Isolation and characterization of behavioural mutants and genes of *Agrobacterium tumefaciens*

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Agrobacterium tumefaciens exhibits an unusual flagellation pattern in the electron microscope. The typical pattern is of two flagella in a polar or sub-polar tuft at one pole only, plus one or two lateral flagella on each side. Twenty independent behavioural mutants were isolated, after Tn5 mutagenesis, and divided into several classes. Fourteen non-motile mutants were differentiable into non-flagellate and flagellate subclasses, one non-chemotactic mutant appeared to have a defect in the intracellular signalling pathway, and one other mutant displayed an unusual pattern of polar cell pairing. Four mutants with impaired swarming ability were also isolated. Root colonization ability was impaired in the non-motile mutant for which this property was tested. From all but three of the mutants, the Tn5 insertion sites, including flanking sequences, have been cloned and used as probes to isolate a set of seven cosmids from a wild-type *A. tumefaciens* library. No hybridization to *Escherichia coli* or *Pseudomonas* DNA was detected for the *A. tumefaciens* mutant flanking sequences, but varying degrees of similarity to *Rhizobium meliloti* behavioural genes were detected. By complementation of *A. tumefaciens* behavioural mutants and hybridization of mutant flanking sequences, one cosmid insert was shown to contain at least nine behavioural loci, and another, linked to it on the chromosome, at least three. This suggests that, as in other organisms, behavioural genes are clustered in the *A. tumefaciens* genome.

Introduction

Bacteria exhibit behaviour, in their responses to external stimuli. The best-characterized of these is chemotaxis, the response to a temporal gradient of attractant or repellent (Bourret *et al.*, 1989; Macnab, 1987*b*; Stewart & Dahlquist 1987; Stock *et al.*, 1990; Taylor & Lengeler 1990). For chemotaxis, the organism must be motile. In the enteric bacteria, it is estimated that approximately 60 genes contribute to this phenomenon, although the bulk are concerned with flagellar structure (Macnab, 1987*a*). Evidence is accumulating to suggest that chemotaxis play a major role in the ecology of many bacteria. For instance, non-motile *Rhizobium* mutants are less competitive nodulators than their wild-type parents (Ames & Bergman, 1981), and non-chemotactic *Agrobacterium* mutants are impaired in virulence under certain conditions (Hawes & Smith, 1989; Hawes *et al.*, 1988).

Crown gall occurs on plants after infection of wounded tissue by *Agrobacterium tumefaciens* (Howard & Citovsky, 1990; Zambryski *et al.*, 1989). Laboratory experiments have indicated that chemotaxis towards plant exudates is an initial step in this interaction (Ashby

et al., 1987, 1988; Loake *et al.*, 1988; Shaw *et al.*, 1988*a*). It is therefore of importance to investigate motility and chemotaxis in this organism. In this paper, we describe the creation of Tn5-induced mutants defective in chemotaxis and motility, and the isolation and partial characterization of their corresponding genes.

Methods

Bacterial strains and growth media. The parent strain used to create the behavioural mutants was the highly motile (Loake *et al.*, 1988) strain *A. tumefaciens* C58C1 Rif^r (van Larebeke *et al.*, 1974) periodically enriched for motile bacteria by passage through swarm plates containing 0.2% agar (Loake *et al.*, 1988). The construction of *A. tumefaciens* C58C1(pGV2201) carrying a virulent nopaline Ti-plasmid, with a streptomycin resistance gene inserted into the right end of the T-DNA, was described previously (Leemans *et al.*, 1981). *Escherichia coli* 1830(pJB4J1) and pLAFR3 (Staskawicz *et al.*, 1987) were obtained from Dr M. Daniels (Sainsbury Laboratory, Norwich, UK). *A. tumefaciens* GM19023 (Rosenberg & Huguet, 1984) cured of its Ti- and cryptic plasmids was obtained from Dr J. Denarie (Toulouse, France). *Rhizobium meliloti* and the cosmids pRZ2 and pRZ4 (Ziegler *et al.*, 1986), carrying *Rhizobium* behavioural genes, were obtained from Dr K. Bergman (Boston, MA, USA). *R. leguminosarum* biovars *viciae* and *phaseoli* were obtained from Professor A. Johnston (Norwich).

