a 0.8% agarose gel. The DNA fragments were Southern blotted onto nitrocellulose. The resulting blot was probed with α-^{32}P-dCTP random primer labeled *mot* -1 Tn5 flanking sequences under conditions of high stringency.

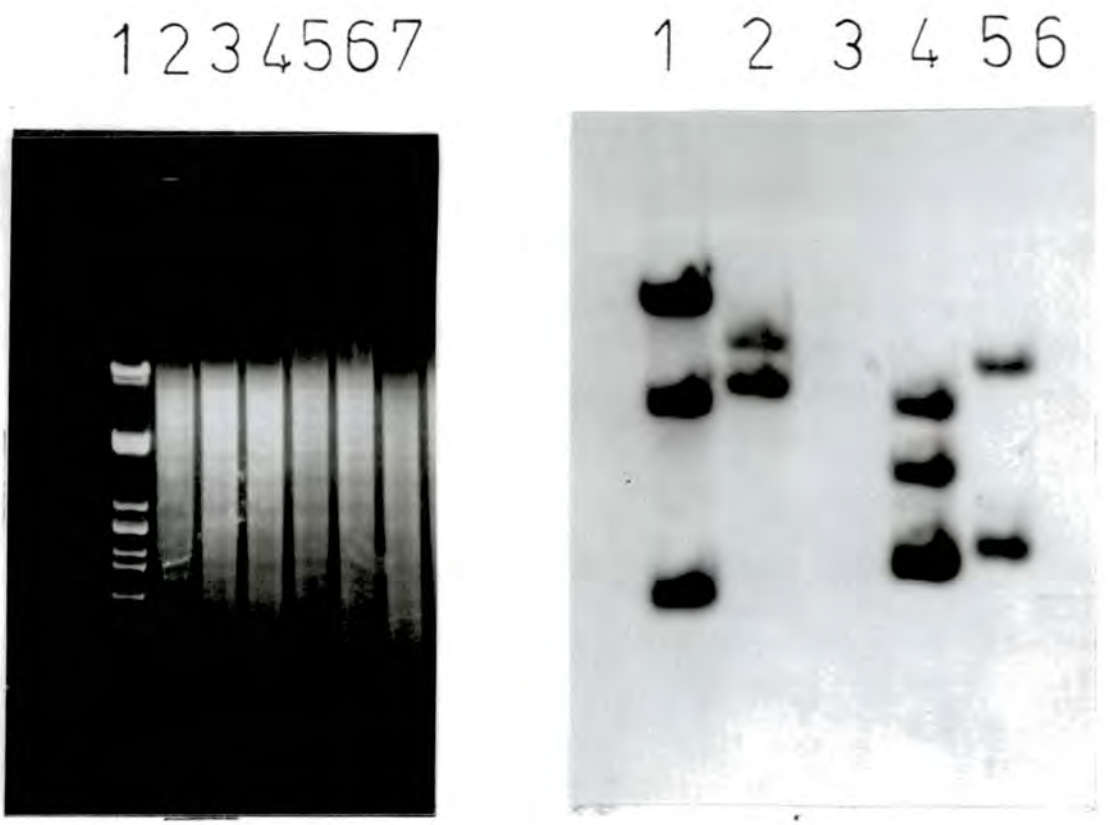


Fig.6.7. *mot-1* flanking sequences show similarity to *R.meliloti* genomic DNA. 0.8% agarose gel (left) of, Lane 2; *R.meliloti* , Lane 3; *A.tumefaciens* , Lane 4; *E.coli* , Lane 5; *R.meliloti* , Lane 6; *A.tumefaciens* and Lane 7; *E.coli* genomic DNA digested with *Bam* HI Lanes 2-4 or *Hind* III Lanes 5-7. Lane 1; contains *Pst* I digested λ DNA. The DNA was transferred to nitrocellulose paper and probed with [α-^{32}P]-dCTP labelled *mot-1* flanking sequences (right).

From Fig.6.8. single bands of identical size were detected in the C58C/ and GM19023 lanes. Therefore, *mot-1* and probably all other *A.tumefaciens* behavioural genes are not located on the cryptic plasmid. As GM19023 has also been cured of its Ti-plasmid, *A.tumefaciens* behavioural genes must be located on the chromosome.

There was no detectable similarity, at least at this high stringency, between *R.leguminosarum* , *R.leguminosarum* biovar *viciae* , *Pseudomonas reactans* , *Pseudomonas talassii* and *E.coli* genomic DNA and the *mot-1* Tn5 flanking sequences.

6.9. *A.tumefaciens mot-1* and *che-1* Tn5 flanking sequences are similar to cloned *R.meliloti* behavioural genes

Bergman and coworkers produced a number of spontaneous *R.meliloti* behavioural mutants (239). These mutants all mapped to a single *fla-che* region on the chromosome. Most of the mutants could be complemented by either one of two contiguous cosmids pRZ2 and pRZ4 (239).

In view of the similarity detected between the *A.tumefaciens mot-1* Tn5 flanking sequences and sequences on the *R.meliloti* chromosome, pRZ2 and pRZ4 were also screened for similarity. As pRZ2 and pRZ4 contain a number of defined chemotaxis genes, similarity to any given pRZ2 or pRZ4 restriction enzyme fragment could result in the correlation to a specific *R.meliloti* chemotaxis gene.

Gradient purified pRZ2 and pRZ4 cosmid DNA was digested with *Eco* RI, the endonuclease which possesses the most useful number of sites in the cloned *R.meliloti fla-che* region, and hence best resolves the defined genes onto separate fragments. The digested DNA was electrophoresed through 0.8% agarose gels and transferred to nitrocellulose filters by Southern blotting. The blots were probed with either [α-^{32}P]-dCTP random primer labelled *mot-1* or *che-1* Tn5 flanking sequences under conditions of high stringency.

