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Development of Effective Chromosomal Gene Transfer Systems and Mapping of Agrobacterium tumefaciens 15955 Chromosomal Loci

Diane L. Pischl
Loyola University Chicago

DEVELOPMENT OF EFFECTIVE CHROMOSOMAL GENE TRANSFER SYSTEMS AND MAPPING OF *AGROBACTERIUM TUMEFACIENS* 15955 CHROMOSOMAL LOCI

by

DIANE L. PISCHL

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VITA

Diane Pischl was born December 22, 1952, in Lebanon, Indiana. She is the eldest of four children born to William and Arline Hayden. After graduating from Waveland High School in Waveland, Indiana, Diane enrolled in DePauw University, from which she received an A. B. degree in Bacteriology in June, 1975. Following her marriage later that year to John Pischl, Diane worked as a clinical microbiologist in Fort Knox, Kentucky, and in Elmhurst, Illinois. In 1978, she began graduate studies in the Department of Microbiology at Loyola University of Chicago. During her tenure there, she was awarded a number of Basic Science Fellowships and a Schmidt Dissertation Fellowship. She is a member of the American Association for the Advancement of Science and of the American Society for Microbiology.

Diane is the co-author of three publications:

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LIST OF ABBREVIATIONS

bp	base pairs
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
kb	kilobases
LPS	lipopolysaccharide
MNNG	N-methyl-N'-nitro-nitrosoguanidine
RNase	ribonuclease
SDS	sodium dodecylsulfate
Tris-HCl	tris hydroxy(aminomethane) hydrochloride

PHENOTYPE DESIGNATIONS

Superscript	Phenotype
r	resistant
s	sensitive
+	prototrophic
-	auxotrophic

CHAPTER 1

INTRODUCTION

The role of Agrobacterium tumefaciens in the inception of crown gall disease of plants has been under investigation since 1907, when it was identified as the etiologic agent (Smith et al., 1907). This organism, a normal inhabitant of the soil rhizosphere, enters the plant at a wound site, establishes an infection and induces the plant to form tumors or "galls". The disease derives its name from the fact that the galls are often seen at the root crown of the host plant.

The crown gall system has for many years been of considerable interest as a model of cancer, for the diseased plant tissue in many respects is similar to an animal tumor. It shows nonself-limiting growth, cellular dedifferentiation, apolar as well as chaotic cell division, autonomous growth in vitro in the absence of the hormones required by normal tissue, and the tissue maintains its tumorous phenotype when grafted onto the root stock of a healthy host plant (Jensen, 1910; Smith, 1916a,b; White et al., 1942). Along with these properties, crown gall tissue has also acquired the ability to synthesize from normal plant constituents one or more novel low molecular weight carbon compounds called opines. In fact, crown gall tumors are commonly classified on the basis of the opines that they synthesize, with each class of tumor named for the predominant or earliest-identified opine. The four major groups of opines which

have been identified so far include the octopine, nopaline, agropine and succinamopine groups (Nester, 1984, and references therein; Dahl, et al., 1983; Chang et al., 1983; Chilton et al., 1984).

In addition to its value in cancer research, crown gall disease is also of considerable importance in agriculture and horticulture. A broad range of dicotyledonous plants are susceptible, including 643 species distributed among 331 different genera (DeCleene et al., 1976). Recently it has also become possible to establish experimental infections in some species of monocots as well (Hooykaas-Van Slogteren et al., 1984). The economic losses are significant. In 1976 alone, crop losses in the United States from A. tumefaciens infections were estimated at $23 million, an amount second only to losses caused by Pseudomonas glycinea (Kennedy et al., 1980). The crown gall system is even more important agriculturally as a potential tool to effect genetic engineering of plants, and at present considerable effort is being directed toward developing that potential (Bevan et al., 1983b; Herrera-Estrella et al., 1983a; Zambryski et al., 1983a; Fraley et al., 1983). The current expansion in crown gall research is also reflected in the ready availability of recent reviews (Bevan et al., 1982; Caplan et al., 1983; Zambryski et al, 1983b; Verma et al., 1984; Hooykaas et al., 1984a; Nester, 1984).

One aspect of crown gall tumorigenesis that has received relatively little attention is the analysis of A. tumefaciens chromosomal genes that make contributions to tumorigenesis. This

study is intended to facilitate such an investigation. Chapter 2 contains a selective review of the experimental evidence leading to the current understanding of events involved in the induction and proliferation of crown gall tumor cells. It also develops the rationale for a chromosomal genetic analysis of virulence determinants. The remaining chapters describe the development and application of methods of transferring and mapping chromosomal genes. It is hoped that this work will lead to the development of more powerful methods of chromosomal genetic manipulation in Agrobacterium.

CHAPTER 2

REVIEW OF RELATED LITERATURE

Background

Many of the early studies of crown gall tumorigenesis dealt with the diseased plant host. One conclusion drawn from such investigations was that, for reasons that were not entirely clear, a wound was required for tumor formation (Riker, 1923a). Braun subsequently studied the role of the wound by comparing the tumorous response of host cells not wounded prior to infection with that of cells wounded at various times before inoculation (Braun, 1952). To do so, he provided a uniform 24-h transformation period by making use of an observation that incubation at a temperature of 32°C. prevents transformation without inhibiting cell multiplication (Riker, 1926; Braun, 1947). He inoculated sets of Kalanchoe plants at various times after wounding, incubated them at 25°C. for 24 h and then shifted them to 32°C. to prevent further transformation events. The plants which had been wounded 48-72 h prior to infection developed the largest tumors, while those that had not been wounded prior to infection showed only an occasional tumorous response. He interpreted these results to mean that the inoculated plant is not immediately susceptible to transformation, but must first undergo a process of "conditioning," which seemed to be linked to the wound healing process (Braun, 1952). The phase of maximum susceptibility appeared to coincide with the period shown by histological studies of

wounded plant tissue (Braun et al., 1948) to immediately precede cell division. These findings raised the possibility that transformation was linked to a specific phase of the host cell cycle, perhaps the S phase. This proposed link between transformation and host cellular DNA synthesis received additional support when subsequent studies showed that compounds such as iododeoxyuridine or mitomycin C, which interfere with DNA synthesis, were able to prevent tumor formation (Lang et al., 1966; Gribnau et al., 1969).

Other early studies were directed toward defining the contributions of the bacterium in tumor induction. For example, Riker's histological studies of infected tissue showed that the bacteria were present only in intercellular spaces and vessels, indicating that the organism was noninvasive (Riker, 1923b). Second, studies by Lippincott et al. (1966, 1969b) of naturally-occurring and UV-induced auxotrophs suggested a requirement for bacterial growth and metabolism during tumorigenesis. Using a quantitative pinto bean leaf assay in which the number of tumors induced is directly proportional to the size of the bacterial inoculum, these investigators showed that auxotrophs induced relatively fewer tumors than did wild-type bacteria and that either reversion of the auxotrophic marker or applications of the required nutrient to the host plant restored virulence to wild-type levels. A third finding was that bacterial RNA synthesis was essential for tumorigenesis. Braun et al. (1966) and Beiderbeck (1970b) showed that application of RNAse or rifampin prior to inoculation of conditioned plants prevents tumor formation.

However, rifampin was ineffective with drug-resistant bacteria or if applied more than 24 h after inoculation (Beiderbeck et al., 1970a).

Binding

A number of studies have shown that another stage of tumorigenesis involves binding of the bacterium to a susceptible host plant cell. The first evidence of binding was the observation that the addition of avirulent or heat-killed bacteria to the inoculum caused a proportionate reduction in the number of tumors that formed on pinto bean leaves (Lippincott et al., 1969a). This competitive inhibition appeared to be specific for Agrobacterium strains, and was observable only if the coinfecting bacteria were added prior to or less than 15 minutes after inoculation. These results suggested that the inhibitory cells were able to compete with virulent bacteria for a limited number of plant cell receptor sites, and that they need not be viable to do so. Similar observations were reported by other investigators (Kerr, 1969; Manigault, 1970; Glogowski et al., 1978).

In initial attempts to characterize the bacterial components involved in binding, Whatley et al. (1976) demonstrated that the inhibitory activity was localized in the lipopolysaccharide (LPS) layer. Lippincott et al. (1980) subsequently reported that the polysaccharide portion of the LPS was inhibitory, but that the lipid A moiety was not. Other investigators have characterized the bacterial binding component by fluorescent microscopic techniques (Banerjee et al., 1981). They observed that the quenching effect caused by the interaction of fluorescein isothiocyanate-tagged LPS with

plant cell wall components could be prevented by preincubation of the wall components with either β-D-galactose or N-acetyl-galactosamine. Such results suggested that binding involves both of these components which form the polysaccharide side chains projecting from the bacterial cell surface.

Additional support for the binding theory comes from both quantitative and electron microscopic demonstrations that A. tumefaciens binds in vitro to plant cells from suspension culture (Matthysse et al., 1978; Ohyama et al., 1979). One drawback of such in vitro studies is the uncertainty involved in making extrapolations to the in vivo situation. For this reason, Matthysse et al. (1982) examined a wide variety of host plants for the degree of correlation between their in vivo susceptibility to infection and their ability to act as substrates for bacterial binding in vitro. With the exception of the soybean (a monocot), only hosts that were susceptible to in vivo infection would permit in vitro binding, which suggested that the in vitro system provided a valid assessment of bacterial attachment proficiency.

Lippincott et al. (1977) have also used the in vivo competitive inhibition assay of tumorigenesis on pinto bean leaves to investigate the plant cell receptor recognized by A. tumefaciens. They found that isolated cell walls but not membranes from pinto bean leaves were competitively inhibitory. However, cell walls from which the pectin had been extracted appeared to be depleted of their inhibitory

capacity, while the pectic extract itself remained inhibitory (Rao et al., 1982). These results suggested that pectin (a polymer of α-1,4-linked D-galactopyranosyluronic acid units interspersed with 1,2-linked rhamnopyranose units), constitutes the receptor. This interpretation should, however, be viewed with some caution, since pectic substances are known to elicit synthesis of the antimicrobial phytoalexins, whose effect on tumor-inducing bacteria is not known.

Genetic Studies of Crown Gall Tumorigenesis

The first clear indication that crown gall disease has a genetic basis came from the observations that there appeared to be different classes of agrobacteria with respect to the particular opine(s) they catabolized, and that the identity of the opines synthesized in the tumor correlated not with the host plant but with the opines catabolized by the inciting bacterium (Petit et al., 1970). A second hint came with the finding of in planta transfer of oncogenicity from virulent bacteria to saprophytic A. radiobacter strains (Kerr, 1971). The key to understanding the genetic nature of the disease was the discovery of large plasmids ranging in size from 145 to 236 kilobases (kb) in tumorigenic strains (Zaenen et al., 1974). That these plasmids confer tumorigenicity was subsequently shown by demonstrating that strains that acquired virulence during in planta crosses also gained a plasmid and that strains that lost virulence when incubated at elevated temperatures also lost the plasmid (van Larebeke et al., 1975; Watson et al., 1975). Acquisition or loss of the plasmid was also accompanied by gain or loss of the ability to catabolize opines

such as octopine or nopaline, which indicated that opine utilization is another trait specified by these tumor-inducing (Ti) plasmids (Watson et al., 1975; Bomhoff et al., 1976).

Further genetic analysis of octopine- and nopaline-type Ti plasmids was conducted by transposon insertion and deletion mutagenesis (Koekman et al., 1979; Holsters et al., 1980; DeGreve et al., 1981). This approach permitted the mapping of the following Ti plasmid loci: (1) onc, including a cis-active site and several trans-acting oncogenicity and opine biosynthetic functions, (2) vir, containing numerous trans-acting functions required for virulence, (3) opine catabolic functions, (4) tra, encoding conjugal transfer functions that are expressed in the presence of certain opines, (5) replication and incompatibility functions, (6) determinants of plant host range, (7) agrocin-84 sensitivity in nopaline plasmids and (8) bacteriophage AP-1 exclusion.

Transfer and Integration of Bacterial DNA

The foundation for understanding the molecular basis of transformation of normal plant cells to crown gall tumor cells was laid by Chilton et al. (1977), who showed by DNA reassociation kinetics that crown gall but not normal cells contained DNA sequences homologous with a particular restriction fragment from the Ti plasmid of the inciting bacteria. These sequences in the tumor that showed homology with the plasmid were designated T-DNA, and the corresponding plasmid sequences were called the T-region.

Combined with the prior observation that opine synthesis in the tumor is specified by the Ti plasmid, the finding that some but not all plasmid sequences appeared in the tumor raised the possibility that there was some specificity to the T-DNA. Indeed, the observation by Merlo et al. (1980) that four independent tumor lines induced by strain B6-806 each contained DNA sequences that were able to accelerate the rate of reassociation of a particular fragment of plasmid pTiB6-806 supported this suggestion. That various tumor lines harbor nearly identical sets of T-DNA fragments became clear when they were examined by Southern blotting techniques. With this approach tumor DNA was digested with a restriction endonuclease, fractionated by agarose gel electrophoresis, denatured and blotted to nitrocellulose. After radiolabelled Ti plasmid fragments were hybridized to the bound DNA, autoradiography of the blots revealed that independent tumor lines induced by a single strain of A. tumefaciens each contained nearly identical sets of T-DNA fragments. These fragments could be grouped in three classes: (1) junction fragments containing the right or left edge of T-DNA covalently attached to plant DNA sequences, (2) internal fragments entirely homologous with Ti plasmid sequences, and (3) fusion fragments consisting of the right or left end of T-DNA sequences joined to T-DNA sequences that usually mapped as internal fragments. Fusion fragments were suggested to arise either by integration of tandem T-DNA copies or through the formation of circular polymers of T-DNA sequences (Lemmers et al., 1980; Chilton et al., 1980).

Octopine tumors were shown by these techniques to contain a 13.5 kilobase (kb) segment designated T_L-DNA; some in addition contained a noncontiguous 6 or 7 kb T_R-DNA (Thomashow et al., 1980b; DeBeuckeleer et al., 1981; Ooms et al., 1982a). In most tumor lines the right and left boundaries of T_L-DNA mapped to one pair of T-region fragments. In nopaline tumors the T-DNA was integrated as a single colinear segment 23 kb in size, and the boundaries appeared to be less variable than those of octopine T_L-DNA (Lemmers et al., 1980; Chilton et al., 1980; Zambryski et al., 1980). Fusion fragments were occasionally noted in both types of tumors. In addition, the T-DNA sequences in each tumor appeared to coincide with a region of nucleotide sequence homology in octopine and nopaline plasmids (Drummond et al., 1978). Thus, the results of Southern blot analyses indicated that there was considerable specificity to the T-DNA transfer and integration process.