Pseudomonas talassii and *P. reactans* were obtained from Dr A. M. Ashby (Cambridge, UK). Bacterial cultures were grown in either *Rhizobium* Initiation Medium (RIM) (Aguilar *et al.*, 1988), L-broth or Minimal A (MinA) supplemented with glucose (Miller, 1972; Shaw *et al.*, 1988a) and after centrifugation, washed and resuspended in chemotaxis medium (CM) (Adler, 1973; Ashby *et al.*, 1987, 1988). Antibiotic concentrations and all conjugations were as previously described (Leemans *et al.*, 1981; Shaw *et al.*, 1988a).

Chemotaxis assays. The capillary assay (Adler, 1973) was employed, using a 60 min incubation time. Bacteria attracted into capillaries were plated out onto selective media (Ashby *et al.*, 1987, 1988). Alternatively, blindwell assays (Shaw *et al.*, 1988a) in triplicate were performed, the bacterial number attracted into the attractant chamber being measured in a Coulter counter.

Microscopy. After washing and resuspension in chemotaxis medium, motility of each strain was checked using a Nikon Optiphot microscope utilizing phase-contrast optics, and a Hitachi HV-720K CCTV camera. Behaviour and speed measurements were made during slow-motion playback of recorded motility, using a Ferguson 3V23 video recorder, a Panasonic WJ-810 time-date generator and a Hitachi VM-920K video monitor.

Electron microscopy. Samples (20 μ l) of bacteria were allowed to air-dry onto Formvar-coated grids, then placed upside down onto 50 μ l drops of saturated uranyl acetate for 30 s, washed by placing on several successive drops of distilled water, and air dried. Grids were observed in a Philips EM400, and photographed.

Isolation of chromosomal DNA. DNA was isolated using a scaled-up version of the mini-prep method for *Agrobacterium* chromosomal DNA (Dhaese *et al.*, 1979). The DNA was subsequently purified by centrifugation in a caesium chloride gradient, dialysed and stored at -20°C .

DNA manipulation. Isolation of plasmid DNA, restriction enzyme digestions, vector phosphorylations, ligations, transformation of *E. coli*, and screening for recombinants were done by established protocols (Maniatis *et al.*, 1982). To isolate each Tn5 insertion site, 28 μ g of *Eco*RI-cleaved chromosomal DNA from each of the behavioural mutants was ligated to 4 μ g of similarly-cut pUC18 or 19 and transformed into *E. coli* DH5 α , with selection for ampicillin and kanamycin resistance. The wild-type *A. tumefaciens* C58C1 library was constructed in pLAFR3. Total DNA from *A. tumefaciens* C58C1 (cured of its Ti-plasmid) was partially digested with *Bam*HI, and 20–25 kb fragments size-selected. These were ligated to *Bam*HI-cut pLAFR3 arms, packaged *in vitro* into phage heads, and introduced into *E. coli* HB101. Each of the cosmid clones was maintained separately, the library consisting of 1200 separate clones, which is in excess of the number of clones (820) required for 99% probability of finding any particular gene.

Southern blotting. Probes were labelled using the Amersham Multi-prime kit. Restriction enzyme digests of bacterial DNA were separated by electrophoresis through 0.8% agarose gels, and blotted onto nitrocellulose or nylon. Hybridization was performed at 65°C in $6 \times \text{SSC}$, and the blots washed at 65°C in $1 \times \text{SSC}$.

Pulsed field gel electrophoresis. Bacteria were immobilized, lysed and their DNA restriction enzyme digested in 1% (w/v) low-melting-point agarose plugs (Flanagan *et al.*, 1989). Plugs were placed in the wells of a 1.5% agarose gel, and DNA separated on a Bio-Rad CHEF DRII orthogonal-field electrophoresis apparatus, using a pulse time of 7 s at 150 V for 20 h.

Virulence assays. Two-week-old sunflower seedlings were inoculated with 20 μ l of an overnight culture of bacteria, grown for 2–3 weeks in a 1:1 peat/vermiculite mixture, and monitored for tumour growth.

Root colonization. The assay used was based on a published protocol (de Weger *et al.*, 1987). Two-week-old potato plantlets, with 2–3 cm roots, were dipped into overnight bacterial cultures, and planted in soil in PVC tubes resting on a bed of vermiculite. After two weeks of further growth, during which time the plants were kept moist by watering the vermiculite bed, they were removed from the soil, and their roots cut into 1 cm sections. Ten sections from each root position were disrupted by agitation with fine glass beads, and the resultant slurry diluted and plated onto appropriate agar.

Results

Motility and flagellation in *A. tumefaciens*

Motility and flagellation in *A. tumefaciens* has not been extensively studied. Thus, it was crucial to establish the pattern of flagellation and motility of the wild-type parent, *A. tumefaciens* C58C1, prior to mutagenesis. We have previously reported that this organism has a slightly unusual manner of motility, more reminiscent of *Rhizobium* than *E. coli* (Loake *et al.*, 1988). Thus *A. tumefaciens* exhibits long runs with few tumbles, and appears to move by unidirectional clockwise flagellar rotation (Loake *et al.*, 1988).