The *mot-1* Tn5 flanking sequence hybridized to the 11.5 Kb *Eco* RI fragment of pRZ4 (Fig.6.9.). This fragment contains the behavioural genes *che-1* , *che-2* , *che-3* and *mot-206* . Therefore, *mot-1* of *A.tumefaciens* may be similar to *mot-206* of *R.meliloti* . However, other as yet undefined *mot* genes could also be present on the

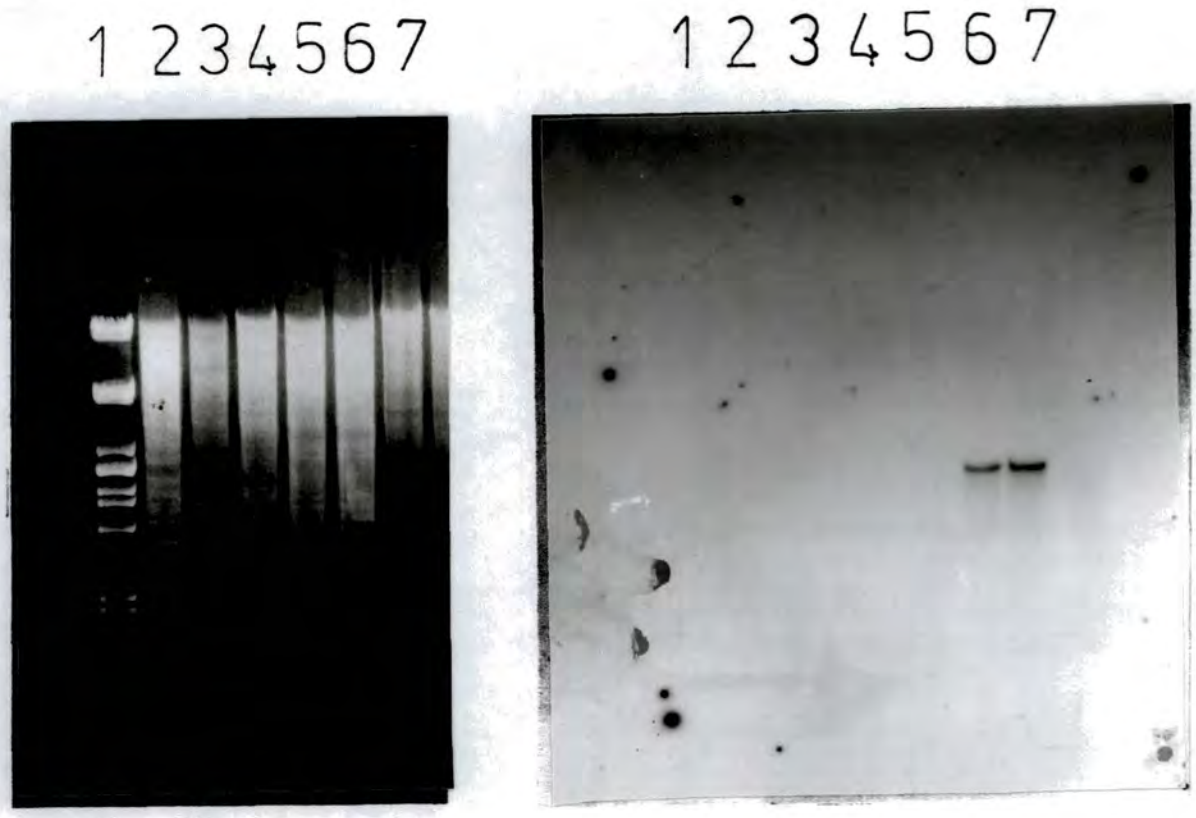


Fig.6.8. *A.tumefaciens* genes are not encoded by the cryptic plasmid. 0.8% agarose gel (left) of, Lane 2; *P.reactans* , Lane 3; *P.talassii* , Lane 4; *R.leguminosarum* , Lane 5; *R.leguminosarum* biovar *viciae* , Lane 6, *A.tumefaciens* GM19023, Lane 7; *A.tumefaciens* C58C*1*, genomic DNA digested with *Hind* III. Lane 1; contains λ DNA digested with *Pst* I. The DNA was Southern blotted and probed with [α-^{32}P]-dCTP labelled *mot-1* flanking sequences (right).