The specificity of T-DNA boundaries was further borne out by analysis of nucleotide sequences at the junctions of plant and bacterial DNA. In nopaline Ti plasmids a 25 base pair (bp) sequence was repeated (with a few nucleotide changes) in direct orientation on either side of the T-region sequences (Zambryski et al., 1980). In four nopaline tumor lines the right T-DNA boundary occurred either at the first nucleotide of the repeat or one nucleotide internal to the repeat (Zambryski et al., 1980, 1982; Yadav et al., 1982). The left endpoint in these tumors varied, occurring in one case within the repeat and in the others at a site between 86 and 93 bp inside the

repeat. For octopine Ti plasmids a related 25 bp sequence was found directly repeated on either side of the T-region DNA, and in two octopine tumors examined the T_L-DNA endpoints occurred within or very near the repeat on the right side (Holsters et al., 1983) and within or 57 bp internal to the repeat on the left side (Holsters et al., 1983; Simpson et al., 1982). These findings suggested that the imperfect direct repeats function to determine the endpoints of T-DNA, perhaps by serving as recognition signals for T-DNA transfer or integration.

The role of these repeats has been studied in more detail. Octopine plasmids deleted for the right side of T_L were found to be nontumorigenic and could not be complemented in trans (Ooms et al., 1982b; Leemans et al., 1981). However, plasmids deleted for the left side of T_L or for T_R remained virulent, as did plasmids lacking the right side of T_L but retaining T_R. For nopaline plasmids, deletion of the right T-region border abolished virulence, but deletion of the left border had no effect on tumorigenicity (Joos et al., 1983a; Wang et al., 1984). Along with the above data on junction sequences, these results suggested that the right border repeat was required for tumorigenicity, but that sequences near T_R in octopine plasmids could also meet that requirement. The requirement for the left repeat appeared to be minimal.

The results of two other experiments have shown that the T-DNA border repeats must be present to allow normal T-DNA transfer and integration. Joos et al. (1983b) demonstrated that a borderless Ti

plasmid was unable to transfer and integrate its T-DNA except when cointegrated with another plasmid harboring functional border repeats. In the complement of that experiment, Zambryski et al. (1983b) showed that a Ti plasmid containing border repeats but deleted for all internal T-DNA sequences except the dispensable nopaline synthase gene (which served as a T-DNA marker) remained proficient at T-DNA transfer, as indicated by the synthesis of nopaline in the plant.

The requirement for the right border repeat has been unequivocally established in two recent studies. Shaw et al. (1984b) constructed a set of plasmids with partially overlapping deletions extending rightward from a site about 150 nucleotides to the left of this repeat. A deletion extending to within 3 bp of the repeat had no effect on tumorigenicity, whereas a deletion extending 39 nucleotides beyond the repeat abolished virulence. Wang et al. (1984) further showed that insertion of a chemically synthesized right repeat sequence in its native orientation restored tumorigenicity in a right-borderless nopaline plasmid. In contrast, insertion of the repeat in the opposite orientation yielded a plasmid with strongly attenuated tumorigenicity.

Whether specific host nucleotide sequences act as preferred integration sites is not known, but the answer to this question may be important for understanding the mechanism of T-DNA integration. In preliminary investigations, the host sites of insertion were

studied by Southern analytic techniques. Cloned border fragments, when hybridized to genomic blots of normal tobacco DNA gave either smears or numerous discrete bands on autoradiographs, indicating that the T-DNA was integrated in or near repetitive host sequences (Yadav et al. 1980; Thomashow et al., 1980b; Zambryski et al., 1980; Holsters et al., 1982, 1983). However, in tumor lines from sunflower plants, labelled junction fragments hybridized to only a limited number of genomic fragments, indicating that the T-DNA had integrated into unique or low repetitive plant sequences (Ursic et al., 1983). These studies were limited by their relatively low level of resolution. Additional information regarding insertion site specificity can be obtained by extensive nucleotide sequence analyses.

Although the precise sequence of events leading to T-DNA transfer and integration is still unclear, some inferences can be made at this time. First, it appears that T-region border sequences are required in cis, while other Ti plasmid functions can act in trans (Zambryski et al., 1983b; Joos et al., 1983b; Leemans et al., 1981). The strict cis requirement for the right 25 bp repeat suggests that it may function as a recognition site for a restriction endonuclease (Joos et al., 1983b; Shaw et al., 1984b; Wang et al., 1984; Ooms et al., 1982b). Second, these T-DNA recognition signals may actually function in the bacterium rather than in the plant. This possibility arises from the observation that the boundaries of T-DNA in tumor tissues produced by introducing naked plasmid DNA into plant cell protoplasts tend to differ from those of tumors produced

by infection with whole bacteria (Krens et al., 1982; Draper et al., 1982). Third, T-DNA may integrate as a linear segment of DNA. Previously, the presence of fusion fragments in tumor DNA was taken as evidence that the substrate for integration was a circular DNA molecule. However, the recent discovery that at least some such fragments contain plant DNA sequences between the tandem copies of T-DNA has strengthened the idea that fusions arise by rearrangements secondary to the initial integration event (Holsters et al., 1983). Fourth, since the right 25 bp repeat is far more active in one orientation than in the other, the process of T-DNA transfer and integration must be highly polarized (Wang et al., 1984). Characterization of the nucleotide sequences of various border repeats has revealed that all repeats differ from one another at two adjacent nucleotides, and that right repeats differ from left repeats at two additional nucleotides (Wang et al., 1984). The most obvious explanation is that the latter two nucleotides are functionally important for determining the polarity of T-DNA transfer and integration. Finally, it can be said that, in at least some cases, sequences outside the T-DNA can be transferred to the plant. Joos et al. (1983b) showed that cointegrate plasmids containing insertions of T-DNA onc and opine synthetic genes some distance away from the border repeats were able to induce opine synthesis in plant tissue at a low frequency. These results are consistent with models postulating transfer to the plant of a complete copy of the Ti plasmid. Thus, there are several interesting clues to T-DNA processing,

but it is not yet clear exactly how these bacterial sequences are transferred and integrated into plant DNA.

T-DNA Gene Expression

There have been a number of indications that T-DNA encodes multiple gene products that are expressed in the tumor. For example, RNA transcripts homologous to Ti plasmid DNA are detectable in tumor tissue (Drummond et al., 1977; Gurley et al., 1979; Yang et al., 1980). The various transcripts were found to differ from one another in relative abundance, which suggested that their expression was controlled at individual promoters within T-DNA, rather than at exogenous promoters in the surrounding plant DNA. To test this, Gelvin et al. (1982) hybridized two sets of probes to polyadenylated tumor RNA. One probe was a junction fragment containing both T-DNA and plant DNA sequences. The other consisted of similar T-DNA sequences but lacked the adjoining plant sequences. In each case, the transcripts which hybridized to the larger probe were the same size as those detected with the smaller T-DNA probe, indicating that little if any expression of T-DNA occurred by initiation of transcription at external plant promoters.

Further evidence that T-DNA controls its own expression has been obtained by nucleotide sequence analysis of regions that are actively transcribed in the tumor (Bevan et al., 1983a; Depicker et al., 1983; DeGreve et al., 1983; Heidekamp et al., 1983; Barker et al., 1984; Gielen et al., 1984; Klee et al., 1984; Goldberg et al.,

1984; Lichtenstein et al., 1984; Sciaky et al., 1984). Each region contains open reading frames surrounded by eukaryotic-like transcriptional control signals. Sequences similar to the eukaryotic consensus sequences CCAAT and TATAAA (Breathnach et al., 1981) occur at appropriate positions upstream from the initiation codon, and polyadenylation signals follow the termination codon. In addition, deletion mutagenesis of sequences upstream from the octopine or nopaline synthase genes has revealed that these T-DNA genes carry all the sequences necessary for their expression (Koncz et al., 1983; Shaw et al., 1984a). Furthermore, expression of chimeric genes constructed by fusing the promoter region from the T-DNA nopaline synthase gene to heterologous coding sequences has been demonstrated in plant cells (Herrera-Estrella et al., 1983b; Bevan et al., 1983a; Fraley et al., 1983). This acquisition by a prokaryotic bacterium of gene expression signals that function in a wide range of eukaryotic plants is an intriguing evolutionary puzzle.

The specific transcripts synthesized in octopine and nopaline tumor tissues have been mapped with respect to T-DNA location, size and polarity (Murai et al., 1982a; Willmitzer et al., 1982, 1983). Transcripts mapping to the left-hand portion of nopaline T-DNA (outside the region of conserved plasmid sequences) were unlike octopine transcripts. However, octopine and nopaline transcripts mapping to the central conserved region appeared to be similar to each other in size and polarity (Willmitzer et al., 1982, 1983). Moreover, similar pairs of octopine and nopaline transcripts hybridized to the same

regions of the octopine plasmid. These transcripts in octopine tumor tissues were given the designations 1, 2, 4, 5, 6a and 6b, with similar numbers followed by a subscript "n" for the nopaline tumor transcripts. On the right side of octopine T-DNA a transcript shown by *in vitro* translation and immunoprecipitation to encode octopine synthase was mapped (Schröder *et al.*, 1981; Murai *et al.*, 1982b; Willmitzer *et al.*, 1982). The nopaline synthase transcript likewise maps to the right side of nopaline T-DNA (Bevan *et al.*, 1983a; DePicker *et al.*, 1983; Holsters *et al.*, 1980). Thus, the functional organization of the central region of octopine and nopaline T-DNA's appears to be closely conserved, while the right and left sides of each had been allowed to diverge.

Genetic studies of T-DNA were facilitated by the use of site-directed mutagenesis techniques. Specific T-region DNA fragments previously subcloned into small plasmids were mutagenized in *E. coli* and then transferred back into *A. tumefaciens*, where by homologous recombination they replaced the corresponding wild-type sequences (Garfinkle *et al.*, 1981). Such mutations constructed in an octopine plasmid were subsequently tested for their effect on tumorigenicity. No single mutation abolishing virulence was detected. However, mutations in any of three loci did alter the morphology of the tumors (Garfinkle *et al.*, 1981; Ooms *et al.*, 1981). For example, tumors with numerous shoots were produced when the plasmid contained a transposon insertion within a 3 kb region designated *tms*. In addition, tumors with roots were induced by plasmids with a insertions in

the tmr locus, while unusually large tumors resulted from mutations in the tml region. Similar tumor morphology loci were also described for a nopaline Ti plasmid (Joos et al., 1983a).

Octopine plasmid tumor morphology loci were further studied by analyzing plasmids that contained multiple mutations (Ream et al., 1983). Two classes of double mutants, tml tmr and tml tms, remained virulent, but the third class, tmr tms, was completely avirulent on most plants and induced only a very weak response on others. The triple mutant, also nontumorigenic, was nevertheless able to induce the synthesis of opines at the host inoculation site. These observations indicate that either the Tmr^+ or the Tms^+ gene product alone is sufficient to induce tumor formation in most plants, and that the loci involved in T-DNA transfer and integration are controlled independently from loci affecting tumor morphology.

Ooms et al. (1981) noted that the morphology of tissue produced by tmr or tms mutants resembled that of normal plant tissue grown in vitro on relatively high concentrations of either auxin or cytokinin. High levels of auxin (which regulates cell enlargement) are known to stimulate shoot production, while relatively high levels of cytokinin (which controls cell division) promote root proliferation (Skoog et al., 1957). Crown gall tissue, unlike normal tissue can grow in vitro in the absence of these plant hormones (Braun et al., 1958). Therefore, Ooms et al. (1981) reasoned that the T-DNA tumor morphology loci must encode products that alter the levels of these hormones in the plant. In support of this explanation, Akiyoshi et

al. (1983) observed that, compared to wild-type tumor tissue, tms and tmr tissues contained relatively low levels of auxin and cytokinin, respectively. Furthermore, it was also shown that applications of the appropriate hormone could restore the mutant tumor morphology returned to normal (Ooms et al., 1981; Joos et al., 1983a).

Three observations strongly suggest that these tumor morphology loci encoded structural gene products. First, each mutation results in the loss of a single polyadenylated transcript (Leemans et al., 1982; Joos et al., 1983b). A mutation in tms resulted in the loss of either transcript 1 or 2, while insertions in tmr abolished transcript 4 and tml mutations likewise prevented the formation of either transcript 6a or 6b. Second, some tumor morphology mutations could be complemented in trans by coinfection of a strain harboring a Ti plasmid with the wild-type form of the gene in question (Inzé et al., 1984). Third, DNA sequence analysis has revealed the presence of extended open reading frames in these regions (Klee et al., 1984; Sciaky et al., 1984; Lichtenstein et al., 1984; Goldberg et al., 1984; Heidekamp et al., 1983). Combined with the evidence of variations in auxin and cytokinin levels in mutant tumor tissues, these observations suggested that the products of the tumor morphology genes are enzymes involved in auxin and cytokinin metabolism.

Recently, two products of the tumor morphology genes were functionally identified by enzymatic assays of cell extracts made

from strains harboring the genes cloned into expression plasmids. Extracts from a strain harboring the Tmr^+ gene 4 (corresponding to transcript 4) contained an activity which could convert isopentenyl-pyrophosphate to isopentenyladenosine monophosphate, the first step of the cytokinin biosynthetic pathway in plants (Barry et al., 1984; Akiyoshi et al., 1984). Similarly, the product of gene 2 in the tms locus was shown to be indoleacetamide hydrolase, an enzyme which catalyzes the formation of the auxin indoleacetic acid from indole-acetamide (Schröder et al., 1984; Thomashow et al., 1984). Although such analysis of tms gene 1 has not yet been reported, the results of two studies support the idea that it also is involved in auxin metabolism. First, a portion of the predicted amino acid sequence showed homology with residues that form the adenine binding site of the Pseudomonas enzyme p-hydroxybenzoate hydroxylase (Klee et al., 1984). This observation is significant because adenine is also a component of auxins. Second, it was shown that although mutations in tms gene 2 could be complemented only by applications of α-naphthalene acetic acid (NAA), mutations in gene 1 could be complemented by either NAA or its precursor α-naphthalene acetamide (Inzé et al., 1984). Therefore the product of gene 1 is likely to be an enzyme whose product is acetamide.

The experimental evidence summarized above thus strongly points to a model of transformation in which T-DNA gene products alter the levels of auxin and cytokinin in the host, thereby disrupting regulatory systems that control cell growth and division. The end result

is an uncontrolled proliferation of cells synthesizing the opines that serve exclusively as carbon, nitrogen and energy sources for the inciting bacteria.

Vir Region Genes

That other loci outside the T-DNA are also essential for tumorigenicity was revealed by mutagenesis of octopine and nopaline Ti plasmids (Ooms et al., 1980; Garfinkle et al., 1980; Holsters et al., 1980; DeGreve et al., 1981). Such loci, designated vir (virulence), are located in regions of nucleotide sequence homology (Drummond et al., 1978) between octopine and nopaline plasmids. This evidence that vir region functions have been highly conserved received further support with the observation that nopaline plasmid vir genes were able to complement octopine plasmid T-DNA in trans, and octopine vir genes likewise could complement nopaline T-DNA (Hoekema et al., 1983; Hooykaas et al., 1984a).

