Combined Genetic and Physical Map of the Complex Genome of *Agrobacterium tumefaciens*

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A combined genetic and physical map of the *Agrobacterium tumefaciens* A348 (derivative of C58) genome was constructed to address the discrepancy between initial single-chromosome genetic maps and more recent physical mapping data supporting the presence of two nonhomologous chromosomes. The combined map confirms the two-chromosome genomic structure and the correspondence of the initial genetic maps to the circular chromosome. The linear chromosome is almost devoid of auxotrophic markers, which probably explains why it was missed by genetic mapping studies.

Most of the work on *Agrobacterium tumefaciens*, since its identification as the causal agent in crown gall disease of dicotyledonous plants at the turn of the century, has rightfully focused on the mechanism of tumor induction (52; for recent reviews of all aspects of the disease, see references 2, 10, 23, 39, and 58). The virulence mechanism turns out to be unique among interactions between prokaryotic pathogens and eu-

some of ~3 Mbp and one linear chromosome of ~2.1 Mbp (1, 31). This chromosome organization appears to be a conserved trait throughout the genus (32). While multiple chromosomes have been found in some other eubacteria (7–9, 43, 54, 57), we were interested in the discrepancy between the initial genetic maps and the more recent physical mapping data. We hypothesized that the original genetic mapping techniques somehow

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Pertinent characteristics	Reference(s)
A. tumefaciens		
A348	Prototrophic biovar 1 strain; C58 derivative cured of pTiC58, with pTiA6 introduced by mating; Rf^r	22
Plasmids		
pUT::Tn5(pfm)	λ*pir*-dependent replicon carrying a mini-Tn5 derivative with rare restriction sites and a separate transposase gene; plasmid confers Ap^r, and Tn5(pfm) confers Kan^r and Cm^r; maintained in *E. coli* S17-1	15, 60
R68.45	Broad-host-range conjugable plasmid; confers Cb^r, Tc^r, and Kan^r	27
pG644	Cosmid clone of *A. tumefaciens* chromosomal sequences containing the *att* gene cluster in pVK102; confers Kan^r	36, 41
pCP13.101	Cosmid clone of *A. tumefaciens* chromosomal sequences containing the *cel* gene cluster in pCP13; confers Tc^r	21, 40
pRK290	Broad-host-range mobilizable RK2-based plasmid; parent plasmid to pCP13 and pVK102 cosmid vectors; confers Tc^r	17
pRK2013	Helper plasmid for mobilization of RK2-based plasmids carrying the *rlx* locus; ColE1 replicon; confers Kan^r	19

karyotic hosts. Since most of the virulence genes lie on the Ti plasmid, the chromosomal complement of *A. tumefaciens* has been relatively understudied.

Initial chromosomal maps for *A. tumefaciens*, based on chromosome mobilization and recombination of genetic markers, suggested a single circular chromosome (11, 29, 44, 47, 48). However, recent physical mapping data strongly suggests that *A. tumefaciens* has two chromosomes, one circular chromo-

missed the linear chromosome. To test this hypothesis, we constructed a combined genetic and physical map of the *A. tumefaciens* genome by collecting a large number of transposon-mediated auxotrophic mutations, using a transposon carrying rare restriction sites, and then physically mapping the transposon insertions by using pulsed-field gel electrophoresis (PFGE). Our results confirm the two-chromosome genome organization, and we found that almost all the auxotrophic markers lie on the circular chromosome. We put forward an explanation for the discrepancy between the initial genetic maps and the physical mapping data and suggest some hypotheses for the gene organization in this bacterial species.

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TABLE 2. Biochemical complementation and physical mapping of Tn5(pfm)-induced auxotrophic mutations