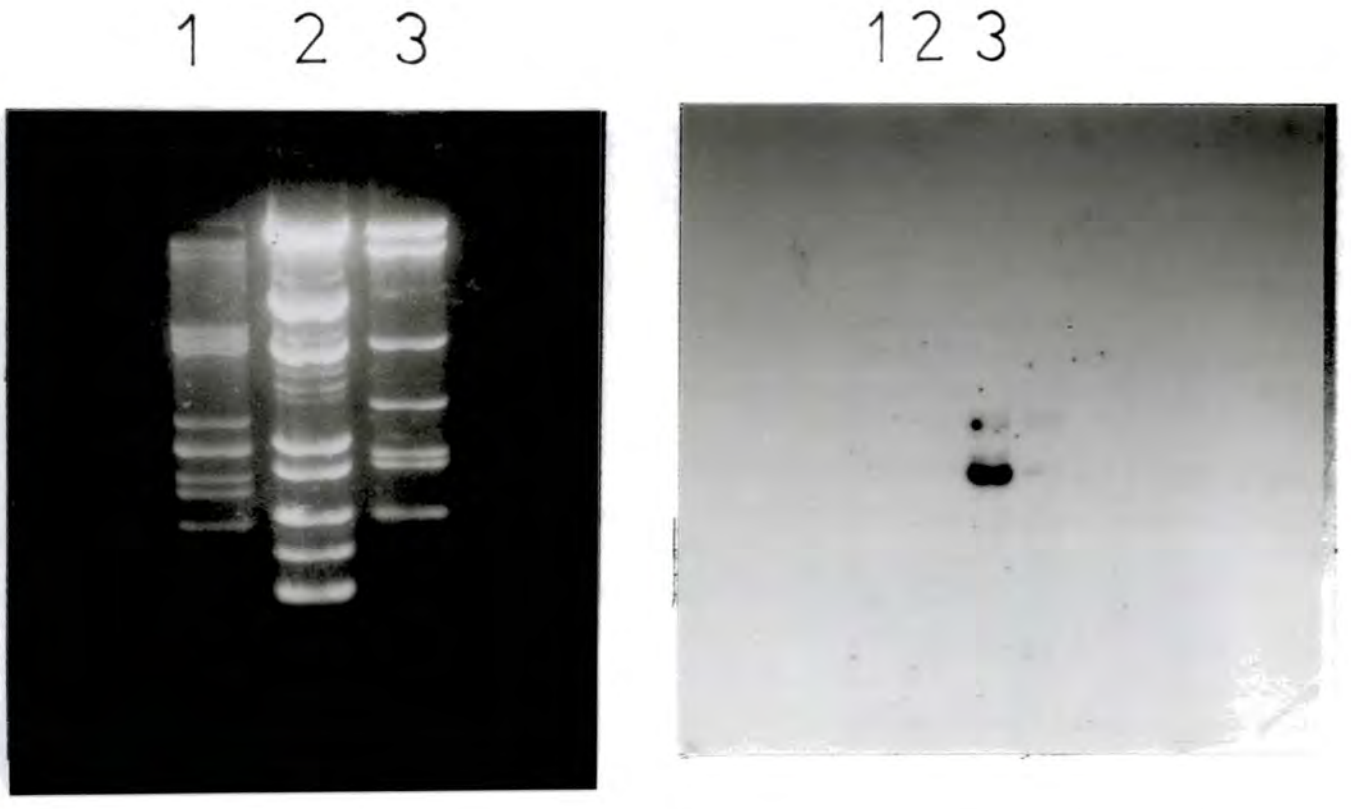


Fig.6.9. *A.tumefaciens mot-1* Tn5 flanking sequences show similarity to cloned *R.meliloti* behavioural genes. 0.8% agarose gel left of, Lane 1; λ DNA digested with *Pst* I. Lane 2; pRZ2, and Lane 3; pRZ4, both digested with *Eco* RI. The DNA was Southern blotted and probed with [α-^{32}P]-dCTP labelled *mot-1* Tn5 flanking sequences.

fragment. Complementation of *R.meliloti mot-206* with the wild-type *A.tumefaciens mot-1* clone would therefore be required to show conserved functionality.

The *che-1* flanking sequence hybridised to the 3.5-kb *Eco* RI fragment of pRZ4 (Fig.6.9.1.). Although no *R.meliloti* behavioural genes have been defined on this fragment as yet this result suggests that a *che* gene possessing similarity with *che-1* of *A.tumefaciens* may be present.

Neither flanking sequence showed any homology to pRZ2.

6.10. Isolation of wild-type *mot-1*

The C58C/ pLAFR3 gene library was screened for the presence of *mot-1* .

1024 cosmid clones were picked from the stored gene library and grown overnight on L-agar. Each clone was then patched onto a grid marked on a large square of nitrocellulose placed on a petri plate containing tetracycline L-agar and grown overnight. The resulting bacterial colonies were lysed and bound to the filter using the method of Grunstein and Hogness (255). The library was screened with 0.5μg of [α-^{32}P]-dCTP random primer labelled *mot-1* Tn5 flanking sequences.

A single positive clone was detected. This clone was picked and retested. Gradient purified recombinant pLAFR3 DNA was isolated from the positive clone and mapped for *Pst* I, *Eco* RI, *Hind* III and *Bam* HI sites (Fig.6.10.). The resulting fragments were transferred to nitrocellulose by Southern blotting and the blot probed with [α-^{32}P]-dCTP random primer labelled *mot-1* Tn5 flanking sequences (Fig.6.10.).

The wild-type *mot-1* gene was contained in an *A.tumefaciens* DNA insert of 23-kb. The cloned fragment had an abundance of *Pst* I sites indicative of coding regions. The *mot-1* Tn5 flanking sequence hybridized to 0.95-kb *Eco* RI, 1.9-kb *Pst* I, 7.1-kb *Bam* HI and 10.5-kb *Hind* III endonuclease fragments. This positive clone was termed pDUB1900.