Octopine plasmid vir region mutants have been characterized genetically by testing for complementation in trans by cloned plasmid fragments. With this approach, 11 vir expression units mapping in 6 physically separate loci (virA-virF) have been distinguished (Iyer et al., 1982; Hille et al., 1982; Klee et al., 1983; Hooykaas et al., 1984a). Some virA mutants showed an altered tumor morphology (small tumors, root proliferation), which suggested that this vir gene product influences plant hormone metabolism. The complementation analysis showed that virB contains at least 6 contiguous expression units, since mutations that mapped to the same virB expression unit

failed to complement one another, but did complement mutations in other virB units. So far, virB as well as virC mutations have resulted in the complete loss of tumorigenicity on every host tested. Adjacent to virC but mapping outside the region of conserved sequence is the octopine plasmid-specific virO locus, which appears to encode a product that controls host range, as does the virF product (Hille et al., 1984b; Hooykaas et al., 1984a). The virE locus may encode a diffusible product that acts outside the bacterium (Otten et al., 1984).

The nopaline plasmid vir region has been less well characterized. To date, 6 complementation groups, one of which showed host range restriction, have been identified (Lundquist et al., 1984).

One question yet to be fully answered is whether vir gene products act in the bacterium or in the host plant. So far, DNA sequences homologous to the vir region have not been detected in tumor tissue (Chilton et al., 1977; Thomashow et al., 1980a). One possible approach to this question is to study the DNA sequences of vir genes to determine whether they contain eukaryotic or prokaryotic expression control signals. A second approach, for which some positive results have been reported, is to ask whether vir DNA contains sequences that can drive the expression of a promoterless gene in a prokaryote (Close et al., 1984; Hille et al., 1984a). This strategy has the disadvantage that the direct selection for promoter-like sequences can lead to false positive results.

The functions of vir region gene products have not yet been elucidated. Some genes, particularly virA, may be involved in hormone metabolism and may be required in the early stages of tumor induction for such functions as conditioning of the plant cells. Pertinent to this line of reasoning is the report of an insertion in vir that simultaneously inactivated both tumorigenicity and the ability of the bacterium to release the auxin indoleacetic acid (IAA) into the culture medium (Liu et al., 1982). In addition, the loss of tumorigenicity by A. tumefaciens strains that acquire the IncW plasmids pSa or R388 has been attributed by some investigators to a substantial reduction in the levels of IAA released (Chernin et al., 1984). However, others have suggested that these plasmids interfere with attachment (New et al., 1983).

Vir region gene products may also be required for recognition and binding to host plant cells. Mutations in such genes should show trans complementation, but they may not necessarily show complementation by coinfection. Other vir genes may be involved in DNA transfer and integration. Such gene products could be DNA-binding proteins that recognize a particular sequence(s) on the Ti plasmid. Clearly there is a need for further study of vir genes and their products.

Chromosomal Virulence Determinants

The studies cited above have described genes on the Ti plasmid that function in tumorigenesis. There is in addition a substantial body of evidence that products of the chromosome are also required.

One of the first indications of chromosomal virulence determinants came from competitive binding assays by Lippincott et al. (1969a). These authors demonstrated that some Ti plasmidless A. tumefaciens or A. radiobacter strains, when added to the bacterial inoculum, were able to inhibit tumor formation by virulent bacteria. They also showed that the degree of inhibition observed was proportional to the number of plasmidless bacteria added to the inoculum. Similarly, in the in vitro systems, binding of A. tumefaciens to plant cells appeared to depend in part upon chromosomal gene products, since lipopolysaccharide extracts from Ti plasmidless strains were able to inhibit binding (Matthysse et al., 1978; Whatley et al., 1978).

The involvement of chromosomal gene products in binding is best illustrated by studies of mutants containing a chromosomal insertion of transposon Tn5 that inactivates both tumorigenicity and in vitro binding ability (Douglas et al., 1982). Such mutants were also unable to competitively inhibit tumor formation upon coinfection, and they failed to adsorb two Agrobacterium phages. For one mutant these phenotypes were shown to be genetically linked to one another by demonstrating that replacement of wild-type chromosomal sequences with homologous Tn5-containing sequences from the mutant resulted in the loss of tumorigenicity, binding ability and phage sensitivity. Further analysis of these nonbinding avirulent mutants showed that each could be complemented in trans by a single cloned wild-type chromosomal restriction fragment 11 kb in size (E. W. Nester, personal communication). Mapping experiments subsequently revealed that

the various Tn5 insertions were clustered in two regions, one 5 kb and the other 2 kb in length. These results indicated that multiple genes are involved in attachment. Interestingly enough, the 2 kb region shows nucleotide sequence homology with DNA from species of Rhizobium, a closely related organism that also interacts with plants and is phylogenetically related to Agrobacterium. Furthermore, preliminary results suggests that some genes on this fragment were concerned with cell wall biosynthesis (Nester, 1984). In addition to the above Att^- mutants, these investigators recovered several avirulent mutants that remained proficient at binding, a finding which suggested that chromosomal genes are also involved in other stages of tumorigenesis (Douglas et al., 1982).

Although host range mutations most frequently map to the Ti plasmid (Thomashow et al., 1980c; Loper et al., 1979), there are several indications that genes on the chromosome also affect this property. For example, Hamada et al. (1980) found that two isolates of A. tumefaciens B6 had lost the ability to induce tumors on kalanchoe. Transfer of the Ti plasmids from these strains into another chromosomal background resulted in transconjugants that were fully tumorigenic, indicating that a chromosomal mutation was most likely responsible for avirulence in the parent strains. A second indication of such chromosomal determinants came from a survey of host range variation in grapevine isolates by Knauf et al. (1982), who reported that transconjugants derived from two field isolates were more restricted in host range than were the parent strains. A third

line of evidence is that several chromosomal transposon insertion mutants have shown loss of tumorigenicity on some hosts (Garfinkle et al., 1980; Hooykaas et al., 1984a).

There also exist a number of other avirulent mutants that have not been functionally characterized but are believed to harbor chromosomal mutations (Sciaky et al., 1978; Douglas et al., 1982; S. K. Farrand, unpublished results; S. B. Gelvin, personal communication). Such a conclusion is based on observations that transconjugants bearing the Ti plasmids from these strains are virulent.

At present there is little evidence regarding the functions of other chromosomal virulence determinants, but several possibilities can be suggested. One role may be the synthesis and release of plant growth regulatory substances (such as indoleacetic acid) that may be required to initiate the conditioning process or to induce the plant cells to become amenable to take up and incorporate foreign DNA. The report by Liu et al. (1979) that the synthesis of indoleacetic acid is in part chromosomally determined supports this idea. Another possibility is that genes involved in flagellar motion and chemotactic response may be used to guide the bacterium to an environment containing susceptible wounded plant cells. There has, however, been one report of a nonmotile mutant that remained tumorigenic (Bradley et al., 1984). In addition to these specialized virulence functions, the bacterium may also utilize the existing DNA synthetic machinery to facilitate transfer of plasmid or T-DNA to the plant cell. Polymerases, ligase, topoisomerases and single-strand DNA-binding pro-

teins are just some of the chromosomal gene products that may function at this stage. Finally, some chromosomal gene products may play a role in overcoming plant defense reactions, thereby allowing the bacterium to establish itself in the host wound site (Staples *et al.*, 1981).

Summary and Perspectives

Crown gall tumorigenesis begins with the entry and establishment of *A. tumefaciens* at a wound site in the plant. The bacteria may elaborate a number of substances that facilitate establishment or induce host cellular repair processes. After interacting with specific receptors in the host cell wall, the bacteria transfer a copy of either the Ti plasmid or T-DNA sequences, possibly in a manner resembling the conjugal transfer of plasmids between bacteria. Specific T-DNA sequences then become integrated into host chromosomal DNA by a mechanism that may involve binding of an endonuclease at the 25 bp direct repeats surrounding the T-DNA. The subsequent expression of T-DNA genes results in the synthesis of novel carbon compounds that serve as specific growth substrates for virulent bacteria, and of plant growth regulatory substances that induce a rapid proliferation of host cells synthesizing these opines. Thus, *A. tumefaciens* creates its own ecological niche by the genetic engineering of plant cells.

It is obvious from the information given above that many of the details of crown gall tumorigenesis have yet to be discerned. How-

ever, it has been firmly established that gene products from two regions of the Ti plasmid are essential for tumorigenicity. One region, the T-DNA, carries both a *cis*-acting site thought to be required for transfer or integration, and *trans*-acting *onc* functions necessary for maintenance of the transformed state. The other, the *vir* region, encodes *trans*-active products that may function during the initial steps of tumorigenesis. In addition to these plasmid-determined components, a number of products of the *Agrobacterium* chromosome are required for virulence. Some appear to function during recognition and attachment, while others may regulate host range. Further analysis of avirulent chromosomal mutants has until now been hampered by the lack of a convenient chromosomal gene transfer system. The remaining sections of this work describe the development of a system designed to facilitate such investigations.

CHAPTER 3

MATERIALS AND METHODS

Some materials and methods used in this study are described in Chapters 4 and 5. However, for the sake of brevity in material intended for publication, some of the details were omitted in those sections. Where appropriate, a more complete description is given below.

Bacterial strains and plasmids

A complete listing of bacterial strains and plasmids used is given in Table 1 (pages 31-36).

Media

Agrobacterium tumefaciens was grown on AB minimal agar containing, in grams per liter: dibasic potassium phosphate, 3; monobasic sodium phosphate, 1; ammonium chloride, 1; magnesium sulfate, 0.3; potassium chloride, 0.15; calcium chloride dihydrate, 0.01; ferrous sulfate heptahydrate, 0.0025; glucose, 5, Bacto agar (Difco), 15 (Chilton et al., 1974). Where appropriate, L-amino acid and nucleotide supplements were added at 25 and 10 µg/ml, respectively. Antibiotics were added at the levels described in Table 2. Escherichia coli was grown on nutrient agar or on EM minimal agar containing, in grams per liter: dibasic potassium phosphate, 3; monobasic potassium phosphate, 1; ammonium chloride, 5; ammonium nitrate, 1; sodium sul-

TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Relevant characteristics[a]	Source
A. tumefaciens		
15955	Prototroph, virulent, octopine Ti plasmid	E. W. Nester
15955-45	15955(R68.45)	Hamada *et al.*, 1979
SA101	*rif-101*; spontaneous mutation of 15955	This study
SA101-37	SA101(pDP37)	This study
SA5004	SA101 *trp*::Tn*5*	This study
SA5004-37	SA5004(pDP37)	This study
SA5004-38	SA5004(pDP38)	This study
SA5013	SA101 *ade*::Tn*5*	This study
SA5013-37	SA5013(pDP37)	This study
SA5013-38	SA5013(pDP38)	This study
SA5017	SA101 *ser/gly*::Tn*5*	This study
SA5017-37	SA5017(pDP37)	This study
SA5017-38	SA5017(pDP38)	This study
SA5017N-37	SA5017-37 *nal-101*	This study
SA5017SE-37	SA5017-37 *str-101 ery-101*	This study
SA5017-24	SA5017(pDDP24)	This study
SA5018	SA101 *leu*::Tn*5*	This study
SA5018-37	SA5018(pDP37)	This study
SA5018-38	SA5018(pDP38)	This study
SA5018-24	SA5018(pDDP24)	This study

TABLE 1 (cont'd)

Strain or plasmid	Relevant characteristics	Source
SA5019	SA101 ura::Tn5	This study
SA5019-37	SA5019(pDP37)	This study
SA5019-38	SA5019(pDP38)	This study
SA5039	SA101 trp::Tn5	This study
SA5039-37	SA5039(pDP37)	This study
SA5039-38	SA5039(pDP38)	This study
SA122	Ti plasmidless SA101 (homooctopine-resistant)	S. K. Farrand
SA12201	Leu$^-$ SA122 (MNNG mutagenesis)	S. K. Farrand
SA12203	Leu$^-$ Rif^r Ery^r SA12201 (spontaneous mutations)	This study
SA12203-24	SA12203(pDDP24)	This study
A3	his-101 (MNNG mutagenesis of 15955)	E. W. Nester
A25	his-101 met-101 (MNNG mutagenesis of A3)	E. W. Nester
SA11	Rif^r Str^r A25 (spontaneous mutations) his-101 met-101	Hamada et al., 1979
SA11-45	SA11(R68.45)	Hamada et al., 1979
A74	his-101 met-101 thr/glu (MNNG mutagenesis of A25)	E. W. Nester
SA74-3	Ery^r A74	This study
A63	his-101 met-101 ilv-101 (MNNG mutagenesis of A25)	E. W. Nester

TABLE 1 (cont'd)

Strain or plasmid	Relevant characteristics	Source
SA63-2	Gen^r A63 (spontaneous mutant)	This study
SA6300	his-101 met-101 ilv-101 trp-101 (MNNG mutagenesis of A63)	This study
SA6351	Ery^r SA6300 (spontaneous mutation) his-101 met-101 ilv-101 trp-101	This study
SA6355	Rif^r Nal^r SA6351 (spontaneous mutations) his-101 met-101 ilv-101 trp-101 Ery^r	This study
SA6361	Ery^r Str^r Nal^r SA6300 (spontaneous mutations) his-101 met-101 ilv-101 trp-101	This study
SA6302	his-101 met-101 ilv-101 leu-101 (MNNG mutagenesis of A63)	This study
SA6303	his-101 met-101 ilv-101 trp-201 (MNNG mutagenesis of A63)	This study
C58	Prototroph; virulent (pTiC58)	E. W. Nester
NT1	Ti plasmidless C58 (heat-cured)	E. W. Nester
SA117	Leu$^-$ NT1 (MNNG mutagenesis)	This study
SA115precursor	His$^+$ Met$^+$ A25 (spontaneous reversions)	S. K. Farrand
SA115	Arg$^-$ SA (MNNG mutagenesis)	S. K. Farrand

TABLE 1 (cont'd)

Strain or plasmid	Relevant characteristics	Source
E. coli		
CE89-084	K-12 Δ*trpE ilvD*::Tn*5*	D. Berg
CE89-084-35	K-12 CE89-084(pDP35)	This study
1231	K-12 *leuB6 serB thi-1 hsdR lacY supE44*	C. Colson
SE1231	1231(R68.45)	This study
1830(pJB4JI)	K-12 *met-63 pro-22 nal* [Gm^r Km^r (Tn*5*)]^b	Beringer *et al.*, 1978
JA200	K-12 Δ*trpE thr leu lacY recA*	P. Matsumura
A767	K-12 *hisA ara gal malA xyl mtl sup*	P. Hartman
B463	K-12 *hisB ara gal malA xyl mtl sup*	P. Hartman
F860	K-12 *hisF ara gal malA xyl mtl sup*	P. Hartman
D4314	C strain *hisD thy*	P. Hartman
SF8	*leuB6 thr-1 thi-1 trp recBC lop-11 supE44 lacY rpsL*	M. Nomura
C600	*leuB6 thr-1 thi-1 hsdR lacY supE44 rpsL*	P. Matsumura
SK2266	*leuB6 metB1 thr-1 hsdR2 lacY supE44*	S. Kushner
WA946	*argG6 sbcB15 hsdR4 gal endA thi* $T1^r$ Tet^r	B. Bachmann
UB281(pMR5)	*pro met nal* [Tc^r Ap/Cb^r Km^r rep^{ts}]^b	Robinson *et al.*, 1980