When observed by electron microscopy (see Methods) *A. tumefaciens* displays a slightly unusual flagellation pattern (Fig. 1, Fig. 2, top left). The typical pattern is of a polar or slightly sub-polar tuft of two flagella, at only one pole, plus one or two lateral flagella on each side, amounting to a total of four to six flagella. It is not apparent whether the lateral flagella are symmetrically arranged or not. This does suggest a polarity of the cell, as far as flagellation is concerned. However, all the flagella display the sinuous curves typical of a helical filament. Higher magnification of the flagellar filament reveals it to be approximately 10–12 nm in diameter.

Mutagenesis and selection of behavioural mutants

Preliminary chemical mutagenesis with ethyl methane-sulphonate revealed that mutants impaired in swarming ability could be isolated from *A. tumefaciens* at a frequency of 6×10^{-4} (data not shown). Experiments using a number of Tn5 suicide vectors demonstrated that pJB4JI had the highest transfer frequency from *E. coli* to *A. tumefaciens*. *E. coli* 1830(pJB4JI) was conjugated with *A. tumefaciens* C58C1 Rif^r, and plated onto L-agar containing rifampicin and kanamycin. Transconjugants, isolated at a frequency of 10^{-6} , were screened for behavioural defects in L-swarm agar, containing rifampicin, kanamycin and 0.2% agar.

Twenty-two putative mutants, showing impaired swarming ability, were picked, subcultured twice, and retested. These mutants were isolated at a frequency of

Fig. 1. Electron micrograph of *A. tumefaciens* C58C1, stained with uranyl acetate. The cell body is approximately 1.2 μm in length.

Table 1. *Classes of behavioural mutants*

Class	Subclass	Features	Mutant no.	Gene locus
Non-motile	Possess flagella	Normal flagella*	5	<i>mot-5</i>
			14	<i>mot-14</i>
		Abnormal flagella*	1	<i>mot-1</i>
			4	<i>mot-4</i>
			6	<i>mot-6</i>
			9	<i>mot-9</i>
		No polar tuft	12	<i>mot-12</i>
	No visible flagella		3	<i>fla-3</i>
		7	<i>fla-7</i>	
		8	<i>fla-8</i>	
		10	<i>fla-10</i>	
		11	<i>fla-11</i>	
		15	<i>fla-15</i>	
		19	<i>fla-19</i>	
Motile	Non-chemotactic	Tumbles incessantly	2	<i>che-2</i>
	Tandemly paired cells	Poor motility	17	<i>tpc-17</i>
	Small swarms		18	<i>ssw-18</i>
			20	<i>ssw-20</i>
			21	<i>ssw-21</i>
			22	<i>ssw-22</i>

* As judged from electron micrographs.

1.8×10^{-2} per kanamycin-resistant transconjugant, which is within the expected range for a complex phenotype encoded by approximately 1% of the total genome. All of the mutants were sensitive to the antibiotics whose resistance is encoded by pJB4JI (except kanamycin) and could not retransfer their kanamycin resistance. This suggests that the mutations are due to insertion of Tn5 into the *A. tumefaciens* genome.

To confirm that impaired swarming ability was not due to a metabolic defect, growth in culture of the 22 putative mutants was assessed. All but two of the candidates (nos 13 and 16) grew at a similar rate to their wild-type parent, and these two were thus discarded. Of the remaining 20, 15 appeared to be incapable of swarming, and five produced intermediate-sized swarms.

Phenotypic analysis of behavioural mutants

The putative behavioural mutants were subjected to further analysis by phase-contrast and electron microscopy (see Methods; Table 1). The former confirmed that 14 of the non-swarming mutants were non-motile. One of them, however, (no. 2), proved to be motile, but to tumble excessively, and was thus categorized as a presumptive non-chemotactic mutant (*che-2*). This mutant has a defect in the intracellular signalling pathway,

showing reduced methylation of methyl-accepting chemotaxis proteins (MCPs) (A.P.B., G.J.L. & C.H.S., unpublished).