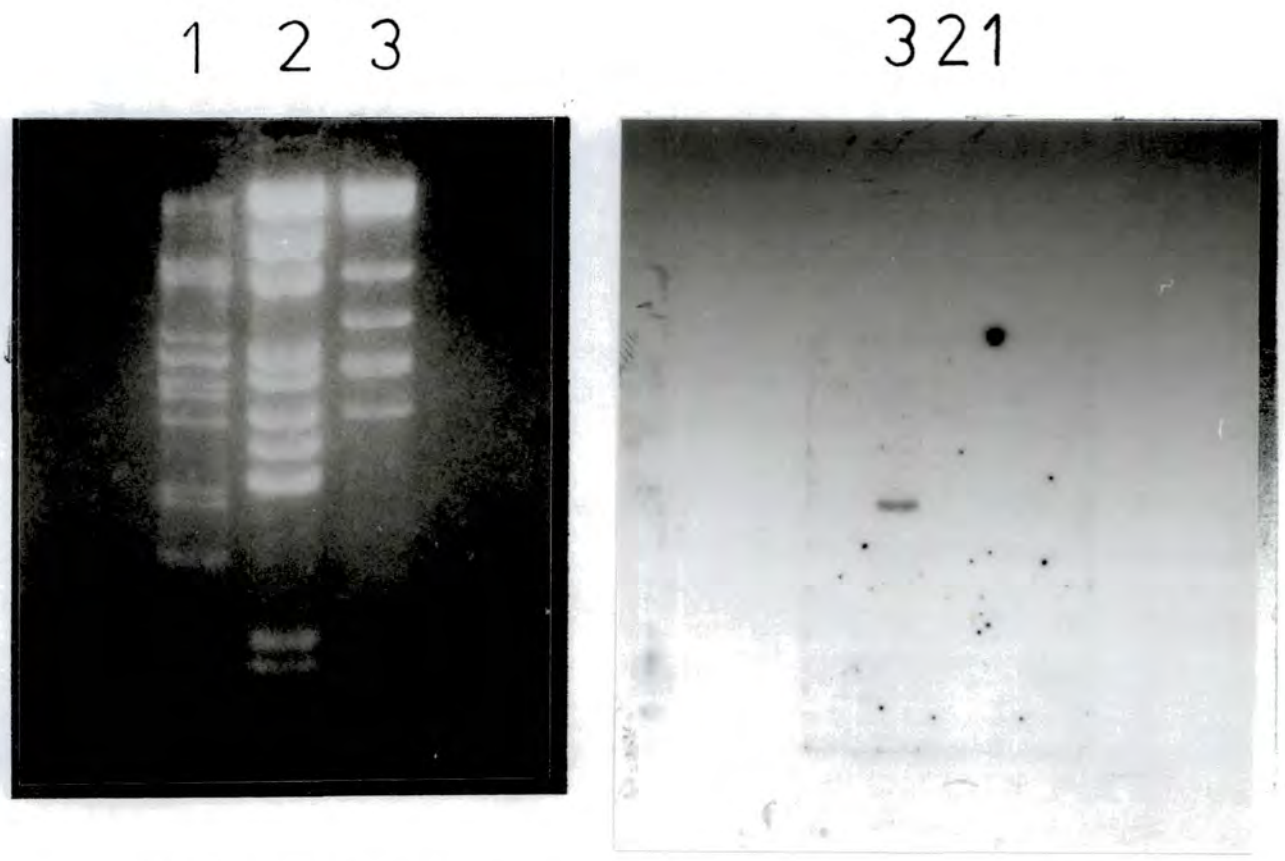


Fig.6.9.1. *A.tumefaciens che-1* Tn5 flanking sequences show similarity to cloned *R.meliloti* behavioural genes. 0.8% agarose gel (left) of, Lane 1; λ DNA digested with *Pst* I, Lane 2; pRZ2 and Lane 3; pRZ4, both digested with *Eco* RI. The DNA was Southern blotted and probed with [α-^{32}P]-dCTP labelled *che-1* Tn5 flanking sequences (right).