TABLE 1 (cont'd)

Strain or plasmid	Relevant characteristics	Source
P. aeruginosa		
PAO25(R68.45)	*leu-10 arg-F10* (R68.45)	F. Mondello
PAO403	*trp-54 res-1 chl-13 sm-1 mod-1*	A. Chakrabarty
PAO1161	*leu-38 res-10*	A. Chakrabarty
PAO38-1	*leu-38 rif*	?
Plasmids		
RP4	Km^r Tc^r Ap/Cb^r (57 kb)	S. Falcow
pJB4JI	IncP1 Gm^r Km^r (contains Tn*5*)	Beringer *et al.*, 1978
R68.45	Km^r Tc^r Ap/Cb^r (59 kb)	F. Mondello
pDP35	Km^s R68.45 (59 kb)	This study
pDP37	pDP35::Tn*5*	This study
pDP38	pDP35::Tn*5*	This study
pDDP24	Tc^r Ap/Cb^r (pDP35 with 13.8 kb *A. tumefaciens* DNA inserted at *BamHI* site)	This study
pACYC184	Cm^r Tc^r (3.9 kb)	Chang *et al.*, 1978
pDP108	Cm^r Tc^s (pACYC184 with 6.3 kb *A. tumefaciens* DNA inserted at *HindIII* site)	This study

[a]Abbreviations: rif, rifampin; leu, leucine; ery, erythromycin; trp, tryptophan; ade, adenine; ser, serine; gly, glycine; nal, nalidixic

TABLE 1 (cont'd)

acid; str or sm, streptomycin; ura, uracil; MNNG, nitrosoguanidine; his, histidine; met, methionine; thr, threonine; glu, glutamate; ilv, isoleucine-valine; gen or gm, gentamicin; arg, arginine; thi, thiamine; hsd, host specificity determinant; lac, lactose; pro, proline; rec, recombination; ara, arabinose; gal, galactose; mal, maltose; xyl, xylose; mtl, mannitol; sup, suppressor; thy, thymine; lop, ligase overproduction; rps, ribosomal protein subunit; tc or tet, tetracycline; ap, ampicillin; cb, carbenicillin; km, kanamycin; rep, replication; ts, temperature-sensitive; sbc, suppressor recB recC; end, endonuclease; T1, bacteriophage T1; res, restriction; chl or cm, chloramphenicol; mod, modification; kb, kilobases. Superscript r, s or - denotes resistant, sensitive or auxotrophic phenotype, respectively.

[b]Characteristics in brackets are plasmid-specified.

Table 2. Concentrations of antibiotics used.

Antibiotic	Concentration (μg/ml)		
	A. tumefaciens[a]	*E. coli*	*Ps. aeruginosa*
Ampicillin	50	100	
Carbenicillin	50		300
Chloramphenicol		25	
Erythromycin	10[b]		
Gentamicin		5	
Gentamicin[b]	25[b]		
Kanamycin	25	25	
Nalidixic acid	100[b]		
Rifampin	25[b]		
Streptomycin	500[b]		
Tetracycline	1	10	

[a]Strain 15955 and its derivatives.

[b]Values are for chromosomal resistance markers; spontaneous mutations were assumed to be chromosomally located.

fate, 2; magnesium sulfate heptahydrate, 0.1; glucose, 5; Bacto agar, 15 (Clowes et al., 1968). Liquid cultures were grown in L broth, which contained, in grams per liter: Bacto tryptone (Difco), 10; Bacto yeast extract (Difco), 5; sodium chloride, 5 (Lennox, 1955). The pH of the L broth was adjusted to 7.2-7.4 prior to sterilization. Stock cultures were maintained on nutrient agar, which contained, in grams per liter: nutrient broth (Difco), 8; Bacto agar, 15 and, where appropriate, an antibiotic to select for maintenance of a plasmid or transposon. Chalk agar used for 3-ketolactose tests contained, in grams per liter: Bacto yeast extract, 10; glucose, 20; calcium carbonate, 20, and Bacto agar, 20 (Bernearts and De Ley, 1963). Lactose agar contained, in grams per liter: lactose, 10; yeast extract, 1, and Bacto agar, 20 (Bernearts and De Ley, 1963).

Chemicals

Most reagent-grade chemicals were manufactured by Mallinkrodt, Inc. Optical-grade cesium chloride was purchased from Harshaw Chemical Company. Amino acids, antibiotics, lysozyme, pronase, Tris-HCl (tris[hydroxymethyl]aminomethane hydrochloride), EDTA (ethylenediamine tetraacetic acid, disodium salt), and MNNG (N-methyl-N'-nitro-nitrosoguanidine) were all purchased from Sigma Chemical Company. SeaKem HGT agarose was purchased from FMC Corporation.

Buffers

TE buffer contained 50 mM Tris-HCl and 20 mM EDTA at pH 8.0. TES buffer contained 30 mM Tris-HCl, 50 mM sodium chloride and 5 mM EDTA at pH 8.0. Standard saline citrate (1X SSC) buffer contained

150 mM sodium chloride and 15 mM trisodium citrate dihydrate (Marmur, 1961). AB buffer contained all the components of AB minimal medium except glucose and agar. Tris-maleic buffer contained 50 mM Tris-HCl and 50 mM maleic acid at pH 6.0, and 1 g/l ammonium sulfate, 100 mg/l magnesium sulfate heptahydrate, 5 mg/l calcium nitrite and 0.25 mg/l ferrous sulfate heptahydrate (Adelberg et al., 1965). STET buffer contained 8 % (w/v) sucrose, 5 % (v/v) Triton X-100, 50 mM EDTA and 50 mM Tris-HCl at pH 8.0. LTE buffer contained 10 mM Tris-HCl and 0.1 mM EDTA at pH 8.0.

Strain constructions

Spontaneous mutants. Spontaneous antibiotic-resistant mutants were isolated by spreading 0.1 ml of an overnight culture of the parent strain on nutrient agar containing the appropriate antibiotic. A single-colony isolate appearing on this media was transferred to nutrient agar, from which subclones were tested for antibiotic resistance phenotype. A stock culture of the mutant strain was prepared from a representative subclone. Strains with a second resistance marker were constructed by repeating this procedure with a different antibiotic.

Nitrosoguanidine mutagenesis and auxotyping. Auxotrophic derivatives of Agrobacterium tumefaciens strains A63 and NT1 (Chapters 4 and 5, respectively) were isolated by a modification of the method of Klapwijk et al. (1975). A 30 ml culture of early exponential-phase cells was prepared by diluting an overnight preculture

to a density of 5-10 Klett units (Klett-Summerson colorimeter, red filter) and incubating at 29°C. to a density of 65 Klett units. Cells were collected by centrifugation, then washed twice with and resuspended in two-thirds volume of Tris-maleic buffer. Following incubation for 3 h at 29°C. in the presence of 250 μg/ml MNNG, bacterial cells were collected by centrifugation, washed twice with and resuspended in L broth to a turbidity of approximately 10 Klett units. The suspension was divided into 1 ml portions that were incubated overnight (New Brunswick Scientific Co., Inc. model R-2 reciprocator, 29°C.) and then plated on nutrient agar. Auxotrophs were detected by screening for their ability to grow on AB minimal medium. Their phenotypes were subsequently identified by screening for a particular amino acid, purine or pyrimidine supplement that could restore growth.

Matings

Plasmid transfer. Cross-streak and filter mating techniques are described in Chapters 4 and 5. Liquid matings were initiated by adding equal volumes (usually 0.2 ml) of exponential-phase donor and recipient cultures to L broth (usually 0.6 ml) in a 16 X 125 mm screw-cap tube that was incubated on its side for 3 h at the optimum growth temperature. Control tubes of either donor only or recipient only were treated similarly. At the end of the mating period, cells were collected by centrifugation, resuspended in AB buffer and plated on selective medium.

Chromosomal gene transfer. For optimal results, precultures of donor (in antibiotic-supplemented L broth) and recipient (in L broth) were prepared from stock cultures no more than 24-48 h old. Precultures (grown 18-20 h) were diluted into fresh L broth to a density of 30-35 Klett units (approximately a 1:12 dilution) and incubated with shaking (New Brunswick Scientific Co., Inc. model G86 water bath shaker, 125 rpm) at 29°C. to mid-exponential phase (120 Klett units for the conditions described above). Occasionally the density of one parent culture increased more rapidly than that of the other, so that it was necessary to make a dilution (with L broth that was pre-warmed to allow continued exponential-phase growth). Mating mixtures were prepared by adding donor and recipient (usually 2 ml recipient to provide sufficient numbers of recombinants) to pre-warmed L broth (final volume 10 ml) in a 16 X 125 mm tube. The tube was inverted once and the contents were decanted into a Nalgene type A filter unit (47 mm filter, 0.2 µm pore diameter). Cells were drawn by suction onto the filter, which was then placed bacterial side up on the surface of a pre-warmed nutrient agar plate and incubated 3 h at 29°C. Cells were washed off the filter into AB buffer (10 ml) and collected by centrifugation (DuPont-Sorvall model RC5 centrifuge, SS-34 rotor, 5 min at 5000 rpm). Cell pellets were resuspended in a minimal volume (determined by the number of plates of selective media) of AB buffer and and 0.1 ml portions were plated. Recombinants acquiring an amino acid biosynthetic marker were selected on minimal medium containing all but one of the amino acids required by the recipient. Rifampin-resistant recombinants were selected by the delayed soft

agar overlay technique described in Chapter 5. Since the overlay method was found to give a heavy background growth when used to select recombinants resistant to streptomycin or erythromycin, they were selected by plating directly on antibiotic medium.

Interrupted matings

Cultures of donor and recipient were grown and mating mixtures prepared as described above. To obtain a sufficient number of recombinants, the volume of the recipient culture added to the mating mixture was 1 ml for each time point to be assayed. Mating pairs were allowed to form by drawing cells onto the surface of a membrane filter (see Chromosomal gene transfer), placing the filter on the surface of a pre-warmed nutrient agar plate and incubating 5 min at 29°C. (Mylroie et al., 1977). The cells were then very gently resuspended in pre-warmed L broth and incubated at 29°C. with gentle shaking (New Brunswick Scientific Co., Inc. model G86 water bath shaker, 100 rpm). The mating period was considered to begin the moment that the mating pairs were resuspended in broth. Portions (1 ml) of the sample were removed at various time points, diluted into 9 ml AB buffer and vortexed 2 min (American Scientific Products Vortex-Genie Mixer S8223-1, high speed) to break up mating pairs. Cells were then collected by centrifugation, resuspended in AB buffer and plated on selective media as described above. Since some recombinants were recovered on the zero-time plates, the titer at each time point was corrected by subtracting the initial titer.

Isolation of plasmid DNA

Small plasmid mini-preps. For recombinant plasmids constructed from small, high copy-number vectors such as pACYC184 (Chang and Cohen, 1978), the rapid method of Holmes and Quigley (1981) yielded sufficient DNA from 1.5 ml L broth cultures to permit electrophoretic analysis. Cells were collected by centrifugation (Fisher model 235 micro-centrifuge, 3 min) and resuspended in 100 µl STET buffer. Lysozyme (stock solution 10 mg/ml in 50 mM Tris-HCl, 50 mM EDTA at pH 8.0, prepared immediately before use) was added (final concentration 1 mg/ml) and digestion was allowed to proceed for 5 min at 4°C. The samples were then placed in a water bath (100°C.) for 50-60 sec to precipitate proteins and high molecular-weight DNA. The gelatinous precipitate was concentrated by micro-centrifugation (10 min) and separated from the supernate by decanting. Plasmid DNA was precipitated from the supernate by adding one volume 95 % ethanol and placing the samples on powdered dry ice for 5 min. The precipitate, collected by micro-centrifugation, was redissolved in 30 µl distilled water. Samples to be digested with a restriction endonuclease were treated with RNase (1µl of stock mixture [20 mg/ml RNase A and 600 units/ml RNase T_1 in 150 mM sodium chloride, 10 mM sodium acetate pH 5.2], 20-30 min at 37°C). Five to ten µl of a sample was sufficient for agarose gel electrophoresis.

Minipreps for larger plasmids. For larger, low copy-number plasmids derived from R68.45, plasmid DNA was isolated from 1.5 ml L broth cultures by the alkaline lysis technique of Birnboim and Doly,

(1979). Cells collected by centrifugation (Fisher model 235 micro-centrifuge) were resuspended in 0.2 ml of freshly prepared solution I (50 mM glucose, 10 mM EDTA pH 8.0, 25 mM Tris-HCl pH 8.0, 2 mg/ml lysozyme) and incubated for 30 min on ice. To this was added 0.4 ml solution II (0.2 N NaOH, 1 % [w/v] sodium dodecyl sulfate [SDS], pH adjusted to 12.4) to ensure cellular lysis and to denature the DNA. After incubation for 5 min on ice, 0.3 ml of solution III (3 M sodium acetate pH 4.8) was added and the samples incubated for 60 min on ice to precipitate high molecular-weight DNA. The gelatinous precipitate was concentrated by micro-centrifugation (10 min) and separated from the supernate by decanting. Plasmid DNA was precipitated from the supernate by adding one volume of 95 % ethanol and incubating 30 min at -20°C. The precipitate, collected by centrifugation, was redissolved in 0.2 ml solution IV (100 mM sodium acetate, 50 mM Tris-HCl pH 8.0). The DNA was again precipitated with 2 volumes of ethanol and a 5 min incubation on powdered dry ice, collected by centrifugation and redissolved in 25 µl distilled water. Samples for restriction endonuclease analysis were treated with RNase as described above.

Preparative scale isolation of small plasmids. Small high copy-number plasmids were isolated from E. coli by preparation of cleared lysates (Davis et al., 1980). Cells from a one-liter L broth culture were collected by centrifugation (DuPont-Sorvall model RC5 centrifuge, GS-3 rotor, 10 min at 10,000rpm, 4°C.), washed in 250 ml cold TE buffer and resuspended in 20 ml of a buffer containing 15 %

(w/v) sucrose, 50 mM Tris-HCl and 50 mM EDTA at pH 8.5 (cold). The suspension was divided among four 30-ml Corex centrifuge tubes, lysozyme (fresh stock solution, 10 mg/ml) was added (final concentration 1 mg/ml), and the tubes were incubated on ice for 5 min. Five ml of Triton solution (0.1 % [w/v] Triton X-100 in 50 mM Tris-HCl and 50 mM EDTA at pH 8.5) was added to each tube and the samples were incubated 30-60 min at 37°C. and 5-10 min at 55°C. to facilitate lysis. Cellular debris and chromosomal DNA were removed by centrifugation (DuPont-Sorvall model RC5 centrifuge, SS-34 rotor, 60 min at 19,000 rpm, 4°C.). The nonviscous portion of the supernate was collected and adjusted in volume to 27.5 ml. Cesium chloride (26.13 grams) was dissolved in the sample and 2.75 ml ethidium bromide solution (10 mg/ml) was added. To minimize precipitation at this step, the flask containing the sample was constantly swirled and the dye added slowly from a pipette held with its tip just below the surface of the liquid. The mixture was allowed to stand 5 min, during which time a thin layer of insoluble material rose to the surface. The sample was loaded into a 39 ml polyallomer Quick-seal ultracentrifuge tube (Beckman no. 342414), care being taken not to transfer any insoluble material. Plasmid DNA was banded by isopycnic centrifugation (Beckman model L8-70 ultracentrifuge; VTi50 rotor; 38,000 rpm for approximately 16 h; 22°C.). Using long-wave ultraviolet irradiation to visualize the positions of the chromosomal (uppermost) and plasmid (lower) bands, chromosomal DNA was removed and plasmid DNA collected with cut-off Pasteur pipettes (separate pipettes for each). The ethidium bromide was removed by extrac-

tion with 20X SSC-saturated isopropanol and the cesium chloride by exhaustive dialysis against LTE buffer.

