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Mapping of *Agrobacterium tumefaciens* Chromosomal Genes Affecting Cellulose Synthesis and Bacterial Attachment to Host Cells

JENNIE L. ROBERTSON, TERESA HOLLIDAY, AND ANN G. MATTHYSSE*

Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599

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Six chromosomal transposon mutations in *Agrobacterium tumefaciens* which result in an inability to synthesize cellulose fibrils were mapped to the vicinity of *trp-2* and *met-6*. Mutations which result in an inability to attach to plant cells, *attC43* and *attC69*, also mapped in this region, as did one Tn*5* mutation which caused overproduction of cellulose. Another cellulose overproduction mutation mapped at a distance and was closely linked to *ilv-13*. The results suggest that there is a region of the *A. tumefaciens* chromosome located near *met-6* which is concerned with the interaction of the bacterium with the host cell surface.

Infections of dicotyledenous plants with *Agrobacterium tumefaciens* result in the formation of crown gall tumors. An early step in tumor formation is the site-specific attachment of the bacteria to the plant host cells (6). This initial reversible loose attachment is followed by the bacterial synthesis of cellulose fibrils, which results in the irreversible tight binding of the bacteria to the host cell surfaces (9).

Transposon mutants which are altered either in the ability to attach to the host cell or in cellulose synthesis have been obtained. All of the nonattaching mutants isolated to date are chromosomal mutants. They can be divided into two groups: (i) *chvAB*, isolated by Douglas et al. (2), and (ii) the *att* mutants isolated by Matthysse (8). The *chvAB* mutations are pleiotropic and affect motility, exopolysaccharide synthesis, ability to transfer pTi by conjugation, and in the case of *chvB*, β-1,2-glucan synthesis (2, 3, 12). The Tn*5* insertions in the *chvAB* mutants map in a few small linked *Eco*RI fragments of the chromosome (3). These insertions have been mapped by Miller et al. (11) and by Waelkens et al. (13) to the vicinity of *trp-13* on the map of Hooykaas et al. (5). The *att* mutations isolated by Matthysse are all located in one 12-kilobase chromosomal *Eco*RI fragment (8). The location of this fragment on the chromosome is unknown. The *att* mutants fail to bind to carrot cells and are avirulent. Although they grow at a normal rate in Luria broth and in minimal medium with or without neomycin, they fail to grow in Luria broth in the presence of neomycin (8).

Seven Tn*5* insertions which result in the inability of *A. tumefaciens* to synthesize cellulose have been obtained (7). One of these insertions is in the large cryptic plasmid carried by *A. tumefaciens*. The other six are chromosomal. Each is located in a different sized *Eco*RI fragment. Nothing is known about the relation of these fragments to each other. In addition, three Tn*5* insertions were obtained which resulted in the overproduction of cellulose. All three of these insertions were chromosomal (7).

We wished to determine the locations on the bacterial chromosome of the *att* and *cel* mutations and in particular whether the *cel* mutations were scattered around the chromosome or were grouped in a few locations. There is no equivalent of a convenient Hfr system which can be used to map Tn*5* insertions in *A. tumefaciens*. Thus an R′ mapping system based on the method used by Hooykaas et al. (5) for mapping the chromosome in this bacterium was used.

Table 1 lists the bacterial strains and plasmids used. *A. tumefaciens* and *Escherichia coli* were maintained on Luria agar. For strains containing Tn*5*, 60 μg of neomycin per ml was added to the medium. The nonattaching *A. tumefaciens* mutants *attC43* and *attC69*, which do not grow on Luria broth with neomycin, were maintained on minimal medium (10) with 60 μg of neomycin per ml. *E. coli* 1843(pJB3JI) was maintained on Luria agar with 5 μg of tetracycline per ml. *A. tumefaciens* was grown at 25°C and *E. coli* was grown at 37°C. Amino acids and vitamins when required were added to the minimal medium at a concentration of 20 μg/ml. The concentration of chloramphenicol and rifamycin was 100 μg/ml. The concentration of tetracycline was 5 μg/ml.

For conjugations, bacteria were grown to mid-log phase in Luria broth on a shaker and equal numbers of the parents were mixed in a volume of about 1 ml on the surface of a Luria agar plate. The plate was incubated overnight at 25°C. The bacteria were scraped off the plate into 5 ml of 0.9% NaCl, harvested by centrifugation, and suspended in phosphate-buffered saline (10).