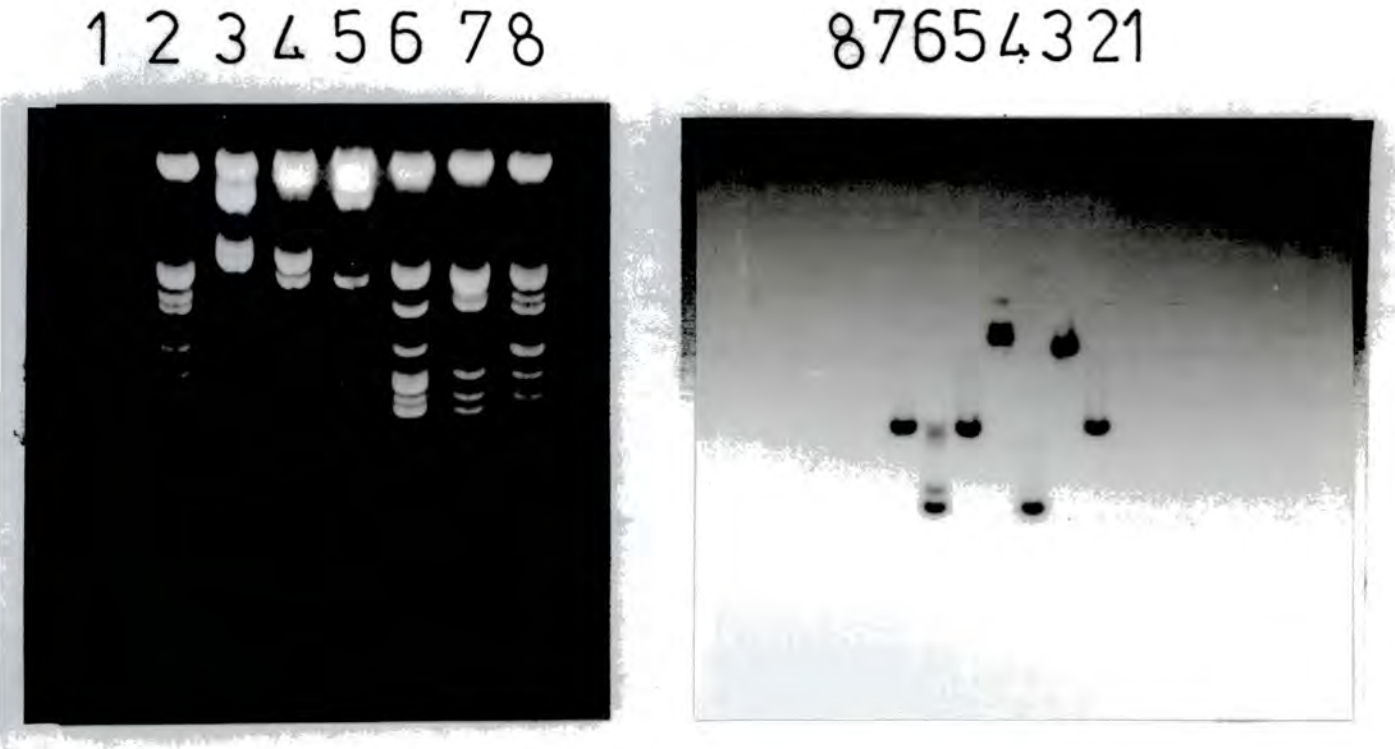


Fig.6.10. Restriction enzyme analysis of pDUB1900. 0.8% agarose gel (left) of, Lane 1, *Pst* I digested λ DNA; and pDUB1900 digested with, Lane 2, *Pst* I; Lane 3, *Bam* HI; Lane 4, *Eco* RI; Lane 5 *Hind* III; Lane 6, *Bam* HI and *Pst* I; Lane 7, *Eco* RI and *Pst* I; Lane 8, *Hind* III and *Pst* I. The DNA was Southern blotted and probed with [α-^{32}P]-dCTP labelled *mot-1* Tn5 flanking sequences.

6.11. Complementation of *A.tumefaciens mot-1* by pDUB1900

A triparental mating HB101/pDUB1900 x ED8767/pRK2013 x C58*C1-mot-1* mobilized pDUB1900 into C58*C1-mot-1* . Transconjugants were selected on rifampicin, kanamycin, tetracycline swarm agar. Rifampicin and kanamycin selecting for C58*C1* and tetracycline for the presence of pDUB1900. The transconjugants swarmed readily compared to the C58*C1-mot-1* controls which formed tight dense colonies. To confirm the transconjugants were *A.tumefaciens* and not contaminating *E.coli* the Bernaerts and Delay test was carried out. The result indicated that all the transformants were *A.tumefaciens* . Furthermore, cosmid DNA isolated from a number of randomly selected transconjugants transformed *E.coli* DH5α to tetracycline resistance at high efficiency.

Therefore, pDUB1900 can complement C58C*1-mot-1* to a wild-type motility and chemotaxis phenotype.

6.12. pDUB1900 complements other *A.tumefaciens* behavioural mutants

As *R.meliloti* behavioural genes map predominantly to a single region on the chromosome and *A.tumefaciens* and *R.meliloti* behavioural genes share extensive similarity, it was possible pDUB1900 encoded other behavioural genes. To assess this possibilty pDUB1900 was mobilized to the remaining *A.tumefaciens* behavioural mutants. Early indications suggest that pDUB1900 may complement a number of other behavioural mutants including; *fla* 1, 3, 5 and 6; and, *mot* 2, 4 and 5. However the Bernaerts and Delay test and the reisolation of pDUB1900 has not been done for any of the apparently complemented mutants. Therefore pDUB1900 appears to contain a behavioural gene cluster similar to that reported for *R.meliloti* .

6.13. Discussion

A.tumefaciens and *R.meliloti* are closely taxonomically related (32). However, their relationship has recently been shown to be more familial than expected.

The *A.tumefaciens* chromosomal virulence loci *chv A* , *chv B* and *psc A* are thought to be involved in the production of the exopolysaccharide β-1-2-glucan

(29,30,31). Such mutants are defective in plant cell attachment and are consequently avirulent.

Homologous sequences to *chv A* , *chv B* and *psc A* were found in the genomes of fast growing rhizobia including *R.trifolii* , *R.meliloti* , *R.leguminosarum* and *R.phaseoli* . These sequences corresponded to the genes *ndv A* , *ndv B* and *exo C* respectively (32,33), which are required for nodule developement on leguminous plants. Complementation experiments showed that the corresponding genes of *A.tumefaciens* and *R.meliloti* are functionally interchangeable (34). Therefore, the attachment process that is necessary for tumour formation by *Agrobacterium* and root nodule formation by *Rhizobium* has been conserved between the two rhizobacteria.

We have isolated a battery of Tn5 induced *A.tumefaciens* behavioural mutants. The Tn5 flanking sequences from two of these mutants *mot-1* and *che-1* were used as molecular probes to screen the genomes of a number of rhizobacteria for similar sequences. Even under conditions of high stringency similar sequences were found in *R.meliloti* . Furthermore, when the cosmids pRZ2 and pRZ4 were screened both *mot-1* and *che-1* Tn5 flanking sequences hybridized to pRZ4. *mot-1* flanking sequences hybridised to the 11.5-kb *Eco* RI fragment of pRZ4 containing the *R.meliloti* behavioural genes *che-1* , *che-2* , *che-3* and *mot-206* . Therefore, *mot-1* of *A.tumefaciens* may be similar to *mot-206* of *R.meliloti* . However, because of the large size of the fragment other *R.meliloti* *mot* genes may be subsequently defined. This problem could be resolved if pDUB1900 could complement *R.meliloti* *mot-206* to a wild-type motility phenotype.