Preparative-scale isolation of larger plasmids. The alkaline lysis method of Casse et al. (1979) was used to isolate larger plasmids. Cells from a one-liter L broth culture were harvested, washed and resuspended in TE buffer (one quarter volume and 5 ml per gram of cells for washing and resuspension, respectively). To this was added lysis buffer (TE buffer containing 1 % [w/v] SDS, pH adjusted to 12.3-12.4; 95 ml per gram of cells). The suspension was mixed 90 sec on a magnetic stirrer (100 rpm), then incubated at 37°C. for 25 min to facilitate lysis. The lysate was adjusted to pH 8.5-8.9 by the rapid addition of 2 M Tris-HCl pH 7.0 (approximately 6 ml) and subsequently stirred for 2 min. Sodium chloride (final concentration 3 % [w/v]) was stirred in and the mixture incubated at room temperature for 30 min. An equal volume of redistilled phenol saturated with 3 % (w/v) sodium chloride was added and the mixture stirred 2 min to precipitate single-stranded DNA and protein. The aqueous upper phase was collected following centrifugation for 5 min at 8000 rpm (DuPont-Sorvall RC5 centrifuge, GS-A rotor), mixed with an equal volume of chloroform solution (chloroform-isoamyl alcohol, 24:1 [w/v]) and again collected by centrifugation. Plasmid DNA was precipitated from the aqueous phase by sequential additions of potassium acetate (final concentration 0.3 M) and 2 volumes of 95 % ethanol, followed by overnight incubation at -20°C. The precipitate was collected by centrifugation (GS-3 rotor, 30 min at 12,000 rpm) and redissolved

in TES buffer (final volume of 3.8 ml for DNA isolated from 1 g of cells). Cesium chloride (4.15 g) and ethidium bromide (0.2 ml of a 10 mg/ml stock solution) were each brought into solution and the sample was loaded into a 5.1 ml polyallomer Quick-seal tube (Beckman no. 342412). For plasmid preparations made from 2 g or more of cells, proportionately greater amounts of reagents were added and multiple sample tubes loaded. Plasmid DNA was banded by isopycnic centrifugation (Beckman model L8-70 ultracentrifuge, VTi65 rotor, 61,000 rpm for approximately 6 h, 22°C.), then isolated and treated as described for small-plasmid preparations above.

Isolation of total DNA. A modification of the method of Marmur (1961) was used to purify total Agrobacterium DNA to be used in cloning. Cells from a one liter L broth culture grown to a density of approximately 100 Klett units were harvested by centrifugation, washed in one-half volume TE buffer and resuspended in 50 ml TE. The cell suspension was digested 1 h at 37°C. with 0.5 µg/ml pronase (stock solution 5 mg/ml dissolved in 20 mM Tris-HCl pH 8.0, 150 mM sodium chloride and preincubated 1 h at 37°C. to digest the contaminating nuclease activity in the preparation). The cells were lysed by adding SDS (final concentration 1 % [w/v]) and incubating at 37°C. (30-60 min) and 55°C. (5 min). Proteins were extracted from the lysate by adding one volume of Tris-saturated (100 mM Tris-HCl pH 8.0) phenol and gently swirling the mixture for 10 min. The two phases of the sample were separated by centrifugation and the aqueous upper phase was retained. Following another phenol extraction, the

aqueous phase was twice extracted with one volume of chloroform-isoamyl alcohol (24:1 [v/v]) to remove residual protein and phenol. Sodium chloride was added to the aqueous portion (final concentration 150 mM) and two volumes of cold (-20°C.) 95 % ethanol were layered over the sample. The DNA was spun out of solution on a glass rod held with its tip at the interface of the two layers. The precipitated DNA was redissolved in 5 ml 0.1X SSC buffer and treated with one hundredth volume of RNase (see small plasmid mini-preps, above) for 45 min at 37°C. The solution was adjusted to 100 mM with respect to Tris-HCl pH 8.0 and digestion was stopped by two extractions with Tris-saturated phenol and one extraction with chloroform-isoamyl alcohol. The aqueous upper phase was removed and 2 volumes of cold ethanol were carefully layered over the sample. The DNA was spun out at the interface of the solutions and redissolved in a minimal volume (about 2 ml) of LTE buffer.

Restriction endonuclease digestion. Restriction endonucleases were purchased from New England Biolabs, Bethesda Research Laboratories, Inc., Pharmacia P-L Biochemicals, or Boehringer Mannheim Biochemicals and used with the buffers recommended by the manufacturers. Digestion was terminated by incubation at 70°C. for 5 min.

Ligation. T_4 DNA ligase was purchased from New England Biolabs or Bethesda Research Laboratories, Inc., or was generously supplied by Dr. Philip Matsumura, (University of Illinos-Chicago). Ligase from the latter source was used in a buffer containing 66 mM Tris-HCl pH 7.9, 33 mM sodium chloride, 10 mM magnesium chloride, 5 mM dithio-

threitol, 0.5 mM adenosine triphosphate and 50 µg/ml bovine serum albumin. Ligase from commercial suppliers was used with the recommended buffer. To the buffer were added restriction endonuclease-digested DNA (final concentration 2 µg/ml vector DNA and 20 µg/ml insert DNA) and ligase (1 unit per µg DNA for ligase from P. Matsumura, 10 units per µg DNA for commercial ligase). Sample tubes were incubated for 18 h in a 15°C. water bath (Haake model FK2 bath and model #E52 temperature circulator).

Agarose gel electrophoresis. Electrophoresis buffer was composed of 89 mM Tris-HCl, 2.5 mM EDTA and 89 mM boric acid (Meyers et al., 1976). Agarose (0.7 % [w/v]) was dissolved in electrophoresis buffer by stirring at 100°C. for 10 min. Five µl of tracking dye (0.07 % [w/v] bromophenol blue, 7 % [w/v] SDS [stock solution 20 %] and 33 % [v/v] glycerol; Meyers et al., 1976) was added to the DNA samples (20-45 µl), which were then loaded under electrophoresis buffer. For restriction endonuclease analysis, DNA samples were generally loaded in a large (21 x 20 x 0.7 cm) gel. A current of 2 mA per cm of gel length was applied for 10-15 min to draw the DNA into the gel. For fractionation, a regulated current of 1 mA/cm was applied for 20-24 h. Supercoiled plasmids were fractionated in either large or small (12 x 11 x 0.7 cm) gels by applying a regulated current of 4 mA/cm for a tracking dye migration distance of 10-12 cm (3-4 h). Recombinant plasmids differing from a 59 kb parent plasmid by as little as 2 kb were readily detectible when the period of electrophoresis was extended (tracking dye migration 15-18 cm).

Photography of gels. Agarose gels were stained overnight in 0.5 µg/ml ethidium bromide at 8°C., destained in distilled water and illuminated with a UV Products, Inc. model C-63 transilluminator (304 nm emission). Gels were photographed with a Polaroid MP4 camera, using Polaroid type 55 film and both UV-17 (nearest the gel) and Wratten no. 23 red filters (f 4.5; 1.5 min exposure for gels with supercoiled DNA, 3.5 min for gels containing restriction endonuclease-digested DNA).

Transformation of E. coli. Plasmid DNA was introduced into competent E. coli cells by the method of Morrison (1979) or as described by Davis et al. 1980). For the Davis technique, cells from a culture grown to a density of 100 Klett units were harvested by centrifugation (DuPont-Sorvall RC5 centrifuge, SS-34 rotor, 5 min at 5000 rpm, 4°C.) and gently resuspended in one-half volume of cold 50 mM calcium chloride dihydrate. After incubating the suspension on ice for 15-30 min, cells were again collected by centrifugation, resuspended in cold calcium chloride (one-twentieth starting volume) and incubated on ice 15-30 min. Two volumes of cells were added to one volume of DNA and the suspension incubated 30 min on ice, 2 min at 42°C.and then 10 min at room temperature. Each sample was diluted with 20 volumes of L broth and incubated 30 min at 37°C. to permit phenotypic expression. Cells were collected by centrifugation and plated on selective medium. The Morrison technique was essentially identical, except that the first resuspension of cells was in 100 mM magnesium chloride and the second in 100 mM calcium chloride.

CHAPTER 4

TRANSPOSON-FACILITATED CHROMOSOME MOBILIZATION IN *AGROBACTERIUM TUMEFACIENS*

Crown gall is a neoplastic disease of plants caused by *Agrobacterium tumefaciens* (Smith and Townsend, 1907). The central role of the *A. tumefaciens* Ti plasmid in tumorigenesis has been well documented (reviewed by Nester and Kosuge, 1981). There is also evidence that chromosomal functions contribute to virulence. For example, insertions of transposon Tn*5* into chromosomal loci appear to confer avirulence (Garfinkle and Nester, 1980). In addition, the Ti plasmidless strain NT1 exhibits site binding activity (Whatley *et al.*, 1978), indicating that chromosomal determinants may govern the ability of the bacterium to adhere to the host plant. Another indication of chromosomally encoded virulence functions from a study of strain 5GlyFe, which is avirulent despite the fact that its Ti plasmid is fully functional (Sciaky *et al.*, 1978). Further characterization of such mutants requires the development of an efficient chromosomal gene transfer system for *A. tumefaciens*.

The IncP1 plasmids RP1, RP4, R68, and R68.45 have been shown to effect chromosome mobilization in a number of gram-negative bacterial genera (Holloway, 1979). The first three R plasmids appear to be nearly identical structurally (Burkardt *et al.*, 1979; Grinsted *et al.*, 1977; Nayudu and Holloway, 1981; Thomas, 1981). Plasmid R68.45

is a derivative of R68 that is able to mobilize the Pseudomonas aeruginosa PAO chromosome (Haas and Holloway, 1976). It harbors two copies of a 2.1-kilobase insertion sequence, IS21, near the kanamycin resistance determinant (Leemans et al., 1980; Riess et al., 1980; Willetts et al., 1981). The transpositional activities of IS21 may be responsible for the ability of this plasmid to mobilize the chromosomal genes of many bacteria at low frequency and from multiple origins (Willetts et al., 1981). In a previous publication, we showed that R68.45 mobilizes the A. tumefaciens 15955 chromosome (Hamada et al., 1979). However, the low mobilization frequencies (at best, about 10^{-7} recombinants per input donor) and the genetic instability of donors limited the efficacy of this system. Recently, similar low frequencies were reported for R68.45-mediated chromosomal gene transfer in A. tumefaciens C58 (Bryan et al., 1982; Hooykaas et al., 1982). To facilitate the characterization of A. tumefaciens chromosomal functions, we undertook to improve the mobilizing ability of R68.45.

The sex factor ability of many plasmids can be enhanced by adding to them a sequence of DNA homologous to a DNA sequence present on the chromosome (Holloway, 1979). One approach has been to use recombinant DNA techniques to insert a chromosomal DNA fragment into the plasmid (Barth,1979; Julliot and Boistard, 1979; Watson and Scaife, 1978); another has been to insert identical transposable elements into each replicon (Chumley et al., 1979; Nayudu and Holloway, 1981). For example, in Vibrio cholerae, the efficiency of chromosome mobilization by plasmid P was improved by the insertion of

Tn*1* into both plasmid and chromosome (Johnson and Romig, 1979). Similarly, insertions of Tn*501* improved the sex factor activity of RP1 in *Rhodopseudomonas sphaeroides* (Pemberton and Bowen, 1981). Our approach was to construct donor strains harboring insertions of the kanamycin resistance transposon Tn*5* in both the chromosome and sex factor plasmid. In this report, we describe the construction and characterization of donor strains that have acquired the ability to mobilize the chromosome efficiently and in an oriented manner.

(Portions of this work were presented at the 82nd Annual Meeting of the American Society for Microbiology [D. L. Pischl and S. K. Farrand, Abstr. Annu. Meet. Am. Soc. Microbiol., 1982, H2, p. 113].)

Materials and Methods

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Table 1 (p.31-36). *Escherichia coli* SE1231(R68.45) was constructed by mating *P. aeruginosa* PAO25(R68.45) with recipient strain 1231. Transconjugants selected for resistance to kanamycin (Km^r) were shown to express other plasmid markers (resistance to tetracycline [Tc] and ampicillin [Ap]) and to harbor a plasmid that comigrated electrophoretically with R68.45 isolated from the donor strain (data not shown). By the method of Haas and Holloway (1976), subclones of one transconjugant were shown to effectively mobilize chromosomal genes to *E. coli* recipients (data not shown).

Isolation of spontaneous drug-resistant mutants. Strain SA101 was derived by plating A. tumefaciens 15955 on nutrient agar containing rifampin (Rif) at 15 µg/ml. The mutant is resistant to rifampin at levels of 25 µg/ml. Strain SA63-2 was isolated by plating strain A63 on nutrient agar containing gentamicin at 10 µg/ml. It is resistant to as much as 25 µg of gentamicin per ml. An erythromycin-resistant derivative of strain A74, designated strain SA74-3, was isolated in a similar fashion. It is resistant to 25 µg of erythromycin per ml.

Media and chemicals. Nutrient agar (Difco Laboratories), L broth, AB buffer, and AB minimal agar have been described previously (Hamada et al., 1979). Amino acids and antibiotics were purchased from Sigma Chemical Co. Amino acid supplements were added at 25 µg/ml. Antibiotics were used at the following concentrations: kanamycin, 25 µg/ml; rifampin, 25 µg/ml; gentamicin, 25 µg/ml for A. tumefaciens, 5 µg/ml for E. coli harboring pJB4JI; erythromycin, 25 µg/ml; ampicillin, 200 (E. coli) or 50 µg/ml (A. tumefaciens; tetracycline, 10 (E. coli) or 1.0 µg/ml (A. tumefaciens).

Isolation of plasmid DNA. For rapid screening of E. coli plasmids, DNA was isolated by the method of Holmes and Quigley (1981). The method of Casse et al. (1979) was used to isolate A. tumefaciens plasmids and for all preparative-scale isolations. In the latter case, plasmid DNA was further purified by isopycnic centrifugation in cesium chloride-ethidium bromide (Farrand et al., 1981).