Phase-contrast microscopy demonstrated a number of features of the intermediate-swarming mutants (Table 1). Mutant no. 17 has a strange phenotype, typified by the appearance of tandemly paired cells, apparently attached at the poles. The attachment appears strong, but extracellular, as both cells can clearly be distinguished in outline. The mutant grows normally, and does not produce filaments or arrays of more than two cells. Thus, it is unlikely to have a defect in cell division or separation. The slow swarming can be attributed to the fact that the tandem pairs swim more slowly and appear more clumsy in their movement than the wild-type. This mutant has been designated *tpc-17* (tandem paired cells). The remaining intermediate swarmer mutants appear indistinguishable from the wild-type in their motility pattern, and chemotaxis in blindwell assays to a complex attractant mixture (data not shown). To denote the uncertainty about their lesion, these mutants have been designated *ssw-18*, 20–22 (small swarms).

Electron microscopy (Figs 2–4) has allowed the division of the 14 non-motile mutants into two subclasses (Table 1): those possessing flagella, which presumably are non-functional in some respect (designated *mot-1*, 4, 5, 6, 9, 12 and 14), and those without observable flagella (designated *fla-3*, 7, 8, 10, 11, 15 and 19). The other

Fig. 2. Electron micrographs of non-motile *A. tumefaciens* C58C1 mutants. Top row: wild-type, and mutants 12 (*mot-12*), 5 (*mot-5*) and 9 (*mot-9*). Bottom row: mutants 14 (*mot-14*), 4 (*mot-4*), 6 (*mot-6*) and 1 (*mot-1*). Bars, 1 μ m.

Fig. 3. Electron micrographs of non-flagellate *A. tumefaciens* C58C1 mutants. Top row: mutants 3 (*fla-3*), 7 (*fla-7*), 8 (*fla-8*) and 10 (*fla-10*). Bottom row: mutants 11 (*fla-11*), 15 (*fla-15*) and 19 (*fla-19*). Bars, 1 μ m.

Fig. 4. Electron micrographs of non-chemotactic and 'small swarming' *A. tumefaciens* C58C1 mutants. Top row: mutants 2 (*che-2*), 17 (*tpc-17*) and 18 (*ssw-18*). Bottom row: mutants 20 (*ssw-20*), 21 (*ssw-21*) and 22 (*ssw-22*). Bars, 1 μm.

mutants, *che-2* and *ssw-*, have flagellar structures indistinguishable from the wild-type (Table 1, Fig. 4).

Biology of the behavioural mutants

To assess the effect of the various mutations upon virulence of *A. tumefaciens*, the Ti-plasmid pGV2201 was conjugated into each of the mutants. When inoculated onto sunflower seedlings (see Methods), with *A. tumefaciens* C58C1 and *A. tumefaciens* C58C1(pGV2201) as controls, the 20 mutants all formed rough tumours indistinguishable from those induced by the wild-type parent harbouring the same Ti-plasmid (data not shown). This result is in agreement with those of Hawes *et al.* (1988), that these types of mutations have no effect on tumorigenicity when large inocula are introduced directly into a wound site. However, we have not tested our mutants for their ability to induce tumours when introduced into the soil surrounding wound sites (Hawes & Smith, 1989).

As a test of the rhizosphere competence of the mutants, two-week-old potato seedlings, with 2–3 cm roots, were dipped into overnight cultures of two mutants (*mot-1* and *ssw-22*) and the wild-type parent, and grown for a further two weeks in conditions of upward capillary watering (see Methods). The numbers of bacteria colonizing defined sections of the roots were determined (Table 2). The wild-type parent and *ssw-22* appeared to be able to colonize all sections of the root analysed. However, *mot-1*, while appearing in similar numbers in the upper parts of the root, seemed to be incapable of colonizing the newly-grown lower reaches, against the flow of water. This is good evidence in favour of motility as a determinant of rhizosphere competence in *A. tumefaciens*.

Genotypes of the behavioural mutants

To assess whether the 20 behavioural mutants were independent in origin, and resulted from a single Tn5 insertion, chromosomal DNA was purified from each, and digested with *EcoRI* (see Methods). The resulting fragments were separated on a 0.8% agarose gel, blotted to nitrocellulose and probed with a radioactively labelled internal *BglII* fragment of Tn5. The results for nine of the mutants are shown in Fig. 5. In each of the mutants analysed, there was a single hybridizing *EcoRI* fragment, ranging from 6.7 to 15 kb. This is to be expected, as Tn5 lacks *EcoRI* sites, so the bands picked out by this analysis represent a genomic *EcoRI* fragment with a Tn5 insert. In most of the cases, the Tn5-containing fragments were of different sizes, but in mutants *mot-4*, *mot-9* and *fla-10* (tracks 3, 8 and 9) they were of the same size. Subsequent