Brown has shown that Tn5 flanking sequences of *fla-5* possess only weak similarity and *mot-4* flanking sequences no similarity at all to sequences present in the *R.meliloti* genome (unpublished data).

Therefore, while undoubtedly the chemotaxis systems of *R.meliloti* and *A.tumefacien* appear to be similar, there is also evidence for subtle differences between the two systems.

Chemotaxis in addition to plant cell attachment seems to be conserved between *A.tumefaciens* and *R.meliloti* . This may reflect a general conservation of genes involved in the plant-microbe ineraction between the two bacteria and perhaps between

rhizobacteria in general.

In enteric bacteria, *B.subtilus* and *R.meliloti* defined functionally diverse behavioural genes were found to be predominately clustered within the genome (289,239).

In *A.tumefaciens* the cosmid pDUB1900 appears to complement a number of behavioural mutants to wild-type motility and chemotaxis. Moreover, Brown has shown that the Tn5 flanking sequences from *fla-5* hybridised strongly to pDUB1900 (unpublished data). Therefore, like other bacteria, behavioural genes in *A.tumefaciens* seem to be clustered on the chromosome.

The isolation of the wild-type *mot-1* gene confirms the integrity of the *A.tumefaciens* cosmid gene library. Moreover, the behavioural genes *fla-2* , *mot-4* and *fla-5* and the cell separation gene *pc-1* have been subsequently isolated from the gene library by Brown, Deakin and Hall (unpublished data). Therefore, the library may serve as a repository for any gene present in the *A.tumefaciens* genome.

A comparison of the nucleotide sequence of the cloned *A.tumefaciens* behavioural genes with the behavioural genes from enteric bacteria may help shed light on the mechanism of chemotaxis and motility in *Agrobacterium* .

7.0 Summary

A range of sugars, many of them characteristic of plant extracts were investigated as potential chemoattractants for *A.tumefaciens* .

A massive and extremly sensitive chemotaxis response was recorded with sucrose, the most abundant plant sugar (265). Chemotaxis towards other carbohydrates resolved four groups of sugars: chemoattractants with peaks at 10^{-6}M (sucrose, glucose and fructose); 10^{-5}M (maltose,lactulose and galactose); 10^{-4}M (raffinose, stachyose and arabinose); and weak or non-attractants (palatinose, lactose, cellobiose and xylose). In descending order the magnitude of the responses were: sucrose >> maltose > lactulose > glucose > galactose/fructose > stachyose/arabinose > raffinose.

In addition to sugars the amino acids valine and arginine were also good attractants with peaks at 10^{-3}M. However, no significant chemotaxis was recorded with alanine, cysteine, methionine or glycine.

In general, the oligosaccharides were stronger attractants than their component monosaccharides. This could be due to the nature of the receptors involved. Alternatively, it could be explained by synergistic responses produced by the degradation of oligosaccharides in the periplasm. In the case of sucrose there is some evidence to support this hypothesis. The failure of *A.tumefaciens* to metabolize other oligosaccharides in the periplasm may account for their behaviour as non-attractants.

As many of these carbohydrates are characteristic of plant extracts, carbohydrate taxis may be involved in attracting *Agrobacterium* to the vicinity of plants, possibly explaining their abundance in the rhizosphere (2,261).

A study of motility in *A.tumefaciens* suggested similarities to *R.meliloti* (237)

rather than *E.coli* . Long runs with few tumbles were the norm. Moreover, bacteria close to the underside of the coverslip turned consistently CW, while those adjacent to the surface of the glass slide moved CCW. These observations suggested that motility in *Agrobacterium* is mediated by unidirectional CW rotation of right handed helical flagella as described for *R.meliloti* (237,264) but not enteric bacteria.

In order to initiate a molecular approach to the study of chemotaxis in *A.tumefaciens* a battery of behavioural mutants was generated using the suicide vehicle pJB4JI (301). This vehicle was the most efficient tested with a transconjugant frequency of 10^{-6}.

A total of 22 mutant candidates were isolated using the miniswarm techninique at a frequency of 1.8 x 10^{-2} per mutagenized bacterium. Two mutants were found to possess metabolic defects and were therefore discarded. Southern blotting revealed that all but two of the putative behavioural mutants were unique.

A detailed phenotypic analysis of all 20 mutants was undertaken. This investigation identified a possible 7 non-motile (*mot*), 7 non-flagellated (*fla*) and 5 non-chemotactic (*che*) mutants. One putative *che* mutant was deficient in cell separation and was termed *pc-1* .

The virulence of all 20 behavioural mutants was tested using conventional assays. None of the mutants were found to be avirulent or attenuated in virulence. However, due to the nature of the assay the role of chemotaxis and motility in virulence (if any) may have been circumvented. Therefore, a more natural assay must be undertaken before motility and chemotaxis can be considered unimportant in virulence.

Using the behavioural mutants *che-5* and *mot-1* the role of chemotaxis and motility in root colonization was assessed.

The results indicated that *mot-1* but not *che-5* showed reduced colonizing efficiency compared to wildtype C58C*1*. Therefore, it appears that motility but not chemotaxis may be important in efficient root colonization by *Agrobacterium* .

This observation could be important in the design of bacterial delivery systems to protect the root against deleterious organisms. For effective biocontrol the vector must colonize the whole root. Therefore, highly motile bacterial vectors would be desirable for use in micro-inoculants.

One behavioural mutant *che-1* was especially interesting, this mutant was motile but tumbled incessantly. Similar mutants have been described for enteric bacteria. Such mutants were found to be aberrant in MCP methylation. Therefore, *che-1* was used to facilitate an investigation into the biochemistry of chemoreception in *Agrobacterium* .