The mapping procedure used was a modification of that of Waelkens et al. (13) and of Hooykaas et al. (5). The modifications were made to allow the mapping of the insertion site of Tn*5*. Hooykaas et al. (5) used plasmid R68.45 to mobilize the chromosome in *A. tumefaciens*. However, R68.45 carries a gene for Neo^r and thus cannot be used to map Tn*5* insertions since transconjugants cannot be selected by resistance to neomycin. Waelkens et al. (13) solved this problem by suggesting the use of pJB3JI, which is a derivative of R68.45 lacking Neo^r. It is able to generate conjugative R′s (1). In addition, Waelkens et al. (13) suggested the use of an *E. coli* intermediate host for the R′s to allow the transfer of R′s from one strain of *A. tumefaciens* to another in cases in which it would not be possible to select the recipient *A. tumefaciens* from a direct cross of the two *A. tumefaciens* strains.

The plasmid pJB3JI was introduced by conjugation from *E. coli* 1843(pJB3JI) into the *A. tumefaciens* Tn*5* mutant to be mapped. The *A. tumefaciens*(pJB3JI) transconjugant was selected by growth on minimal medium containing tetracycline. Transconjugant *A. tumefaciens* was allowed to grow for several generations in Luria broth with tetracycline to

* Corresponding author.

TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristics	Source or reference
A. tumefaciens[a]		
LBA 2095	*leu-8 trp-1*	5
LBA 2132	*met-6 trp-13*	5
LBA 2132C	Spontaneous chloramphenicol-resistant mutant of LBA 2132	This study
LBA 2232	*his-31 nal-2 pyr-12*::Tn*5 rif-8*	5
LBA 2235	*his-32 nal-2 rif-8 trp-22*::Tn*5*	5
LBA 2251	*ilv-13 nal-2 rif-8 trp-22* Neo^s	5
LBA 2288	*trp-2 str-11*	5
LBA 2288R	Spontaneous rifamycin resistant mutant of LBA 2288	This study
C58	Wild type	8
A. tumefaciens mutants		
cel-1	A6::Tn*5* cellulose minus	7
cel-2	A6::Tn*5* cellulose minus	7
cel-3	A6::Tn*5* cellulose minus	7
cel-4	A6::Tn*5* cellulose minus	7
cel-5	A6::Tn*5* cellulose minus	7
cel-13	A6::Tn*5* cellulose minus	7
celB1	A6::Tn*5* cellulose overproducer	7
celB4	A6::Tn*5* cellulose overproducer	7
attC43	C58::Tn*5* attachment minus	8
attC69	C58::Tn*5* attachment minus	8
E. coli		
1843(pJB3JI)	*pro met* Tc^r Neo^s	1
GM4	*pro leu* Tc^s Neo^s	F. Ausubel

[a] Used to map Tn*5* insertions.

provide time for R's to form. The *A. tumefaciens* Tn*5* mutant carrying pJB3JI was then conjugated with *E. coli* GM4. Those *E. coli* cells which received R's carrying Tn*5* were selected by growth at 37°C in Luria broth with neomycin. (*A. tumefaciens* grows poorly at 37°C.) After six or seven successive transfers (1 to 20 dilutions) in liquid medium at 37°C, no *A. tumefaciens* remained in the culture, as judged by the failure of the culture to give any colonies when 0.1 ml was plated onto minimal medium and incubated at 25°C. The *E. coli* GM4 pJB3JI::R'::Tn*5* culture was transferred an additional two or three times. At this stage the *E. coli* culture contained a mixture of R's, all of which carried Tn*5* and various amounts of surrounding *A. tumefaciens* DNA. This mixture of R's was then transferred by conjugation to *A. tumefaciens* LBA 2095, LBA 2132, LBA 2251, and LBA 2288, which carry mapped chromosomal markers. The *A. tumefaciens* transconjugants were selected by growth on minimal medium containing leucine, tryptophan, methionine, histidine, isoleucine, valine, and neomycin. The coinheritance frequency of Tn*5* (Neo^r) and the chromosomal marker was determined by replica plating onto minimal medium plates lacking one of these supplements. Chromosomal markers were located with reference to the map published by Hooykaas et al. (5), since the markers used were among those located on this map. Coinheritance frequencies for the Tn*5* insertion and various chromosomal markers are shown in Table 2. The coinheritance frequencies were used to locate the Tn*5* mutations on the chromosomal map of Hooykaas et al. (5).

The mapping of the *celB4* cellulose-overproducing mutation was complicated by the fact that this mutation was apparently dominant in the heterozygous state. Thus, *A. tumefaciens* strains receiving R's carrying the Tn*5* insertion derived from *celB4* overproduced cellulose and were extremely sticky, clumpy, difficult to work with, and hard to replica plate. Thus the position of *celB4* on the chromosomal map may be subject to error and is only approximate.