Mutation[b]	Biochemical complementation	Restriction fragment abolished by Tn5(pfm) insertion[a] and new fragments created (kb)	
		*Pac*I	*Swa*I
ade-101::Tn5C1	Adenine only	H[c]	B; 855, 35
ade-102::Tn5C2	Adenine or hypoxanthine	A; 760, 660	B; 790, 105
ade-103::Tn5L1	Adenine or hypoxanthine	B; 565, 400	C; 540, 230
ade-104::Tn5C3	Adenine or hypoxanthine	H[c]	B; 790, 105
aah-101::Tn5C4, C5	Adenine + histidine	C; 500, 290	A; 825, 300
aah-102::Tn5C6	Adenine + histidine	A; 1090, 330	A; 840, 285
aat-101::Tn5C7	Adenine + thiamine	A; 970, 450	B; 780, 115
aat-101::Tn5C8	Adenine + thiamine	H; 230, 20	B; 820, 75
cys-101::Tn5C9	Cysteine, thiosulfate, or sulfite	A; 1265, 155	B; 470, 425
cys-102::Tn5C10	Cysteine or thiosulfate	A; 1330, 90	A; 725, 400
glt-101::Tn5C11, C12, C13	Glutamate or glutamine	C; 410, 380	A; 975, 150
gln-101::Tn5C14	Glutamine	A; 1225, 195	B; 485, 410
gln-102::Tn5C15	Glutamine	A; 1050, 370	A; 845, 280
his-101::Tn5C16	Histidine	A; 1090, 330	A; 840, 285
ilv-101::Tn5C17, C18	Isoleucine + valine	E; 295, 90	H[c]
leu-101::Tn5C19, C20, C21	Leucine or α-ketoisocaproate	C; 400, 390	A; 970, 155
met-101::Tn5C22	Methionine or cystathione	A; 1220, 200	A; 710, 415
met-102::Tn5C23, C24	Methionine or cystathione	C; 395, 395	A; 1035, 90
pan-101::Tn5C25	Pantothenate or β-alanine	C; 470, 320	A; 1070, 55
pan-102::Tn5L2	Pantothenate or β-alanine	B; 765, 200	D; 460, 180
prx-101::Tn5C26	Pyridoxine	A; 890, 530	—[d]
ser-101::Tn5L3, L4	Serine, glycine, or threonine	B; 510, 455	C; 680, 90
ser-102::Tn5C27	Serine or glycine	E[c]	H[c]
ser-103::Tn5C28	Serine only	A; 1290, 130	B; 565, 330
thi-101::Tn5C29, C30, C31	Thiamine	C; 530, 260	—
thi-102::Tn5C32	Thiamine	C; 490, 300	A; 840, 285
thr-101::Tn5L5	Threonine	B; 490, 475	C; 660, 110
trp-101::Tn5C33, C34, C35	Tryptophan or anthranilate	E[c]	G; 255, 70
trp-102::Tn5C36, C37	Tryptophan only	C; 400, 390	A; 790, 335
trp-103::Tn5C38	Tryptophan only	H; 235, 15	—
trp-104::Tn5C39	Tryptophan or shikimate	E; 355, 20	—
ura-101::Tn5C40	Uracil or orotic acid	A; 1185, 235	A; 655, 470

[a] *Pac*I and *Swa*I restriction fragments are designated by the letters used in the nomenclative system of Allerdet-Servant et al. (1).

[b] We used the standard genetic nomenclature except for two novel auxotrophic phenotypes, for which we derived new genetic abbreviations (*aah* and *aat*). The C or L designation for each insertion indicates its location on the circular or linear chromosome, respectively.

[c] We were unable to detect and measure the new restriction fragments due to their small size.

[d] —, there was no detectable difference from the wild-type restriction pattern, most probably due to the Tn5(pfm) insertion being too close to the end of a restriction fragment.

nual Crown Gall Meeting [24] and at the Microbial Genomes III Conference [25].)

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The *A. tumefaciens* strain and plasmids used in this study are described in Table 1. *A. tumefaciens* cultures were grown in a modified Luria-Bertani (LB) medium (only 5 g of NaCl/liter) at 30°C. Screens for *A. tumefaciens* auxotrophic mutants were carried out in M9 minimal medium with sucrose as a carbon source (45). The antibiotics carbenicillin, kanamycin, rifampin, and tetracycline were used as needed at 50, 50, 20, and 10 μg/ml, respectively.

Matings. Donor (either *Escherichia coli* or *A. tumefaciens* carrying a plasmid) and recipient (*A. tumefaciens*) strains were mixed by streaking on a modified LB agar plate and incubated at 30°C for 48 h. When necessary (when the donor plasmid was a mobilizable pRK290 derivative), a third strain, *E. coli* carrying pRK2013, was included in the mating. The cell mixture was scraped off, resuspended in M9 minimal medium without a carbon source, diluted, and plated on the appropriate selective medium.