Fig. 5. Hybridization of Tn5 to chromosomal DNA from *A. tumefaciens* mutants. Chromosomal DNA from mutants 2–10 was digested with *EcoRI*, separated on a 0.8% agarose gel, transferred to nitrocellulose, and probed with a ^{32}P -labelled 1.1 kb internal *BglII* Tn5 fragment. Tracks: 1, *che-2*; 2, *fla-3*; 3, *mot-4*; 4, *mot-5*; 5, *mot-6*; 6, *fla-7*; 7, *fla-8*; 8, *mot-9*; 9, *fla-10*. Arrows indicate the sizes of the largest (15 kb) and smallest (6.7 kb) fragments containing Tn5.

Table 2. Root colonization assay

Values represent the mean of 10 replicates of kanamycin and rifampicin doubly resistant (*mot-1* and *ssw-22*) and rifampicin resistant (C58C1) bacteria recovered from root sections.

Strain	No. of bacteria		
	Distance along root (cm):		
	0–1	3–4	6–7
C58C1	1200	1080	511
<i>ssw-22</i>	900	892	713
<i>mot-1</i>	1400	909	19

analysis has demonstrated that these three mutants are due to three separate and distinct Tn5 insertions into the same 9.3 kb *EcoRI* fragment (see below), suggesting that the mutated genes are clustered on the *A. tumefaciens* genome. Similar results, with some common and some distinct fragments carrying Tn5, were observed for the remaining mutants (data not shown). This analysis has helped to demonstrate that all of the mutants are due to separate, single Tn5 insertion events.

Isolation of Tn5-insertion sites and behavioural gene cosmids

The Tn5 insertion site plus flanking sequences from each *A. tumefaciens* behavioural mutant (except *ssw-20-22*) was cloned into pUC18/19 (Table 3), making use of the

fact that Tn5 has no *Eco*RI sites (Jorgensen *et al.*, 1979). Once characterized, the mutant flanking sequences (i.e. those sequences flanking Tn5) were excised by cleavage with *Eco*RI and *Hpa*I, the latter enzyme having two sites in Tn5, 185 bp from each end (Auerswald *et al.*, 1981; Jorgensen *et al.*, 1979). These flanking sequences were used as hybridization probes, to identify their corresponding gene in a cosmid library of wild-type *A. tumefaciens* DNA in pLAFR3 (Staskawicz *et al.*, 1987) (see Table 3 and below).

A. tumefaciens behavioural genes map to the chromosome

The behavioural mutants were created in a strain of *A. tumefaciens* C58C1 cured of its Ti-plasmid (van Larebeke *et al.*, 1974). However, it still possesses the cryptic plasmid pAtC58, and it was possible that the mutations would map to this plasmid. To test this hypothesis, flanking sequences from *mot-1* (pDUB1801) and *mot-6* (pDUB1806) were hybridized against digests of total DNA from *A. tumefaciens* C58C1 and *A. tumefaciens* GM19023, which lacks the cryptic plasmid pAtC58 (Rosenberg & Huguët, 1984). As shown in Fig. 6, both probes hybridized to identical single fragments in the DNA, regardless of the presence of the cryptic plasmid. This is good evidence in favour of the Tn5 insertions in these mutants being in chromosomal genes.

Further evidence in support of this contention was provided by pulsed-field gel electrophoresis. Total DNA from four mutants, *che-2*, *fla-3*, *mot-4* and *mot-6*, was digested with *Dra*I, separated on a 1.5% agarose gel using a CHEF-DR11 orthogonal-field electrophoresis system (see Methods) and then probed with Tn5. As shown in Fig. 7, Tn5 hybridized to a 40–45 kb fragment in each mutant DNA. While it is possible that there is more than one fragment of that size in this region of the gel, mapping of the *A. tumefaciens* genome by this method suggests that this is not so. The mutated loci analysed in this experiment map to two separate cosmids (Table 3 and below) carrying a total of 12 loci. Thus, it is most likely that the bulk of the Tn5 insertions into behavioural genes map to the *A. tumefaciens* chromosome.

Similarity of *Agrobacterium* and *Rhizobium* behavioural genes

Rhizobium and *Agrobacterium* share a number of common features, with respect to motility (Loake *et al.*, 1988) and their interactions with plants (Shaw *et al.*, 1988b). It was therefore of interest to determine whether their behavioural genes share any similarity. Similarly, it was important to ascertain whether there was any similarity

Fig. 6. Hybridization of *mot-1* (a) and *mot-6* (b) flanking sequences to *Hind*III (a) and *Eco*RI (b) digested total DNA from *A. tumefaciens* C58C1 (tracks 1) and *A. tumefaciens* GM19023, which lacks the large cryptic plasmid (tracks 2). Arrows indicate sizes of hybridizing fragments in kb.