Although *met-6* was carried by one of the strains of *A. tumefaciens* used to map the Tn*5* mutations, the location of *met-6* on the chromosome was not determined by Hooykaas et al. (5). Since many of our Tn*5* mutants showed strong linkage to *met-6*, it seemed desirable to determine the location of this mutation. It was apparent from the transconjugant coinheritance frequencies shown in Table 1 that *met-6* showed some linkage to *trp-2*. We were unable to carry out a direct cross to map the location of *met-6* because of the limited number of mapped markers available in *A. tumefaciens* and the difficulty of selecting the desired transconjugants. We observed occasional apparently large R's in the mapping crosses which cotransferred genes located at some distance from each other on the chromosome. To determine whether *met-6* was indeed located near *trp-2* and thus between *his-31* and *pyr-12*::Tn*5* (Fig. 1), the following crosses were carried out. The plasmid pJB3JI was introduced into wild-type strain C58. C58(pJB3JI) was then conjugated with LBA 2232 *his-31 pyr-12*::Tn*5*. Transconjugants were selected for growth on minimal medium containing neomycin and tetracycline. The resulting colonies were LBA 2232, which contained an R' derived from C58 which covered the region of *his-31* and *pyr-12*. These transconjugants were crossed to LBA 2132C *met-6 trp-13*, and the

TABLE 2. Coinheritance frequencies of Tn*5* with various markers

Tn*5* mutation	No. of transconjugants with both markers/total no. of transconjugants (%[a])						
	leu-8	*trp-1*	*met-6*	*trp-13*	*ilv-13*	*trp-22*	*trp-2*
cel-1	0/347 (0)	0/347 (0)	233/233 (100)	0/233 (0)	3/449 (0.6)	15/449 (3)	838/1,077 (78)
cel-2	0/229 (0)	0/229 (0)	691/691 (100)	0/691 (0)	1/693 (0.1)	5/693 (0.7)	19/83 (23)
cel-3	4/56 (7)	0/56 (0)	86/96 (90)	0/96 (0)	7/1,290 (0.5)	0/1,290 (0)	55/340 (16)
cel-4	0/809 (0)	0/809 (0)	701/897 (78)	0/897 (0)	3/66 (5)	0/66 (0)	53/1,241 (4)
cel-5	1/250 (0.4)	0/250 (0)	103/121 (85)	0/121 (0)	0/293 (0)	0/293 (0)	23/302 (8)
cel-13	0/223 (0)	0/223 (0)	390/523 (75)	0/227 (0)	25/418 (6)	56/418 (13)	161/327 (49)
celB1	1/141 (0.7)	1/141 (0.7)	110/133 (83)	0/133 (0)	2/172 (1)	0/172 (0)	21/160 (13)
celB4	1/1,428 (0.07)	0/1,428 (0)	2/284 (0.7)	0/284 (0)	1,213/1,217 (100)	108/1,217 (9)	0/62 (0)
attC43	1/750 (1)	0/750 (0)	82/99 (83)	0/99 (0)	2/223 (0.9)	10/223 (5)	37/141 (26)
attC69	18/1,040 (2)	0/1,040 (0)	587/587 (100)	1/587 (0.1)	1/151 (0.1)	0/151 (0)	74/377 (20)

[a] Percent coinheritance of Tn*5* and marker.