Mutant isolation and characterization. *A. tumefaciens* A348 was mated with *E. coli* S17-1/pUT::Tn5(pfm). *A. tumefaciens* carrying Tn5(pfm) insertions were selected on modified LB medium containing kanamycin [selective for the presence of Tn5(pfm)] and rifampin (selective for *A. tumefaciens*). Single colonies were screened for auxotrophy by plating on M9 and modified LB medium. Potential Tn5(pfm)-induced auxotrophs were tested on M9 plates with various nutrient pools and later supplemented with specific pathway intermediates (13).

Confirmation of the linkage between Tn5(pfm) insertion and auxotrophic mutation. *A. tumefaciens* auxotrophic mutant strains were grown overnight at 30°C in 2-ml cultures. Total genomic DNA was isolated from each strain and

resuspended at ~0.4 to 0.5 μg/ml (16). Approximately 4 to 5 μg (10 μl) of each sample was electroporated into competent wild-type *A. tumefaciens* A348 cells, and the transformed cells were plated on modified LB medium containing kanamycin (5). Three days later, the few resulting colonies were picked from each transformation and streaked onto modified LB plates containing kanamycin, M9 plates containing kanamycin, and M9 plates containing kanamycin and the specific nutrient required by the original *A. tumefaciens* auxotrophic mutant.

PFGE of intact and digested DNAs. Wild-type and mutant *A. tumefaciens* strains used in physical mapping were grown for 48 h at 30°C in 2-ml cultures. Cells were pelleted, suspended in 2% agarose plugs, digested overnight with pronase E (2 mg/ml) at 50°C, and washed (53). Restriction enzyme digest of genomic DNA in the agarose plugs by *Pac*I and *Swa*I (New England Biolabs) were carried out at 25°C for 24 h. PFGE was carried out in a contour-clamped homogeneous electric field apparatus (Bio-Rad), in 0.5× TBE buffer (45 mM Tris, 45 mM borate, 1.25 mM EDTA [pH 8.3]). Restriction enzyme digests of genomic DNA were electrophoresed through 1% agarose gels with a ramp of 40 to 90 s for 22 h at 180 V. Lambda ladder (Bio-Rad) served as size markers.

Plasmid-mediated mobilization of chromosomal markers. R68.45, a conjugable plasmid used in several of the original genetic mapping experiments, was mobilized into several *A. tumefaciens* Tn5(pfm)-induced auxotrophic mutants in independent overnight matings with *E. coli* harboring R68.45. The presence of R68.45 in the *A. tumefaciens* strains was selected by growth on modified LB medium containing kanamycin [selective for the presence of Tn5(pfm)], carbenicillin (selective for the presence of R68.45), and rifampin (selective for *A. tumefaciens*). *A. tumefaciens* strains carrying R68.45 were used as donors in overnight matings on modified LB medium with recipient *A. tumefaciens* strains harboring different auxotrophic mutations. Transconjugants in which the auxotrophic marker of the recipient strain had been replaced by the wild-type coun-

TABLE 3. Physical mapping of Tn5(pfm)-induced mutations with unknown auxotrophic requirements

Mutation[b]	Restriction fragments abolished by Tn5(pfm) insertion[a] and new fragments created (kb)	
	PacI	SwaI
aux-101::Tn5C41	A; 950, 470	A; 1040, 85
aux-102::Tn5L6	F; 210, 140	D; 460, 180
aux-103::Tn5C42	C; 430, 360	A; 1025, 100
aux-104::Tn5C43	C; 470, 320	A; 1070, 55
aux-105::Tn5C44	C; 730, 60	A; 655, 470
aux-106::Tn5C45	A; 1290, 130	B; 495, 400
aux-107::Tn5C46	A; 1250, 170	B; 460, 435
aux-108::Tn5L7	B; 750, 215	C; 440, 330
aux-109::Tn5C47	C; 540, 250	—[d]
aux-110::Tn5C48	A; 940, 480	B; 820, 75
aux-111::Tn5C49	A[c]	B; 640, 255

[a] PacI and SwaI restriction fragments are designated by the letters used in the nomenclative system of Allerdet-Servant et al. (1).

[b] The C or L designation for each insertion indicates its location on the circular or linear chromosome, respectively.

[c] We were unable to detect and measure the new restriction fragments due to their small size.