Methionine auxotrophs when grown under starvation conditions for methionine were found to be motile but non-chemotactic towards sucrose. However, a detectable tactic response could be restored by supplementation with 10^{-5}M methionine. Other amino acid auxotrophs starved for their respective amino acids still remained chemotactic.

The loss of taxis in methionine depleted cells was not due to an effect on protein synthesis but was found to be an absolute requirement for methionine in the chemotactic response. Using methionine analogues, this methionine requirement, was found to be more specifically a requirement for S-adenosyl methioine. In bacteria which possess an MCP system, it is S-adenosyl methionine which provides methyl groups for MCP methylation. A reduction in cellular S-adenosyl methionine, mediated by methionine starvation, results in loss of taxis in these bacteria. These results suggested

that *A.tumefaciens* like enteric bacteria may contain a MCP system.

Because of the exaggerated tumbly phenotype of C58C*1* *che-1* this mutant was compared with wild-type C58C*1* in *in vivo* MCP labelling experiments. *che-1* showed significantly reduced MCP methylation compared to wild-type. This result was slightly surprising. In enteric bacteria tumbly mutants show enhanced MCP methylation and are defective in che B. It is the smooth swimming *che R* mutants that show reduced methylation. Therefore, adaptation in *Agrobacterium* seems to be significantly different from enteric bacteria and more closely resembles that described for *B.subtilus* .

However, reduced MCP methylation can be explained by a number of factors: slow methyl group turnover, lack of available methylation sites and rapid hydrolysis. Therefore, the *che-1* mutation could provoke one of the above. Further studies on the biochemistry of chemoreception will be required to resolve this issue.

Due to the insertion inactivation nature of transposons the generated behavioural mutations were effectively tagged with Tn5. Moreover, the kanamycin resistance determinant encoded by Tn5 provided a positive selectable marker which facilitated the recovery of six mutated behavioural genes.

Isolated Tn5 flanking sequences from *mot-1* and *che-1* were found to possess strong similarity to sequences present in the *R.meliloti* genome. These sequences were found to reside on a cosmid clone pRZ4 (239) containing a number of *R.meliloti* behavioural genes. *mot-1* flanking sequences hybridized to a 11.5 kb *Eco* RI fragment of pRZ4 which contains *che-1* , *che-2* , *che-3* and *mot-206* . Therefore, *mot-1* of *A.tumefaciens* may correspond to *mot-206* of *R.meliloti* . Complementation experiments will be required to demonstrate conserved functionality.

che-1 flanking sequences hybridized to the 3.5 kb *Eco* RI fragment of pRZ4. No behavioural genes have been defined on this fragment as yet. However, this result suggests that an *R.meliloti* *che* gene with similarity to *che-1* may be present.

Brown has shown (unpublished data) that *fla-5* Tn5 flanking sequences show only very weak similarity and *mot-4* Tn5 flanking sequences no detectable similarity to *R.meliloti* genomic sequences. Therefore, while undoubtedly the chemotaxis systems of *A.tumefaciens* and *R.meliloti* are similar they must also be subtly different.

The wild-type *mot-1* gene was isolated from an *A.tumefaciens* cosmid gene library. The 23 kb cosmid clone (pDUB1900) was found to complement a number of other behavioural mutants in addition to *mot-1* . Therefore, it seems *A.tumefaciens* behavioural genes, like those of other bacteria are clustered on the chromosome.

This thesis has laid the ground work for an in depth study of chemotaxis in *A.tumefaciens* .

A system of transposon mutagenesis has been linked to a mini-swarm technique allowing direct screening for mutants post mutagenesis. To date 20 unique behavioural mutants have been identified. Moreover, a rapid recovery of Tn5 tagged behavioural genes has been optimized. Coupled with a C58C*1* cosmid library a system has been created which provides rapid access to any defined behavioural gene.

As pDUB1900 has been shown to complement a number of other behavioural mutants, saturation Tn5 mutagenesis of pDUB1900 may provide an alternative strategy to the above. Moreover, the use of insertion mutagens such as Tn3HoHo or Tn*phoA* may provide information on the transcriptional regulation of defined behavioural genes, or whether their gene products are located in the periplasm. Fur-

ther, the sequencing of *A.tumefaciens* behavioural genes, and a comparison of their nucleotide sequence with behavioural genes from other bacteria may help shed light on the mechanism of chemotaxis in a rhizobacteria.

Further into the future *in vitro* transcription/translation systems should facilitate an analysis of the proteins and protein-protein interactions mediating chemoreception and signal transduction in *Agrobacterium* . This would not only provide an alternative model system to that of enteric bacteria, but also possibly receptors with extremly low Kms which may be useful biotechnologically.

Therefore, there is now a myriad of potential projects to study chemotaxis, where for the first time, the process plays a defined role in the life cycle of the bacterium.