Restriction endonucleases. Restriction endonucleases were purchased from New England Biolabs or from Bethesda Research Laboratories. Digestions were carried out under conditions recommended by the manufacturers.

Endonuclease S1 digestion. HindIII-digested plasmid DNA (5 µg) was treated with 5 U of endonuclease S1 (Bethesda Research Laboratories) at 4°C. in the buffer recommended by the manufacturer. The reaction was terminated after 60 s by the addition of 500 mM EDTA to a final concentration of 25 mM.

Agarose gel electrophoresis. Supercoiled plasmids were examined by electrophoresis in 0.7 % agarose gels (12 X 11 X 0.7 cm). A regulated current of 4 mA/cm was applied. For the separation of restriction fragments, samples were electrophoresed in 0.7 % agarose gels (21 X 20 X 0.7 cm). A regulated current of 4 mA/cm over a period of 20 to 24 h gave effective separation.

Transformation of E. coli. Competent cells of E. coli were prepared immediately before use, essentially by the method of Morrison (1977). After heat shock, cells were incubated in L broth for 30 min at 35°C. to allow expression.

Conjugal plasmid transfer: filter matings. Overnight cultures in L broth plus kanamycin (donor) or in L broth (recipient) were diluted 1:20 in fresh L broth without antibiotics and incubated at optimum growth temperature to mid-exponential phase. For most experiments, the mating mixture contained 1 ml of donor culture, 1 ml of recipient

culture, and 8 ml of L broth. Controls of donor plus L broth and of recipient plus L broth were run in parallel. Bacteria were drawn onto the surface of 2.54-cm, 0.22-μm pore diameter filters (Millipore Corp.). Filters were placed bacterial side up on the surface of a nutrient agar plate and incubated for 3 h at 35 (*E. coli*) or 29°C. (*A. tumefaciens*). Cells were then eluted from the filter and washed once with AB buffer. Samples of the dilutions were spread on selective medium.

Cross-streak matings. Plasmids pDP37 and pDP38 were introduced into *A. tumefaciens* by cross-streak matings on selective medium. A small mass of donor bacteria was streaked with a toothpick across a single linear inoculum streak of recipient bacteria.

Chromosome mobilization. Matings were conducted essentially as described for filter matings, except that overnight cultures were diluted 1:10. Amino acid-independent recombinants were selected by plating on minimal medium containing all but one of the amino acids required by the recipient. Rif^r recombinants were selected by plating on AB medium containing required amino acids plus rifampin. Medium selective for plasmid-harboring transconjugants contained tetracycline at 1.0 μg/ml.

Results

Construction of a Km^S derivative of R68.45. Initial studies showed that although R68.45 could be used to mobilize the *A. tumefaciens* chromosome, the system was inefficient (Hamada *et al.*, 1979). We therefore sought to improve the sex factor ability of this plasmid by adding a transposon to both the plasmid and the chromosome. We elected to work with the kanamycin resistance transposon Tn*5* because it readily transposes with relatively little site specificity (Kleckner, 1977) and because kanamycin resistance is easily selected for in *A. tumefaciens*. However, we first had to inactivate the native kanamycin resistance gene of R68.45. This was done by cutting R68.45 at its single *Hin*dIII site, which is within the kanamycin resistance gene (Thomas, 1981), digesting it with endonuclease S1, and introducing the unligated products by transformation into *E. coli* 1231. Five of twelve transformants expressed the tetracycline and ampicillin resistance determinants of R68.45, but remained sensitive to to kanamycin; seven expressed resistance to all three antibiotics. Four of the Km^S isolates contained a plasmid about 50 kb in size. The fifth transformant harbored a plasmid (pDP35) that was indistinguishable in electrophoretic mobility from that of the 59-kb parent, R68.45. This plasmid appeared to retain full conjugal activity, since donors transferred tetracycline resistance as efficiently as does R68.45 (10^{-2} transconjugants per donor in a 3-h mating). Because it seemed likely that the effect of our *in vitro* manipulations would be localized to the

kanamycin resistance gene of pDP35, we chose to use this element as the target plasmid for Tn*5* insertions.

Construction of Tn*5*-containing plasmids pDP37 and pDP38. *E. coli* CE89-084, which contains Tn*5* in its chromosome (D. Berg, personal communication), was used to donate Tn*5* to pDP35. We expected that after the introduction of pDP35 into strain CE89-084, Tn*5* would transpose onto the plasmid in a fraction of the transconjugant cells. Such Tn*5*-containing plasmids were recovered by mating strain CE89-084-35(pDP35) with the *recA* *E. coli* recipient strain JA200 and selecting for Km^r transconjugants. We then transferred the plasmids from a number of such Km^r isolates into plasmid-free *E. coli* strain 1231 for further characterization. An initial screen showed that each isolate harbored a plasmid of identical size, and that each was slightly larger than pDP35 (Fig. 1). Two particular plasmids, pDP37 and pDP38, were characterized in more detail.

Digestion with *Hind*III or *Hpa*I, both of which cut at each end of Tn*5* (Jorgensen *et al.*, 1979), confirmed the presence of the transposon in pDP37 and pDP38 (Fig. 2). After the insertion of Tn*5*, these plasmids should have acquired novel internal transposon restriction fragments of 3.3 (*Hind*III) or 5.4 kb (*Hpa*I). The original parent plasmid, R68.45, is cut at a single site by either enzyme (Fig. 2, lanes 2 and 7). The absence of a 2.1-kb *Hpa*I fragment (Riess *et al.*, 1980) in this preparation of R68.45 may indicate either that the IS*21* duplication is present in only a very low proportion of plasmid copies

FIGURE 1

Electrophoretic analysis of R68.45 and its derivatives. Plasmid DNA isolated by the method of Casse *et al.* (1979) was analyzed by agarose gel electrophoresis as described in the text. The lanes contain plasmid DNA from: 1, *E. coli* 1231; 2, strain SE1231(R68.45); 3, strain SE1231-35(pDP35); 4, strain SE1231-37(pDP37); 5, strain SE1231-38(pDP38); 6 through 9 four additional Km^r transformants of strain 1231; 10, strain SE1231-35(pDP35).

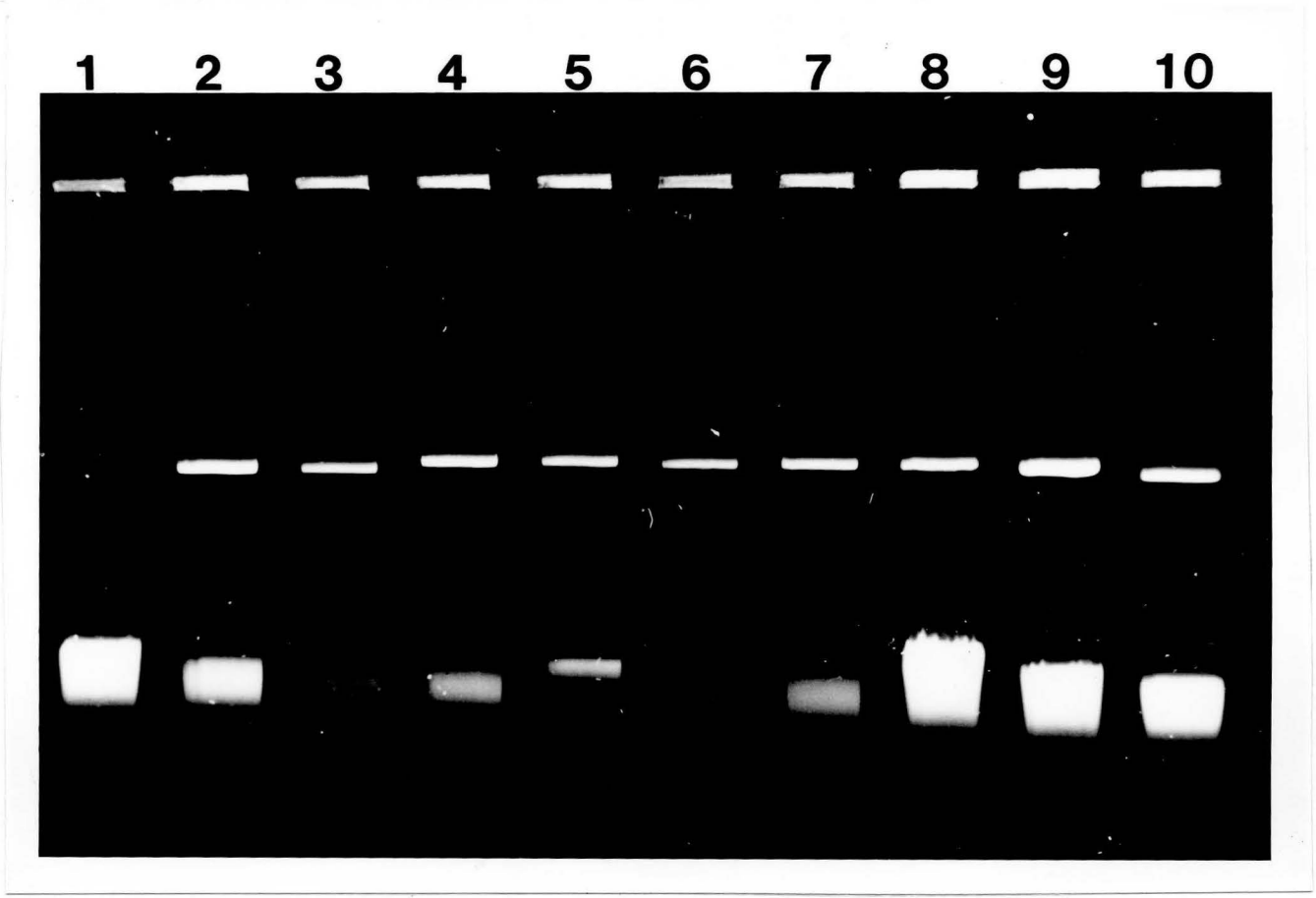
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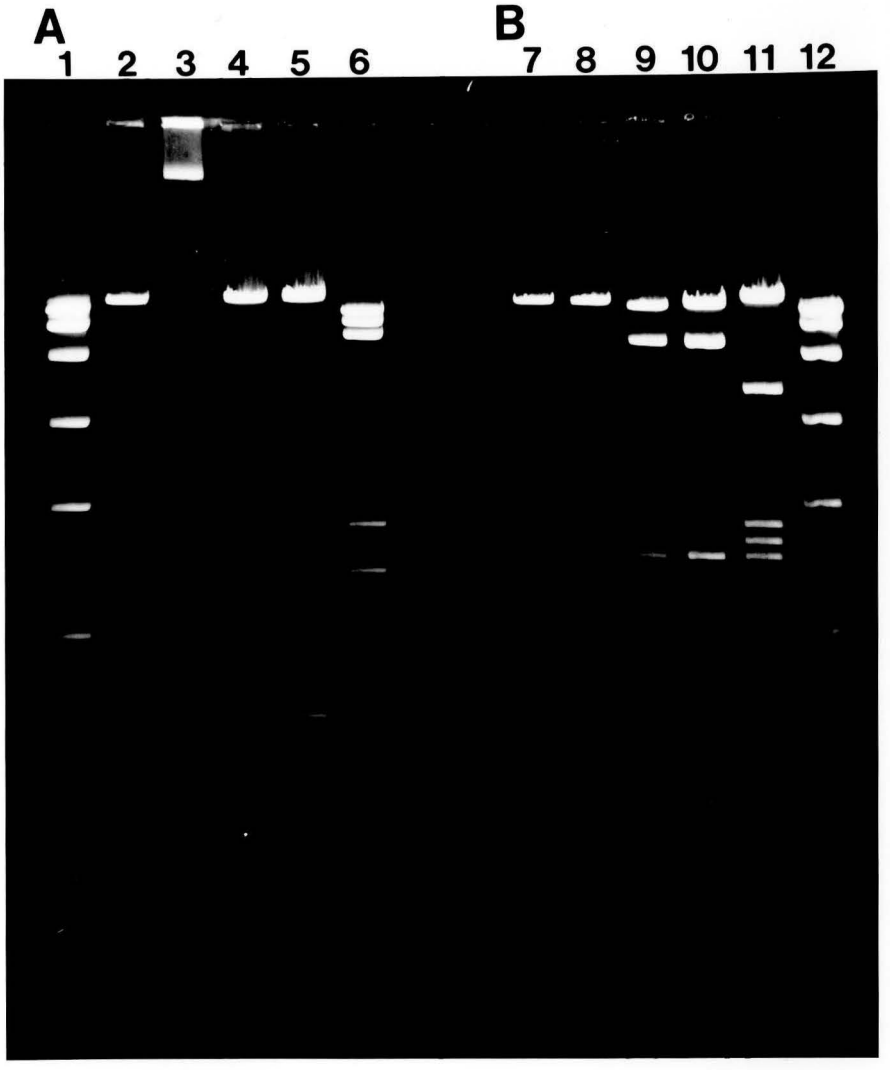
FIGURE 2

Analysis of R68.45 and its derivatives by restriction endonuclease digestion. Purified plasmid DNA was digested and the fragments were separated by agarose gel electrophoresis as described in the text. Lanes 1 and 12 each contain a mixture of HindIII and SalI fragments of λ DNA, the sizes of which are given in kilobase pairs. The remaining lanes contain (A) HindIII digests of: 2, R68.45; 3, pDP35; 4, pDP37; 5, pDP38; 6, pJB4JI; (B) HpaI digests of: 7, R68.45; 8, pDP35; 9, pDP37; 10, pDP38; 11, pJB4JI.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12

and is therefore not readily visible, or that at least a portion of the duplication has been deleted, in which case the parent plasmid would be similar to R68. As expected, pDP35, when treated with HindIII, migrated as a supercoiled plasmid (lane 3), indicating S1 digestion had altered the HindIII recognition site. The HpaI site, however, was retained (lane 8). Both pDP37 and pDP38 acquired two HindIII sites, generating a novel HindIII fragment of 3.2 kb (lanes 4 and 5). This fragment comigrated with a HindIII fragment of pJB4JI (lane 6), a plasmid known to contain Tn5 (Beringer et al., 1978). Similarly, pDP37 and pDP38 acquired a novel HpaI fragment of 5.3 kb (lanes 9 and 10), which comigrated with a HpaI fragment of pJB4JI (lane 11). In addition, pDP37 and pDP38 had two other novel HpaI fragments of approximately 38 and 19 kb.

The sites of the Tn5 insertions in pDP37 and pDP38 were precisely mapped by double digestions with SmaI and PstI. Each enzyme makes several cuts within R68.45 (Nayudu and Holloway, 1981). In addition, SmaI cuts at a single site within Tn5, whereas PstI cuts at several sites, two of which are 0.6 kb from each end (Jorgensen et al., 1979). We expected that an insertion of Tn5 into pDP35 would change the SmaI-PstI fragment pattern in three ways. First, there should be four internal Tn5 fragments, of which the largest, 2.4 kb, should be readily visualized. Second, since Tn5 introduces new SmaI-PstI sites, the plasmid target fragment should be missing. Third, there should be two other novel fragments, consisting of the upstream and downstream segments of the plasmid target fragment plus 0.6 kb of

the Tn5 sequences. Since each extends from a parental plasmid site to a site within Tn5, the sizes of these two fragments should depend upon the exact site of the Tn5 insertion.