[d] There was no detectable difference from the wild-type restriction pattern, most probably due to the Tn5(pfm) insertion being too close to the end of a restriction fragment.

terpart from the donor chromosome were selected by plating dilutions of a mating mixture onto M9-sucrose medium.

RESULTS

Construction of a combined genetic and physical map for A. tumefaciens A348. To build on the results of Allerdet-Servant et al. (1), we first repeated their experiments with A. tumefaciens A348. We obtained identical results for strain A348 (data not shown), which differs from strain C58 only by having a chromosomal rifampin resistance mutation and a different Ti plasmid (22). Next, we devised a strategy for physical localization of genetic markers with digestions by PacI and SwaI, the same enzymes used in the initial physical mapping experiments (1). Tn5(pfm), a minitransposon carrying selectable markers and several rare restriction sites, was introduced by mating into A. tumefaciens A348 (60). From 30 independent matings, approximately 11,000 kanamycin-resistant colonies were replicated onto LB and M9-sucrose minimal media. Of these, 103 were identified as Tn5(pfm)-induced auxotrophs. A total of 56 independently isolated auxotrophs were chosen for further analysis, with 45 eventually being characterized down to the affected biosynthetic pathway and the remainder having unknown requirements (Tables 2 and 3).

To confirm that the auxotrophy was due to the Tn5(pfm) insertion, genomic DNA was individually isolated from a random subset of the auxotrophic strains (aah-102::Tn5C6, gln-102::Tn5C15, met-102::Tn5C23, ser-101::Tn5L4, trp-101::Tn5C34, and aux-102::Tn5L6). Each genomic DNA sample was electroporated into wild-type A. tumefaciens A348, and the transformants were plated on LB medium containing kanamycin to select for cells in which the transposase-less Tn5(pfm) insertion had been recombined into the recipient genome (5). Kanamycin-resistant colonies were then screened for coinheritance of the proper auxotrophic marker. In all cases, the kanamycin-resistant colonies from a given electroporation carried the auxotrophic marker corresponding to the auxotrophic strain whose genomic DNA had been used in that electroporation.

~3 Mbp Circular Chromosome

~2.1 Mbp Linear Chromosome

FIG. 2. Best-fit combined genetic and physical maps of the two A. tumefaciens A348 chromosomes. The PacI and SwaI restriction fragments are designated by the letters used in the nomenclature system of Allerdet-Servant et al. (1). The locations of all auxotrophic and some prototrophic Tn5(pfm) insertions are indicated by their appropriate abbreviations along the outer edge of each map. The small SwaI restriction fragments J, K (doublet), L, and M, which were previously localized by hybridization (1) to the circular (J, K1, and L) and linear (K2 and M) chromosomes, are not shown on these maps, since we were unable to obtain a Tn5(pfm) insertion in any of them.

Tn5(pfm) insertions were mapped by PFGE of PacI- and SwaI-digested genomic DNA. Since Tn5(pfm) carries PacI and SwaI restriction sites, the insertion of the transposon leads to an altered restriction pattern compared to the wild type. A total of 56 Tn5(pfm)-induced auxotrophic mutations and 28 prototrophic Tn5(pfm) insertions were mapped (Tables 2 to 4). The PacI and SwaI digestion patterns were consistent with single Tn5(pfm) insertions in each mutant and served to identify the fragments harboring the transposon (Fig. 1).

Essential features of the map. The results of the mapping were entirely consistent with the findings of earlier studies indicating two independent chromosomes, a 3.0-Mb circle and a 2.1-Mb linear structure (1). Furthermore, the restriction enzyme digestions of the genomic DNA of the various Tn5(pfm) mutants allowed us to order the PacI and SwaI fragments on each of the chromosomes and to localize a large number of transposon insertions (Fig. 2). Insertions were found on both chromosomes. None of the transposon insertions localized to the small SwaI fragments J, K (doublet), L, M, and N. All but one of these small fragments had been assigned to chromo-