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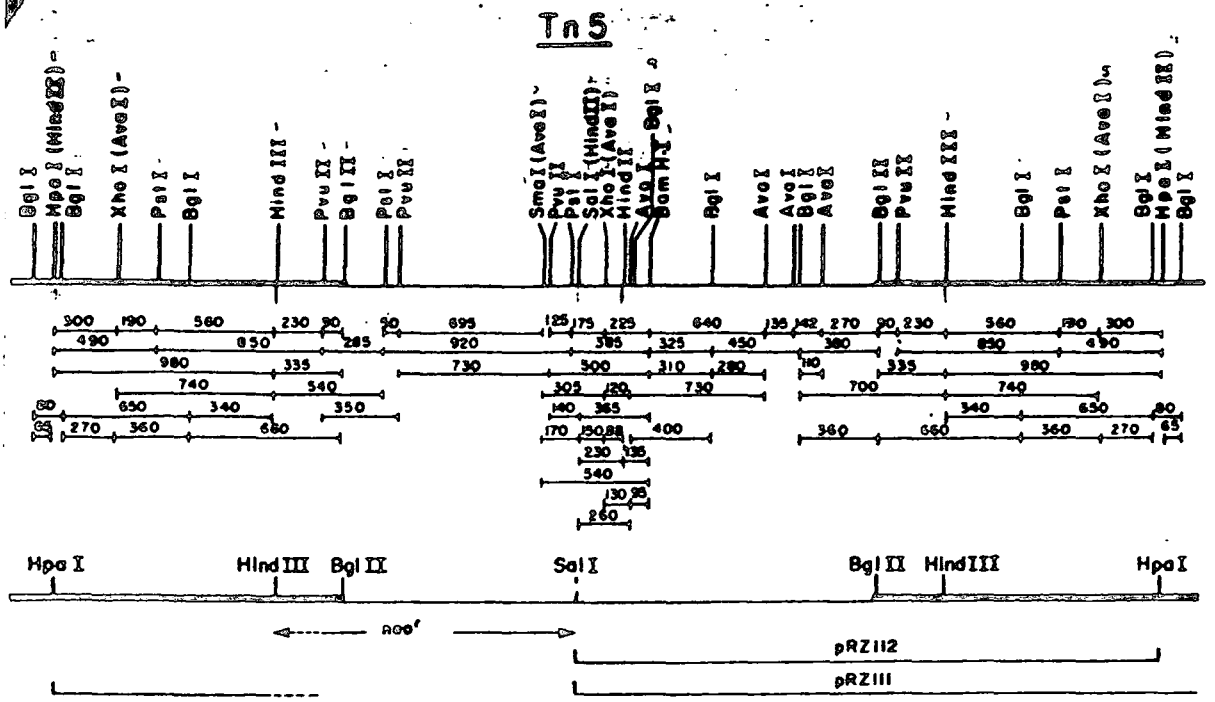

Fig. 3. Cleavage map of Tn*5*. Inverted repeats are indicated by the heavy lines. Distances in base pairs between cleavage sites are indicated by the numbers. The map has been simplified below. Indicated by the horizontal lines are the Tn*5* sequences removed in the construction of pRZ112 and pRZ111 as well as the neo^r coding region defined by these deletions

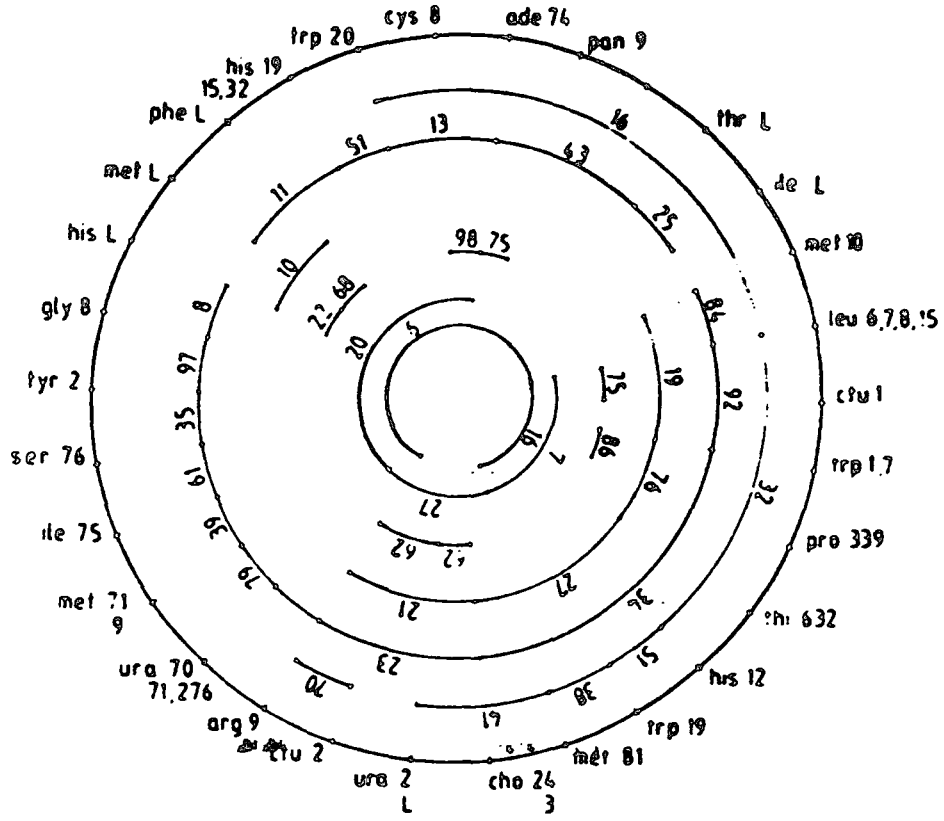

Fig. 1. The chromosomal map of *A. tumefaciens* C58 constructed using the data in Bryan et al. (1982) and in Tables 2 and 4. The numbers below the circle indicate the coinheritance (%) values used to draw the map. Existence of linkage between the markers used for (donor) counterselection and selection, information obtained from tests for allelism and results obtained from incomplete crosses (data not shown) were used in selecting the individual coinheritance frequencies where contradictory values were obtained: *trp* 1013 occupies the same locus as *trp*1