Agarose gel electrophoresis of SmaI-PstI digests showed that Tn5 had inserted in the second largest SmaI-PstI fragment in both pDP37 and pDP38 (Fig. 3, lanes 3 and 4). Novel fragments were 13.8 and 3.5 kb in pDP37 and 13.6 and 3.8 kb in pDP38. On the basis of the SmaI-PstI and HpaI fragment sizes, the sites of insertion were unambiguously identified as being at plasmid coordinate 17.6 in pDP37 and 17.9 in pDP38 (Fig. 4A).

We then determined the orientation of Tn5 in pDP37 and pDP38. To do this, we performed double digestions with SmaI and KpnI, since these enzymes produce the smallest possible plasmid fragment in the region of interest (Fig. 4A). Furthermore, SmaI was particularly useful in that it cuts at a single asymmetric site within Tn5, producing a 2.4-kb left and a 3.1-kb right portion of the transposon (Jorgensen et al., 1979; Fig. 4B). Since there is no KpnI site within Tn5, we expected that SmaI-KpnI digests of our Tn5-containing plasmids would produce two novel fragments whose sizes should be equal to the length of the plasmid sequences plus either the 2.4- or 3.1-kb portion of Tn5 depending on the orientation of the element.

The results of these digestions are shown in Fig. 3. The parent plasmid, pDP35 (lane 5), showed five fragments, two of which

FIGURE 3

Characterization of the Tn5 insertions in pDP37 and pDP38. Lanes 1 and 8 contain a mixture of HindIII and SalI fragments of λ DNA, the sizes of which are given in kilobase pairs. The remaining lanes contain (A) SmaI-PstI digests of: 2, pDP35; 3, pDP37; 4, pDP38; (B) SmaI-KpnI digests of: 5, pDP35; 6, pDP37; 7, pDP38.

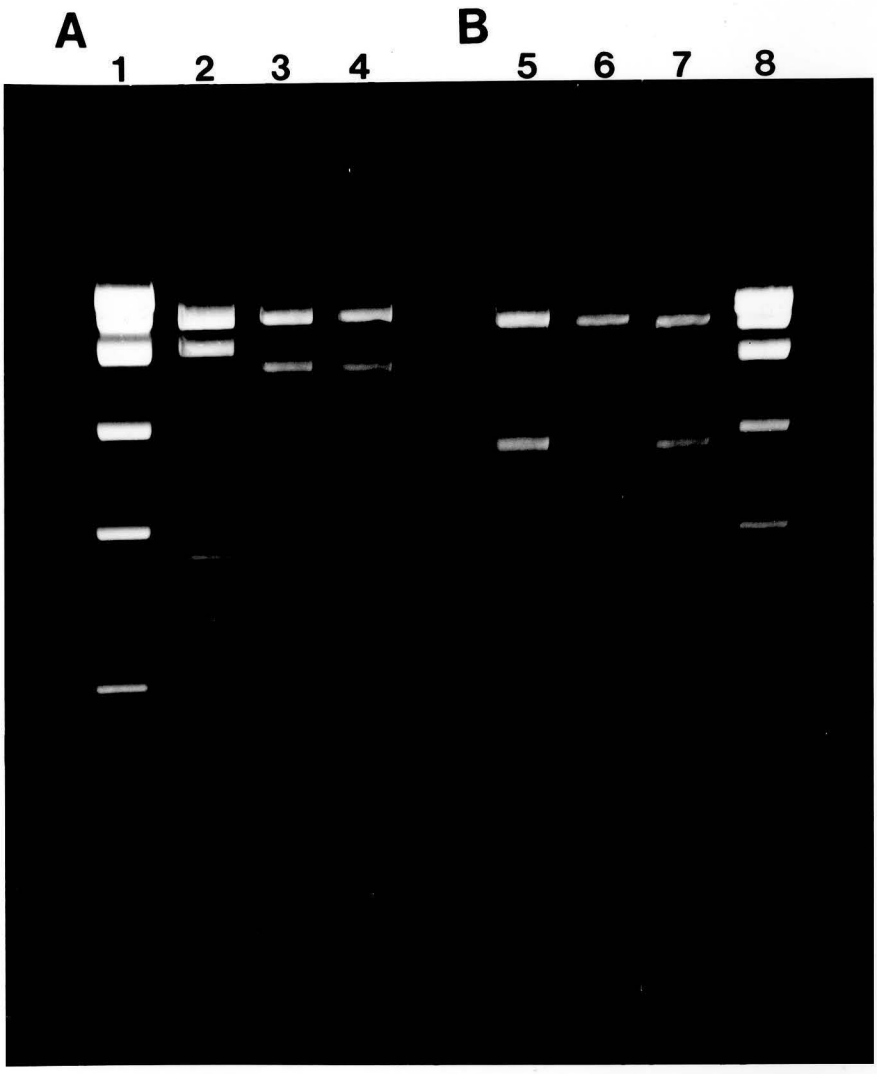
A
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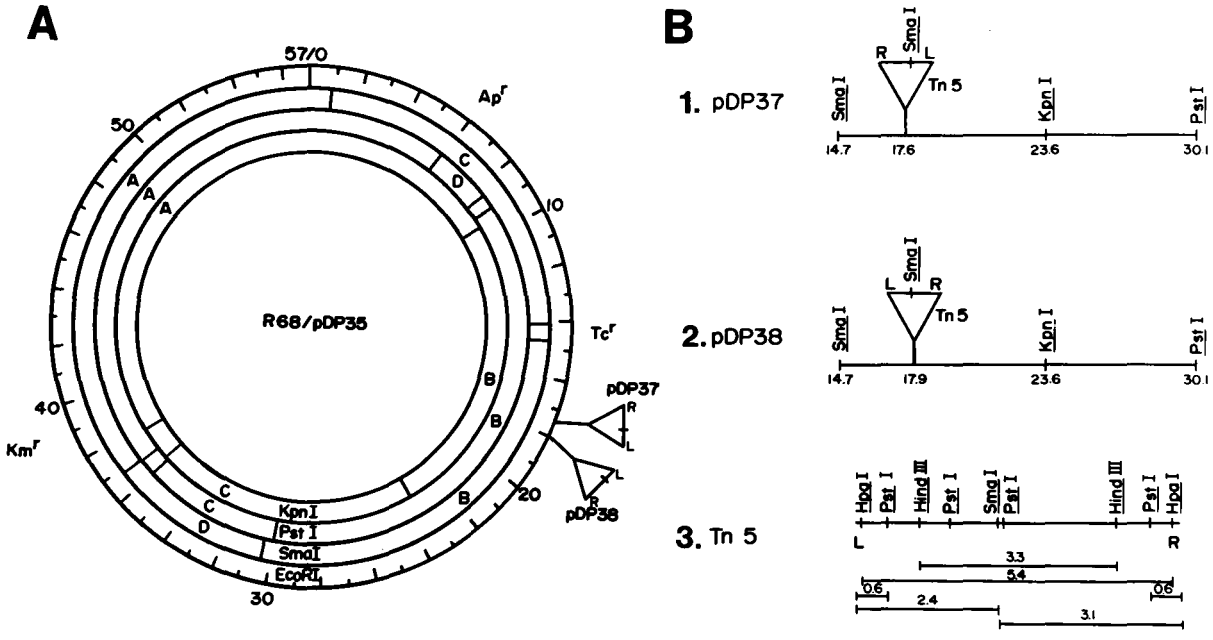
FIGURE 4

Restriction endonuclease maps.

(A) Map of R68 or pDP35, oriented with the single EcoRI site at the top, as developed by Nayudu and Holloway (1981). The locations and orientations of the Tn5 insertions in pDP37 and pDP38 are also shown. The cross-hatches mark the single SmaI site within Tn5 (Jorgensen et al., 1979). The orientation is defined by the relative positions of the left (L) and right (R) arms of Tn5 (Holmes et al., 1981).

(B) Detailed diagram of the SmaI-PstI fragment of B of pDP37 (top) and pDP38 (center). For clarity, Tn5 is shown at the precise locations and in the orientations determined as described in the text. (Bottom) Map of Tn5 showing appropriate restriction sites (Jorgensen et al., 1979). The left (L) and right (R) arms of Tn5 are shown in the standard orientation.


A
57/0
Ap^r
10
20
30
40
50
Km^r
Tc^r
R68/pDP35
A
B
C
D
KpnI
PstI
SmaI
EcoRI
pDP37
pDP38
R
L
B
1. pDP37
Sma I
R
L
Tn 5
Kpn I
Pst I
14.7
17.6
23.6
30.1
2. pDP38
Sma I
L
R
Tn 5
Kpn I
Pst I
14.7
17.9
23.6
30.1
3. Tn 5
Hpa I
Pst I
Hind III
Pst I
Sma I
Pst I
Hind III
Pst I
Hpa I
L
R
3.3
3.4
0.6
0.6
2.4
3.1

migrated as a 9.2-kb doublet. In pDP37 (lane 6), there were a 9.2-kb fragment and two novel fragments of 8.2 and 6.3 kb. In pDP38, the two novel fragments, 8.7 and 5.7 kb, each migrated in doublets (lane 7). These results were confirmed in two other experiments. With these data, the orientations of Tn*5* were determined to be right to left in pDP37 and left to right in pDP38 (Fig. 4B). Thus, we concluded that in these two plasmids Tn*5* had inserted in the same local region, but in opposite orientations.

Construction of *A. tumefaciens* donor strains. We used a two-step approach to construct *A. tumefaciens* donors with enhanced chromosome mobilizing activity. First, to provide a sequence homologous with the sex factor, Tn*5* was inserted into the chromosome. As the transposon donor we used pJB4JI (Beringer *et al*, 1978), a Tn*5*-containing plasmid that readily transfers to, but fails to become established in, *A. tumefaciens* (Garfinkle and Nester, 1980). *Escherichia coli* 1830(pJB4JI) was mated with *A. tumefaciens* SA101. Survivors resistant to kanamycin and rifampin were selected on antibiotic-supplemented nutrient agar and screened for auxotrophy. We assumed that coinheritance of an unselected auxotrophy most probably resulted from insertion of Tn*5* into a chromosomal gene involved in biosynthesis of the required nutrient. Approximately 3% of the Km^r isolates screened were auxotrophic for amino acids, nucleotides, vitamins, or coenzymes. The rates of reversion to prototrophy for five of the auxotrophs were found to be about 10^{-8} per cell.

In the second step of our strategy, we conjugally transferred pDP37 or pDP38 into a number of the A. tumefaciens strains harboring Tn5 insertions. These strains were then tested as donors for chromosome mobilizing activity.

Tn5-facilitated chromosome mobilization. We used strains A63 and A74 as recipients in the test matings. These auxotrophs were derived by nitrosoguanidine mutagenesis of the His^- Met^- strain A25, a strain 15955 derivative (Hamada et al., 1979). To compare the efficiencies of different donors, we expressed the mobilization frequencies as the number of recombinants per input donor.

In preliminary matings between donor strain SA5017-37(pDP37) and recipient strain A63, recombinants were recovered at frequencies of 9×10^{-5} per donor for Ilv^+, 2×10^{-5} for His^+, and 3×10^{-6} for Met^+. Since the frequencies for Ilv^+ and His^+ recombinants were 10- to 100-fold greater than had been observed with R68.45, we asked whether the effect was caused by the presence of the homologous Tn5 elements in both plasmid and chromosome. In one experiment, we compared the mobilizing ability of pDP37 within the background of the Tn5-containing strain SA5017 with that of pDP37 within the Tn5-lacking parent strain SA101. The results (Table 3) showed that, within the strain SA101 background, pDP37 mobilized donor markers at low levels (Ilv^+) or at rates below the limit of detection. In the strain SA5017 background, however, mobilization was enhanced by several orders of magnitude. Furthermore, some markers were transmitted more

TABLE 3. Tn*5* requirement for enhanced chromosome mobilization

Site of Tn*5* examined	Donor Mobilization strain	frequency[a] Val$^+$	Rif^r	His$^+$	Met$^+$	Glu$^+$
Expt. 1[b]						
Chromosome lacking Tn*5*	SA101-37(pDP37)	2×10^{-7}	$<2\times10^{-8}$	$<1\times10^{-8}$	$<1\times10^{-8}$	$<5\times10^{-9}$
Chromosome with Tn*5*	SA5017-37(pDP37)	3×10^{-4}	3×10^{-5}	2×10^{-6}	5×10^{-7}	2×10^{-6}
Expt. 2[c]						
Plasmid lacking Tn*5*	SA5017-35(pDP35)	$<5\times10^{-8}$	$<3\times19^{-9}$	5×10^{-8}	5×10^{-8}	$<3\times10^{-9}$
Plasmid with Tn*5*	SA5017-37(pDP37)	7×10^{-5}	2×10^{-7}	2×10^{-7}	2×10^{-7}	2×10^{-6}

[a]Recombinants were selected on minimal medium supplemented with all but one amino acid required by the recipient or on minimal medium supplemented with all recipient nutrients and rifampin. In experiment 1, the medium also contained gentamicin when the recipient was strain SA63-2 or erythromycin when the recipient was strain SA74-3. The frequency is reported as the number of recombinants per input donor. See Table 1, footnote *a* (p.35), for abbreviations.

[b]Donors shown were mated with strain SA74-3 (selection for Glu$^+$) or with strain SA63-2 (all other markers).

[c]Donors shown were mated with strain A74 (selection for Glu$^+$ and Rif^r) or with strain A63 (all other markers).

frequently than others (compare Ilv^+ and Met^+). In a second experiment, we tested the requirement for the plasmid Tn*5* element. Within the chromosomal background of the Tn*5*-containing donor strain SA5017, we compared marker transfer mediated by the Km^S pDP35 parent plasmid with that mediated by the Tn*5*-containing derivative pDP37. Again we found that when Tn*5* was present, mobilization was markedly enhanced. We conclude that the presence of Tn*5* on both plasmid and chromosome is necessary for enhanced mobilizing ability in our donors.

Polarity of chromosome transfer. We noticed that some Tn*5*-containing donors transmitted certain chromosomal markers more frequently than others (Table 3). This suggested that transfer might be polarized, *i. e.*, unidirectional from a fixed origin. We looked for evidence of polarized transfer in two ways. First, we compared mobilization by pDP37 with that by pDP38 within the same donor background. Donor strains SA5004-37 (harboring pDP37) and SA5004-38 (harboring pDP38) were mated in parallel with recipient strain A63. The pDP37-containing donor mobilized the His^+ marker most frequently, followed in decreasing order by Ilv^+, Rif^r and Met^+ (Table 4). The pDP38-containing donor, however, produced the opposite gradient of transmission frequencies. In a similar experiment, we examined mobilization by pDP37 and pDP38 within the chromosomal backgound of strain SA5017. Again we found that the two plasmids gave opposite gradients of transmission frequencies (Table 4).