TABLE 4. Physical mapping of Tn5(pfm) prototrophic insertions

Insertion[b]	Restriction fragments abolished by Tn5(pfm) insertion[a] and new fragments created (kb)	
	*Pac*I	*Swa*I
Tn5C50	A; 1270, 120	—[c]
Tn5C51	A; 1150, 270	—[c]
Tn5C52	A[c]	B; 585, 310
Tn5C53	C; 770, 20	G[c]
Tn5C54	A; 1230, 190	B[c]
Tn5C55	A[c]	B[c]
Tn5C56	E[c]	H[c]
Tn5C57	—[c]	A[c]
Tn5C58	C[c]	—[c]
Tn5C59	—[c]	A[c]
Tn5C60	—[c]	B[c]
Tn5C61	—[c]	B[c]
Tn5C62	—[c]	B[c]
Tn5L8	F[c]	D; 420, 220
Tn5L9	B; 690, 275	D; 560, 80
Tn5L10	B; 530, 435	C; 555, 215
Tn5L11	B; 510, 455	C; 530, 240
Tn5L12	D; 475, 235	E; 430, 85
Tn5L13	F[c]	D; 430, 210
Tn5L14	B[c]	D; 410, 230
Tn5L15	F; 210, 140	D; 195, 455
Tn5L16	B[c]	C; 450, 320
Tn5L17	D[c]	E[c]
Tn5L18	B[c]	D[c]
Tn5L19	D[c]	E[c]
Tn5L20	B[c]	C[c]
Tn5L21	—[c]	E[c]
Tn5L22	—[c]	C[c]

[a] *Pac*I and *Swa*I restriction fragments are designated by the letters used in the nomenclature system of Allerdet-Servant et al. (1).

[b] The C or L designation for each insertion indicates its location on the circular or linear chromosome, respectively.

[c] We did not characterize the insertion further.

FIG. 1. Example of PFGE of *Swa*I-digested genomic DNA from *A. tumefaciens* A348 strains carrying Tn5(pfm)-induced auxotrophic mutations: *aux-102*::Tn5L6 (lane 1), *met-101*::Tn5C22 (lane 2), *thr-101*::Tn5L5 (lane 3), *aux-105*::Tn5C44 (lane 4), *pan-102*::Tn5L2 (lane 5), *ura-101*::Tn5C40 (lane 6), *ade-104*::Tn5C3 (lane 7), and *ilv-101*::Tn5C18 (lane 8). The positions of the wild-type *Swa*I restriction fragments are shown on the left, in the nomenclature of Allerdet-Servant et al. (1). Only one wild-type restriction fragment is missing in each mutant strain.

somes earlier by Southern hybridization (1), but we were unable to assign specific map positions for them.

Auxotrophic markers were found for almost all biosynthetic pathways, with the major exceptions being the arginine, lysine, and proline pathways. Auxotrophic markers are present on both chromosomes but predominantly (conservative estimate of 37 of 43 loci) on the circle. The essential genes on the circular chromosome are widely scattered, with little evidence of pathway-specific gene clusters. In contrast, we were unable to find auxotrophic markers on over one-third of the linear chromosome. This was not due to the lack of Tn5(pfm) insertions in the linear chromosome, since prototrophic Tn5(pfm) insertions were found on the two chromosomes at comparable frequencies, with 13 hits on the circular chromosome and 15 hits on the linear chromosome (Table 4).

Correspondence of the physical-genetic map to previous genetic maps. It seemed reasonable to suggest that the circular chromosome recognized by physical mapping and further characterized in this study is the same as the circular chromosome from earlier genetic mapping studies. To further anchor our physical and genetic map in comparison to previous genetic maps, we tested whether a methionine biosynthetic gene used in previous genetic mapping studies was the same as any of the methionine biosynthetic genes identified by Tn5(pfm) mutagenesis. The chemically induced auxotrophic mutation *met6* had previously been shown to map very close to both the *att* gene cluster, required for initial binding of *A. tumefaciens* to plant cells, and the *cel* gene cluster, encoding a cellulose bio-

synthesis pathway (48). Both the *att* and *cel* gene clusters have been separately isolated from genomic cosmid libraries (40, 41). To determine if the cosmid clones carrying the *att* and *cel* gene clusters also harbored the wild-type *met6* gene and whether that gene would complement any of our Tn5(pfm)-induced methionine auxotrophs, the *att* gene cluster cosmid pG644, the *cel* gene cluster cosmid pCP13.101, and pRK290, the parent plasmid on which the cosmids are based (17, 21, 36), were independently mobilized into *A. tumefaciens* A348 strains carrying the auxotrophic mutations *met-101*::Tn5C22, *met-102*::Tn5C23, and *met-102*::Tn5C24. The parent plasmid pRK290 and the *att* gene cluster cosmid pG644 failed to complement any of the mutations. However, the *cel* gene cluster cosmid pCP13.101 complemented the *met-102*::Tn5C23 and *met-102*::Tn5C24 mutations, which have transposon insertions at the same location.