Table 3. Properties of behavioural gene cosmids

Mutant	Plasmid containing Tn5-flanking sequences	Cosmid hybridizing to flanking sequences	Cosmid complementing mutant
<i>mot-1</i>	pDUB1801	pDUB1900	pDUB1900
<i>che-2</i>	pDUB1802	pDUB1905	pDUB1905
<i>fla-3</i>	pDUB1803	pDUB1900	pDUB1900
<i>mot-4</i>	pDUB1804	pDUB1900	pDUB1900
<i>mot-5</i>	pDUB1805	—	—
<i>mot-6</i>	pDUB1806	pDUB1905	pDUB1905
<i>fla-7</i>	pDUB1807	pDUB1901/2/3	ND
<i>fla-8</i>	pDUB1808	pDUB1905	pDUB1905
<i>mot-9</i>	pDUB1809	pDUB1900	pDUB1900
<i>fla-10</i>	pDUB1810	pDUB1900	
<i>fla-11</i>	pDUB1811	pDUB1900	pDUB1900
<i>mot-12</i>	pDUB1812	pDUB1900	
<i>mot-14</i>	pDUB1814	pDUB1900	pDUB1900
<i>fla-15</i>	pDUB1815	pDUB1900	pDUB1900
<i>tpc-17</i>	pDUB1817	pDUB1904	ND
<i>ssw-18</i>	pDUB1818	—	—
<i>fla-19</i>	pDUB1819	pDUB1906	ND

ND, Not determined.

between the behavioural genes we have isolated and genes from other Gram-negative bacteria. A number of hybridization experiments were performed in which several different flanking sequences from *A. tumefaciens*

Fig. 7. Hybridization of Tn5 to total DNA from *A. tumefaciens* mutants, digested with *Dra*I and separated by pulsed-field gel electrophoresis. Tracks: 1, *che-2*; 2, *fla-3*; 3, *mot-4*; 4, *mot-6*. Hybridizing bands near the top are residual DNA left in the slots. The bands further down the gel are approximately 40–45 kb in size.

behavioural mutants were used as probes against 'zoo' blots (Figs 8–10). These Southern blots were prepared by transferring to nitrocellulose or nylon, digested, electrophoresed DNA from *E. coli*, *A. tumefaciens*, three strains of *Rhizobium* and two of *Pseudomonas*, and two cosmids, pRZ2 and pRZ4, which represent contiguous regions of the *R. meliloti* genome encoding behavioural genes (Ziegler *et al.*, 1986).

None of the behavioural genes isolated in this work showed any hybridization with *E. coli* DNA (see for example Fig. 8, track 1, and Fig. 9, track 3) or with *P. reactans* and *P. talassii* DNA (Fig. 8, tracks 8 and 9, Fig. 9, tracks 10 and 11). However, varying degrees of similarity were evident with *Rhizobium* DNA. Thus flanking sequences from *A. tumefaciens* *fla-7* (pDUB1807) and *fla-11* (pDUB1811) showed very weak hybridization with *R. meliloti* DNA (Fig. 8, tracks 4 and 5, Fig. 9, tracks 6 and 7) but not with *R. leguminosarum* (Fig. 8, tracks 6 and 7, Fig. 9, tracks 8 and 9). In the case

of *fla-11*, the hybridizing sequences have been localized to a 2 kb *Eco*RI fragment of the cosmid pRZ2 (Fig. 9, track 1). For the *A. tumefaciens* behavioural genes *mot-1* (pDUB1801) and *che-2* (pDUB1802) the similarity to *R. meliloti* genes was much more pronounced (Fig. 10). The hybridizing bands in this case mapped to two adjacent *Eco*RI fragments of pRZ4. The region to which *mot-1* hybridized (the 11.5 kb *Eco*RI pRZ4 fragment) carries several *R. meliloti* *che* genes, a *mot* gene (Ziegler *et al.*, 1986), and the two flagellin genes (Pleier & Schmitt, 1989) but no functions have been ascribed to the 3.5 kb pRZ4 *Eco*RI fragment to which *che-2* hybridized (Ziegler *et al.*, 1986). This suggests the similarity of some of the *A. tumefaciens* behavioural genes and their counterparts in *R. meliloti*, indicating that the flanking sequences we have isolated are indeed from behavioural genes of *A. tumefaciens*.