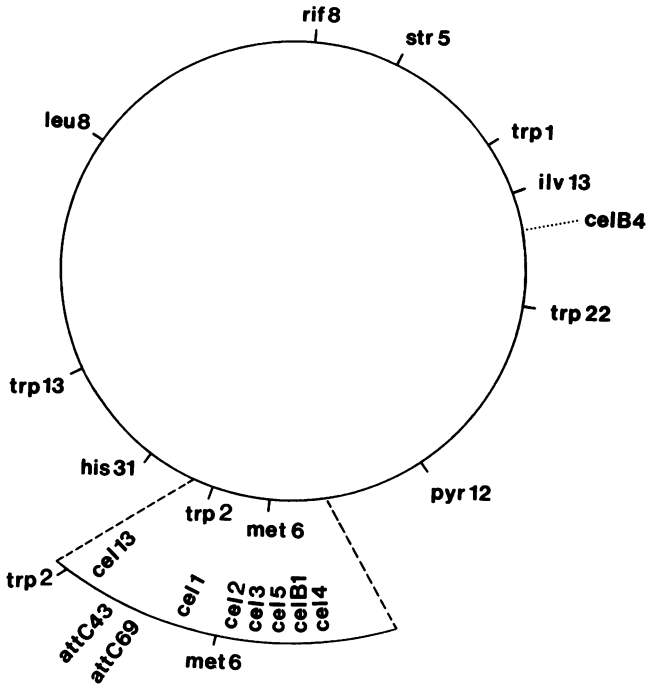

FIG. 1. Chromosomal map of *A. tumefaciens* based on the map of Hooykaas et al. (5). The positions of the markers previously mapped (5) and used in this study are shown, as are the positions of the *cel* and *att* mutations mapped in this paper. The region surrounding *met-6* and *trp-2* has been enlarged for convenience. Tn*5* insertions used were *attC43*::Tn*5*, *attC69*::Tn*5*, *cel-1*::Tn*5*, *cel-2*::Tn*5*, *cel-3*::Tn*5*, *cel-4*::Tn*5*, *cel-5*::Tn*5*, *cel-13*::Tn*5*, *celB1*::Tn*5*, *celB4*::Tn*5*, and *pyr-12*::Tn*5*.

products were selected for growth on minimal medium containing methionine, tryptophan, chloramphenicol, and tetracycline. The resulting colonies were LBA 2132C, which contained the DNA derived from C58 which covered both *his-31* and *pyr-12*. The colonies were replica plated onto minimal medium containing tryptophan, chloramphenicol, and tetracycline. Eighty percent of the transconjugants (188 of 236 scored) had received the *met-6* gene and no longer required methionine for growth. The other 20% might reflect either double recombination events in the origin of this large R′ or in its integration into the recipient chromosome, or the location of *met-6* just outside one end of the *his-31*-to-*pyr-12* region.

The one marker located between *his-31* and *pyr-12* on the map of Hooykaas et al. (5) is *trp-2*. To determine whether *met-6* is between *trp-2* and *his-31* or between *trp-2* and *pyr-12*, the following crosses were carried out. The plasmid pJB3JI was introduced from LBA 2232 *his-31 pyr-12*::Tn*5* into LBA 2132C *met-6 trp-13*, and the transconjugants were selected for growth on minimal medium containing tetracycline. The products were LBA 2132C, which carried either an R′ which covered both *trp-13* and *met-6* or a recombinant chromosome covering this region. As expected, these products were chloramphenicol resistant and neomycin sensitive. These transconjugants were then crossed to LBA 2288R *trp-2*, and transconjugants were selected for growth on minimal medium containing tryptophan, tetracycline, and rifamycin. These transconjugants were replica plated onto medium lacking tryptophan to determine the percentage of the transferred DNA which contained *trp-13* and *met-6* and also contained *trp-2*. All of the transconjugants (1,093 were scored) no longer required tryptophan for growth and thus carried *trp-2* on the DNA which they had received. This result suggests that *trp-2* lies between *met-6* and *trp-13*, although the possibility that *trp-2* lies very close to *met-6* on the side opposite that of *trp-13* cannot be excluded.

The coinheritance frequencies shown in Table 1 and the data described above on the location of *met-6* were used to determine the location of the *att* and *cel* mutations on the chromosomal map of Hooykaas et al. (5). Despite the fact that each of the *cel* mutants is located on an *Eco*RI fragment of a different size, all of the Tn*5* insertions which resulted in a failure to synthesize cellulose fibrils mapped within the region of *met-6* and *trp-2*. One of the cellulose-overproducing mutations (*celB1*) also mapped in this region. Both the *att* mutations mapped are located in the same 12-kilobase *Eco*RI fragment (8). They both mapped to the same region as the *cel* mutations. This region is distant from the suggested locations for the *chvAB* genes, which also affect bacterial attachment to host cells (11, 13). Since many of the *cel* and *att* mutations mapped are apparently located very close to each other, the exact order of the mutations in this region as presented here must be regarded as tentative. It is difficult to resolve mutations so close together by using chromosomal mapping techniques such as R's. Cloning and sequencing of the region will be required to determine the precise location of the mutations. As expected for Tn*5* insertions, all of these mutations were recessive in the heterozygous condition created by the presence of a mutation on an R′. The only mutation involved with cellulose synthesis which did not map in the region of *met-6* and *trp-2* was the cellulose-overproducing mutation *celB4*, which mapped close to *ilv-13*. This mutation was dominant in the heterozygote. Although it is unusual for a Tn*5* mutation to be heterozygous dominant, there is at least one other transposon insertion in *A. tumefaciens* which is dominant (4).

The results of the mapping of these mutations suggest that there is a region of the *A. tumefaciens* chromosome located near *met-6* and *trp-2* which is concerned with the surface interaction of the bacterium with plant host cells and which contains genes involved in bacterial cellulose synthesis and the attachment of the bacteria to host cells.

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