Ability of the circular and linear chromosomes to be mobilized. One possible explanation for the failure of the original genetic mapping experiments to detect both chromosomes may be a reduced ability of the linear chromosome to be mobilized. We used R68.45, the same conjugable plasmid used in many of the original genetic mapping experiments, in experiments to determine if the circular and linear chromosomes each could be mobilized (27). The basic strategy was to mate donor and recipient strains carrying different Tn5(pfm) insertions. The donor strain also harbored R68.45. Chromosome mobilization was determined by selection for recipients in which the Tn5(pfm) insertion site of the recipient strain had been replaced by its wild-type counterpart from the donor strain (Table 5). In a control experiment where the donor (*cys-101*::Tn5C9) and recipient (*cys-101*::Tn5C9) strains carried the exact same transposon insertion, no wild-type transconjugants were found, as expected. This also showed that Tn5(pfm) insertions do not revert at a measurable frequency, which makes sense since this minitransposon lacks a transposase gene (60). In experiments where the donor and recipient strains carried different Tn5(pfm) insertions, the circular and linear chromo-

TABLE 5. Frequency of R68.45-mediated-mobilization and recombination of markers on the circular and linear chromosomes

Mutation in donor strain carrying R68.45[a]	Mutation in recipient strain[a]	Frequency of wild-type transconjugants per recipient cell
cys-101::Tn5C9	cys-101::Tn5C9	$<2.3 \times 10^{-9b}$
gln-102::Tn5C15	cys-101::Tn5C9	6.0×10^{-6}
trp-101::Tn5C33	cys-101::Tn5C9	1.5×10^{-8}
aux-108::Tn5L7	cys-101::Tn5C9	4.2×10^{-6}
cys-101::Tn5C9	thr-101::Tn5L5	1.3×10^{-6}
gln-102::Tn5C15	thr-101::Tn5L5	4.6×10^{-6}
trp-101::Tn5C33	thr-101::Tn5L5	1.3×10^{-6}
aux-108::Tn5L7	thr-101::Tn5L5	8.3×10^{-8}
cys-101::Tn5C9	aux-108::Tn5L7	4.6×10^{-7}
cys-101::Tn5C9	gln-102::Tn5C15	7.7×10^{-6}
cys-101::Tn5C9	trp-101::Tn5C33	$<4.1 \times 10^{-9b}$

[a] The C or L designation for each insertion indicates its location on the circular or linear chromosome, respectively.

[b] No transconjugants were recovered, and so the number given is an estimate of the upper limit possible.

somes were mobilized at comparable frequencies. The only exceptions were cases in which the auxotrophic markers in the donor and recipient strains were near each other on the same chromosome (trp-101::Tn5C33 × cys-101::Tn5C9, cys-101:: Tn5C9 × trp-101::Tn5C33, and aux-108::Tn5L7 × thr-101::Tn5L5).

DISCUSSION

We were able to confirm the two-chromosome genome organization in A. tumefaciens by constructing a combined genetic and physical map of the circular and linear chromosomes (Fig. 2). A strong case can be made that the circular chromosome is the chromosome on which previous genetic maps are based. The previous genetic maps are congruent with one another and are consistent with a circular chromosome (11, 29, 44, 47, 48). Furthermore, the chvAB genes, encoding enzymes involved in extracellular β-glucan production, had been placed on one of the genetic maps and were later shown in the initial physical mapping to hybridize to PacI fragment A and SwaI fragment A of the circular chromosome (1, 11, 18). Finally, we were able to prove that the met6 gene, located on one of the genetic maps, is the same as one of the methionine biosynthetic genes we physically localized to the circular chromosome (48).