Analysis of *A. tumefaciens* cosmids

Initially the cosmid library containing wild-type *A. tumefaciens* DNA was screened with radioactively labelled mutant flanking sequences from pDUB1801 (*mot-1*), pDUB1806 (*mot-6*), pDUB1807 (*fla-7*), pDUB1817 (*tpc-17*) and pDUB1819 (*fla-19*). This resulted in the isolation of seven cosmids (Table 3): pDUB1900 (*mot-1*), pDUB1901/2/3 (*fla-7*), pDUB1904 (*tpc-17*), pDUB1905 (*mot-6*) and pDUB1906 (*fla-19*). pDUB1901/2/3 share a common 14 kb *Bam*HI fragment, and represent overlapping segments of the same chromosomal region. The other four cosmids do not share any common DNA fragments, and we thus conclude that they all contain separate, non-overlapping regions of the *A. tumefaciens* genome. As yet, no other flanking sequences have been shown to hybridize to cosmids pDUB1901–4 and pDUB1906. At present these cosmids thus have only one behavioural gene mapped to them. However, cosmids pDUB1900 and pDUB1905 have subsequently been shown to carry multiple behavioural genes.

The existence of several behavioural genes on cosmids pDUB1900 and pDUB1905 has been determined by two different methods (Table 3): complementation of mutant phenotypes; and hybridization of flanking sequences from other mutants to them. Using pRK2013 to mobilize cosmids from *E. coli* into *A. tumefaciens* behavioural mutants, pDUB1905 has been shown to complement mutants *che-2* (Fig. 11a) *mot-6* (Fig. 11b) and *fla-8*, data confirmed by the hybridization of the corresponding mutant flanking sequences to this cosmid (Table 3).

Restriction mapping using the enzymes *Hind*III, *Bam*HI, *Eco*RI and *Hpa*I of the cosmid pDUB1900 allowed the construction of the map shown in Fig. 12. As

Fig. 8. Hybridization of *fla-7* flanking sequences to *Bam*HI (tracks 3 and 5) or *Eco*RI (all other tracks) digested chromosomal DNA from *E. coli* (1), *A. tumefaciens* (2 and 3), *R. meliloti* (4 and 5), *R. leguminosarum* biovar *viciae* (6), *R. leguminosarum* biovar *phaseoli* (7), *P. reactans* (8), and *P. talassii* (9). Arrows indicate sizes of hybridizing fragments in kb.

this cosmid complements the behavioural mutants *mot-1*, 4, 9 and 14, and *fla-3*, 11 and 15 (Table 3), flanking sequences from these and other mutants were used as hybridization probes against Southern blots of digested pDUB1900 DNA. This confirmed that flanking sequences from *mot-1*, 4, 9, 12 and 14, and *fla-3*, 10, 11 and 15, hybridize to pDUB1900 and served to position the hybridizing sequences on the pDUB1900 map (Fig. 12). The existence of *Hpa*I sites in *Eco*RI fragments A and B of pDUB1900 meant that these sites were also present in the flanking sequences of mutants *mot-4*, 9, 12 and 14, and *fla-10* and 15. This facilitated the accurate positioning of the Tn5 insertions in these mutants. Mapping of the sequences hybridizing to flanking sequences from mutant *fla-3* led to the conclusion that in this mutant there is a 4 kb deletion adjacent to the Tn5 insertion site.

Probing of *Eco*RI-digested total DNA from mutants

mot-4, *mot-9* and *fla-10* with Tn5 revealed that all three mutants had similar-sized hybridizing fragments (Fig. 5). The Tn5 insertions in these three mutants map close together in *Eco*RI fragment B of pDUB1900. The *Eco*RI site that defines the left end of fragment B is in fact from the multiple cloning site of pLAFR3. However, analysis of plasmids pDUB1804 (*mot-4*), pDUB1809 (*mot-9*) and pDUB1810 (*fla-10*), which all contain fragments extending beyond the boundaries of pDUB1900, has confirmed that these Tn5 insertions are all in a common 9.3 kb *Eco*RI fragment of *A. tumefaciens* chromosomal DNA. Mapping confirms that these are three distinct insertions, with 1.2 kb separating the *mot-9* and 4 insertions, and 300 bp between *mot-4* and *fla-10*. Similarly, the Tn5 insertions in *mot-12* and 14 are both in *Eco*RI fragment A and appear to be spaced approximately 50 bp apart. Thus, pDUB1900 carries a number of genes concerned

Fig. 9. Hybridization of *fla-11* flanking sequences to *Hind*III (tracks 5 and 7) or *Eco*RI (all other tracks) digested cosmids pRZ2 (track 1) and pRZ4 (track 2), both of which carry behavioural genes from *R. meliloti*, and chromosomal DNA from *E. coli* (3), *A. tumefaciens* (4 and 5), *R. meliloti* (6 and 7), *R. leguminosarum* biovar *viciae* (8), *R. leguminosarum* biovar *phaseoli* (9), *P. talassii* (10) and *P. reactans* (11). Arrows indicate sizes of hybridizing fragments in kb.

Fig. 10. Hybridization of *mot-1* (a) and *che-2* (b) flanking sequences to *Eco*RI-digested cosmids pRZ2 (tracks 1) and pRZ4 (tracks 2) carrying behavioural genes from *R. meliloti*. Arrows indicate sizes of hybridizing fragments in kb.

with motility and chemotaxis in *A. tumefaciens*, confirming that as in *R. meliloti*, these genes are clustered in the chromosome.

Discussion

This paper describes the isolation and characterization of 20 behavioural mutants of *A. tumefaciens*, and the initial characterization of the corresponding genes. Martha Hawes and coworkers have described a set of Tn5-induced mutants of *A. tumefaciens* (Hawes & Smith, 1989; Hawes *et al.*, 1988) and together with the present work these have served to demonstrate the role of chemotaxis and motility in rhizosphere competence and virulence of this organism.

Analysis of the behavioural mutants and their wild-type parent has highlighted the unusual flagellation pattern. The presence of a polar tuft, at only one pole, and lateral flagella suggests that there is some polarity to the cell. Mutant *mot-12*, which lacks the polar tuft, points to the possibility that in some way the polar and lateral flagella are non-equivalent.

The paucity of non-chemotactic mutants isolated by our methods could be due to the rarity of their genes in the genome, compared to motility genes. However, it is worth pointing out that our method, using a complex attractant mixture during mutant screening, would only

Fig. 11. Complementation of mutants *che-2* and *mot-6* by cosmid pDUB1905. (a) Swarm plates of *A. tumefaciens* C58C1 *che-2* (left) and *A. tumefaciens* C58C1 *che-2*(pDUB1905) (right). (b) Swarm plates of *A. tumefaciens* C58C1 *mot-6* (left) and *A. tumefaciens* C58C1 *mot-6*(pDUB1905) (right).

Fig. 12. Restriction map of the *A. tumefaciens* chromosomal DNA insert in cosmid clone pDUB1900. The top three panels show the restriction maps for *Hind*III, *Bam*HI and *Eco*RI. The *Eco*RI site to the left, and the *Hind*III site to the right of the insert, derive from the multiple cloning site of pLAFR3. Below that are shown the approximate positions of sequences hybridizing to the various mutants (boxes) and the extent of the deletion in *fla-3* (dotted line). At the bottom are shown the cleavage sites for *Hpa*I, and the mapped locations of the Tn5 insertions in mutants *fla-3*, 10 and 15, and *mot-4*, 9, 12 and 14. The Tn5 insertions in *mot-12* and 14 are within 50 bp of each other, below the resolution of the figure.

have led to the isolation of generally non-chemotactic mutants, i.e. those affected in the cytoplasmic signalling pathway. Moreover, some of the non-motile, but flagellated mutants, which we have designated *mot*, may in fact have paralysed flagella due to a defect in the signalling pathway.

Hybridization of *mot-1* and 6 flanking sequences to DNA from an *A. tumefaciens* strain cured of both Ti- and cryptic plasmids confirmed that these particular Tn5 insertions were chromosomal. The pulsed-field gel data suggest that *mot-6* is linked to *che-2*, *fla-3* and *mot-4*. As the flanking sequences from these mutants map to two separate cosmids which together encode *mot-1*, 4, 6, 9, 12 and 14, *fla-3*, 8, 10, 11 and 15, and *che-2*, this suggests that the bulk of the Tn5 insertions in the behavioural mutants are into linked sections of the chromosome.

The isolation of cosmids encoding the behavioural genes demonstrates that these functions are clustered in the *A. tumefaciens* genome, as they are in *R. meliloti* (Ziegler *et al.*, 1986). This will facilitate further characterization, and DNA sequencing. However, the estimates of about 60 genes involved in chemotaxis and motility in *E. coli* (Macnab, 1987*a,b*) suggest that we have not identified all of the genes in *A. tumefaciens*. Saturation mutagenesis of the isolated cosmids followed by homogenotization should aid in identifying linked genes, not yet mutagenised.

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