We can rule out some explanations of why the genetic mapping approaches missed the linear chromosome. First, the chromosome mobilization experiment shows that genetic markers on the linear chromosome can be mobilized by R68.45 at frequencies comparable to those for markers on the circular chromosomes (Table 5). This shows that the linear chromosome is not recalcitrant to conjugation-based mobilization due to its topology. Second, we found prototrophic Tn5(pfm) insertions at similar frequencies for both chromosomes. This discounts the possibility of transpositional bias between the chromosomes. Therefore, we are left with a simple but intriguing hypothesis. Since the genetic mapping approaches mainly used auxotrophic markers and we found that virtually all such markers (~86%) lie on the circular chromosome, it is possible that the small collections of auxotrophic strains used for the genetic maps do not contain any examples of mutations on the linear chromosome. We believe that this hypothesis is robust based on the large number of independent auxotrophic markers, both characterized and uncharacterized, that we physically mapped. The fact that some biosynthetic pathways (arginine,

lysine, phenylalanine, proline, and tyrosine) are not represented in our collection is not worrisome, since it is possible to find such auxotrophic markers for these pathways in A. tumefaciens and since several of these markers were mapped to the circular chromosome by purely genetic approaches (11, 29, 44, 47, 48).

The paucity of auxotrophic markers on the linear chromosome brings up the question of the origin of the two-chromosome state in this genus (32). If the current chromosomes resulted from a splitting of a single ancestral chromosome, one might expect those two chromosomes to have similar densities of auxotrophic markers. To see if this is true for single chromosome genomes, we looked at the distribution of putative auxotrophic markers (i.e., genes involved in amino acid, cofactor or vitamin, and nucleotide biosynthesis that, when mutated, would lead to auxotrophy) in the published genomic sequences of several members of the Eubacteria and Archaea (3, 4, 14, 20, 33, 35, 37, 51, 56). In the genomes analyzed, auxotrophic markers are rarely separated by more than 100 kbp, with the largest gap being less than 300 kbp. An even better comparison is the recent low-resolution sequencing of approximately one-third of the ~0.9-Mb chromosome II of Rhodobacter sphaeroides 2.4.1[T] (8, 9, 54). Putative auxotrophic markers were found at a density slightly lower than but comparable to that for the single-chromosome genomes. In contrast, we found only six auxotrophic markers on the 2.1-Mbp linear chromosome of A. tumefaciens, with approximately one-third of the linear chromosome being devoid of such markers. As detailed genetic maps or complete genomic sequences become available for other species with multiple non-homologous chromosomes, such as Brucella melitensis (31, 43), Burkolderia (formerly Pseudomonas) cepacia (7), and Vibrio cholera (57), it will be interesting to see if they show asymmetry in the distribution of auxotrophic markers between their chromosomes.

One hypothesis to explain the lack of auxotrophic markers on one-third of the linear chromosome is a bias against Tn5(pfm) jumping into this region due to a different base composition. The only data we obtained that can address this idea is the distribution of randomly chosen prototrophic Tn5(pfm) insertions. Of 15 such insertions on the linear chromosome, 4 mapped to the region lacking auxotrophic markers (Table 4). This number closely matches that expected for random insertion of the transposon. While this small data set cannot disprove the hypothesis, it is highly suggestive that transpositional bias is not the cause of the asymmetrical distribution of auxotrophic markers on the linear chromosome.

An alternative hypothesis is the acquisition or evolution of a large cluster of nonessential genes either on the ancestral chromosome before the split into two chromosomes or on the linear chromosome after the split. For example, the linear chromosome may contain a large gene cluster specifically involved in the interaction of A. tumefaciens with plant tissue. This is seen in several animal pathogens, where many virulence genes are clustered into "pathogenicity islands" (28, 38). Of the known A. tumefaciens chromosomal virulence genes, only the chvAB operon, the att gene cluster, the cel gene cluster, and the ros gene have been mapped, and all are located on the circular chromosome (1, 11, 48). To further test this hypothesis, the other known chromosomal virulence genes and non-virulence genes implicated in the plant-microbe interaction need to be localized on the physical map (6, 26, 30, 34, 42, 46, 49, 50, 55).

Ultimately, a fuller understanding of the genetic structure and role of the two chromosomes in the ecology of A. tumefaciens will require genomic sequencing. We have initiated such an effort for the ~710-kbp PacI fragment D of the linear

chromosome. We hope to verify the real size of the auxotrophic marker gap on the linear chromosome. Other benefits will include the identification of (i) additional genes that can be used for structure-function, evolutionary, and comparative genomic studies, (ii) a bacterial telomere, and (iii) genes involved in the interaction of *A. tumefaciens* with plant tissues.

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