TABLE 4. Origin of transfer and polarity of donor segment

Donor strain[a]	Donor genotype	Mobilization frequency (±SD)[b]			
		His^+	Rif^r	Ilv^+	Met^+
SA5004-37	trp::Tn5	$1.2(\pm0.6)\times10^{-5}$	$2.0(\pm0.0)\times10^{-6}$	$3.7(\pm0.1)\times10^{-6}$	$3.2(\pm1.5)\times10^{-7}$
SA5004-38	"	$7.4(\pm2.5)\times10^{-8}$	$2.4(\pm0.4)\times10^{-7}$	$1.6(\pm0.8)\times10^{-7}$	$4.6(\pm1.8)\times10^{-7}$
SA5017-37	ser/gly::Tn5	$5.2(\pm2.8)\times10^{-6}$	$2.0(\pm2.5)\times10^{-5}$	$6.5(\pm4.8)\times10^{-5}$	$5.7(\pm3.8)\times10^{-7}$
SA5017-38	ser/gly::Tn5	$1.4(\pm0.3)\times10^{-7}$	$1.2(\pm0.2)\times10^{-7}$	$1.0(\pm0.3)\times10^{-7}$	$3.6(\pm2.2)\times10^{-7}$

[a]Donor strains SA5004-37 and SA5004-38 were mated in parallel with strain A63 for 18 h; donor strains SA5017-37 and SA5017-38 were mated in parallel with strain A63 for 1 h in one experiment and for 7 h in a second.

[b]Mobilization frequency is given as the number of recombinants per input donor. The values are the mean of two expeiments. See Table 1, footnote a (p. 35) for abbreviations.

Secondly, these same experiments allowed us to compare mobilization by donors that differed only in the site of the chromosomal Tn5 element. Strain SA5017 presumably harbors Tn5 in a gene of the serine-glycine pathway. With pDP37 as the mobilizing plasmid, the gradient of transmission frequencies was $Ilv^+ \rightarrow Rif^r \rightarrow His^+ \rightarrow Met^+$ (Table 4). On the other hand, donor strain SA5004-37, harboring an insertion of Tn5 in a gene of the tryptophan pathway, produced a gradient of $His^+ \rightarrow Ilv^+ \rightarrow Rif^r \rightarrow Met^+$ (Table 4). When pDP38 was the mobilizing plasmid, the same phenomenon was observed (Table 4). In some experiments, we noted that the frequency of formation of Rif^r recombinants was depressed, so that the average transfer frequency for this marker was lower than might have been expected. We believe this to be due in part to problems in expression of the incoming Rif^+ allele (see below). The observation that donors give gradients in transfer frequencies strongly suggests that transfer is polarized.

Acquisition of unselected donor markers. If recombinants selected for the acquisition of certain donor markers coinherit other unselected markers, then we should be able to construct a linkage map for these markers. To test for coinheritance, we screened recombinants selected in three separate matings between donor strain SA5017-37 and recipient strain A63. Recombinants selected for any given marker acquired other markers at a detectable frequency (Table 5). There was also a strong relationship between the relative abundance of each class of selected recombinants and the particular markers co-

TABLE 5. Acquisition of unselected donor markers[a]

Selected marker	Mobilization frequency[b]	No. of selected recombinants scored[c]	%Coinheritance[d]			
			Val^+	Rif^r	His^+	Met^+
Val^+	4.9×10^{-5}	260	100.0	36.2	6.9	2.3
Rif^r	2.4×10^{-5}	330	99.7	100.0	1.2	0.3
His^+	2.6×10^{-6}	206	34.5	17.8	100.0	17.0
Met^+	4.3×10^{-7}	206	50.5	40.0	91.7	100.0

[a]Results of three independent matings of donor strain SA5017-37 and recipient strain A63. See Table 1 footnote a for abbreviations.

[b]Number of recombinants per input donor; mean of three experiments.

[c]Recombinants selected for acquisition of a single marker were tested for ability to grow on various media selective for each additional donor marker.

[d]Number of recombinants showing growth on test medium divided by number scored, multiplied by 100.

inherited by each class. The most numerous class of selected recombinants, Ilv^+, had acquired unselected rifampin resistance at a moderately high frequency of 36.2%. However, His^+ and Met^+ were acquired at the low frequencies of 6.9 and 2.3%, respectively. Rif^+ recombinants, the second most numerous class, domonstrated a very high rate of coinheritance of Ilv^+ (99.7%), but a very low rate for His^+ and Met^+. The third most numerous class of selected recombinants, His^+, coinherited other markers at moderate levels, from 34.5% for Ilv^+ to 17.0% for Met^+. Finally, the least numerous class, Met^+, coinherited the other markers at moderate (40.0% Rif^r) to high (91.7% His^+) levels.

Characterization of plasmids in recombinants. In E. coli gene transfer systems that were developed by supplying IncP1 plasmids with sequences homologous to the chromosome, most recombinants also inherit the intact R plasmid (Grinter, 1981; Watson and Scaife, 1978). We tested whether this was also true in our system. Nearly 700 recombinants from five separate matings were screened for acquisition of either the Tn5 marker (resistance to kanamycin) or the plasmid marker (resistance to tetracycline). Each was inherited at high frequency (98% for Km^r and 96% for Tc^r). These data introduced the possibility that chromosome transfer was mediated by R-prime plasmids, rather than by an Hfr-like donor. In E. coli, recombinants formed from F-prime plasmids, when grown under nonselective conditions, undergo recombination and deletion events in the region of diploidy and are therefore unstable in phenotype (Low, 1972). We examined 100 A. tumefaciens recombinants selected for acquisition of

a proximal marker and 100 recombinants selected for a distal marker. In each case, 97 of the recombinants stably maintained their phenotype after growth under nonselective conditions. This experiment showed the recombinants to be quite stable and provided no genetic evidence for R-primes.

Discussion

Plasmid R68.45 has been reported to possess low-efficiency chromosome mobilizing ability in two strains of A. tumefaciens (Bryan et al., 1982; Hamada et al., 1979; Hooykaas et al., 1982). In this study, we were able to construct plasmids with improved mobilizing efficiency. Following a strategy that has been successful in other bacterial gene transfer systems (Chumley et al., 1979; Johnson and Romig, 1979; Pemberton and Bowen; 1981), we supplied the plasmid with a nucleotide sequence homologous to a sequence present on the chromosome. The resulting donor strains showed enhanced gene transfer ability, a trait that was stably retained.

When Tn5 was used to develop homology, this element appeared to confer a unique origin and polarity to the gene transfer system (Table 4). Plasmids with oppositely oriented Tn5 elements generated opposite transmission gradients (Table 4: compare donor strains SA5004-37 and SA5004-38). Donors harboring Tn5 at different sites in the chromosome appeared to generate transmission gradients in which the order was permuted (compare strains SA5004-37 and SA5017-37). The single inconsistency was the frequency of formation of Rif^r

recombinants, which we believe resulted from the difficulty of selecting for a recessive character. Furthermore, donors with oppositely oriented Tn*5* elements in the chromosome also appeared to generate opposite transmission gradients when the same sex factor was used (compare strains SA5004-37 and SA5017-37). The data from these experiments support the idea that in each of the donors the chromosome is mobilized from a single origin, and that this origin is fixed by the site of the chromosomal Tn*5* element. The direction of chromosome transfer would then be determined by the orientation of the Tn*5* elements with respect to the plasmid sequence *oriT* (Chumley *et al.*, 1979; Grinter, 1981; Willetts *et al.*, 1981). Thus, donor strain SA5004-37 transmits His^+ as a proximal marker, followed by Rif^r/Ilv^+, and then by Met^+ as the distal marker (Table 4). On the other hand, donor strain SA5017-37, which we postulate to contain a chromosomal Tn*5* element in the opposite orientation, transmits Ilv^+ proximally, followed by Rif^r, His^+ and Met^+ (Table 4).

Coinheritance frequencies can be used to map chromosomal markers. In a polarized gene transfer system, coinheritance frequencies are greatest among recombinants for the distal marker, because the entire chromosomal segment preceding the selected marker is necessarily transferred. From the data (Table 5), we concluded that Met^+ is transmitted from strain SA5017-37 as a distal marker, since overall coinheritance was greatest when this marker was selected. The His^+ marker appears to lie next to Met^+, with Rif^r and Ilv^+ proximal to His^+. Since Rif^r recombinants coinherited Ilv^+ (99.7%) more

frequently than Ilv^+ recombinants coinherited Rif^r (36.2%), we believe that Ilv^+ is most probably the proximal marker. Thus, we place the order of markers on the SA5017-37 chromosome as follows: origin of transfer → Ilv^+ → Rif^r → His^+ → Met^+. This marker order is also supported by the mobilization frequencies (Tables 4 and 5).

A very high proportion of recombinants selected for the acquisition of a distal marker (Met^+ when SA5017-37 was the donor) coinherited all unselected markers (see above). This suggests that the markers are all linked, which we find surprising since it is unlikely that four such markers should be in close physical association on the donor chromosome. In *E. coli* F-mediated chromosome transfer, markers more than three map units apart are inherited as though they are unlinked (Hayes, 1968). The behavior of these *A. tumefaciens* recombinants suggests that (i) large segments of the chromosome can be transferred and (ii) the frequency of recombination is low.

Both the chromosomal and plasmid Tn*5* elements are required for efficient polarized transfer (Table 3). This suggests that the Tn*5* sequence provides a site for homologous recombination, leading to the formation of Hfr-like donors. Similar interpretations have also been made for the analogous RP4-λ hybrid plasmid (Grinter, 1981) and the F'ts114*lac*::Tn*10* plasmids (Chumley *et al.*, 1979) of *E. coli*. Because the *A. tumefaciens* donors require both Tn*5* elements, the mechanism of mobilization may be unlike that for R68.45, which has been proposed to occur by the formation of a plasmid-chromosome cointegrate during transposition (Willetts *et al.*, 1981).

Alternatively, mobilization might be effected by donors harboring R-prime plasmids. However, the evidence does not support this mechanism. First, we observed that pDP37 and pDP38 generated polarized and inverted transmission gradients. In contrast, R-primes should be a mixture of plasmids containing chromosomal sequences from either side of the point of integration (Low, 1972), and the donor population should generate gradients which are neither inverted nor polarized. Second, physical analysis of plasmids in recombinants failed to demonstrate R-primes (data not shown). Finally, the high degree of genetic stability of both proximal and distal recombinants conflicts with the predictions of the R-prime model. In this respect, the A. tumefaciens gene transfer system appears to be unlike that mediated by P::Tn1 plasmids in V. cholerae, in which recombinants for proximal markers are stable, but recombinants selected for distal markers segregate (R. D. Sublett, J. Cone and R. A. Finkelstein, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, D39, p. 54).

In the E. coli Hfr and P. aeruginosa R68.45 chromosome mobilizing systems, recombinants generally do not acquire the intact plasmid (Haas and Holloway, 1976; Hayes, 1968). However, in other E. coli IncP1 plasmid mobilizing systems recombinants do inherit the plasmid (Grinter, 1981; Watson and Scaife, 1978). The A. tumefaciens recombinants appear to resemble the latter, since nearly all were resistant to tetracycline. We believe that the R plasmids are most probably acquired as a result of additional mating events with mem-

bers of the donor population harboring only autonomous plasmids. This would also suggest that each donor population contains a significant proportion of cells with the sex factor in the autonomous state. The ease with which the sex factor plasmid could be isolated from such populations supports this notion (data not shown).

The properties of plasmids pDP37 and pDP38 should make them useful as sex factors in other bacterial genera. Their broad host range, multiple drug resistance markers, and efficient transfer ability should permit their use in nearly any gram-negative bacterium (Thomas, 1981). Since they show gene transfer activity for all of the markers tested in A. tumefaciens (Table 4), they should function as general chromosome mapping plasmids. All that would be required is the construction of a set of donors, each of which contains a unique insertion of Tn5 in the chromosome. Furthermore, since these two plasmids generate opposite transmission gradients (Table 4), they should function as complementary sex factor plasmids. Genes that are transferred by pDP37 as proximal markers should be transferred by pDP38 as distal markers. Finally, since gene transfer appears to originate at the site of the chromosomal Tn5 element (Table 4), these plasmids could be used to map the site and orientation of that insertion (Chumley et al., 1979).

CHAPTER 5

CHARACTERIZATION OF Tn5-FACILITATED DONOR STRAINS AND DEVELOPMENT OF A CHROMOSOMAL LINKAGE MAP FOR AGROBACTERUIM TUMEFACIENS

Agrobacterium tumefaciens is the gram negative soil organism which induces crown gall tumor formation in many dicotyledonous plants. While the A. tumefaciens Ti plasmid is clearly a primary determinant of virulence (Nester and Kosuge, 1981), certain functions encoded by the chromosome must also participate, since some insertions of Tn5 into chromosomal loci are sufficient to confer avirulence (Garfinkle and Nester, 1980). To permit us to study such chromosomal virulence determinants, we have undertaken to develop an effective chromosomal gene transfer system. We began working with R68.45, a plasmid which has been reported to mobilize chromosomal genes in many gram negative bacteria, including A. tumefaciens strain C58 (Bryan et al., 1982; Hooykaas et al., 1982). In strain 15955, however, this plasmid showed inefficient mobilizing activity (Hamada et al., 1979; D. L. Pischl and S. K. Farrand, unpublished results). We therefore investigated whether a transposon-facilitated mobilizing system would demonstrate more efficient gene transfer activity (Pischl and Farrand, 1983; Chapter 4). We constructed donor strains that contain a copy of Tn5 in both the sex factor plasmid (a derivative of R68.45) and in the chromosome (Pischl and Farrand, 1983; Chapter 4). In addition to an improved transfer frequency such donor strains have acquired the ability to

transmit the chromosome in a polarized fashion. Furthermore, we observed a gradient in the frequency with which different classes of selected recombinants acquired other unselected markers. This observation suggested that it would be possible to develop a chromosome map from such coinheritance frequencies. Here, we further characterize the Tn*5*-facilitated mobilization system, discuss our attempts to isolate other types of chromosome mobilizing plasmids, and present a map of the *A. tumefaciens* strain 15955 chromosome.

Materials and Methods

Bacterial strains and plasmids. Strains and plasmids are listed in Table 1. The construction of derivatives of strain SA101 harboring chromosomal insertions of Tn*5* has been described (Pischl and Farrand, 1983; Chapter 4). These isolates were assigned sequential strain numbers, beginning with SA5000. Donor strains, constructed by introducing pDP37 or pDP38 into a Tn*5*-containing SA101 derivative, were assigned a strain designation to indicate both plasmid and chromosomal background, *e.g.*, strain SA5017-37 is strain SA5017 into which pDP37 has been introduced. Spontaneous single-step antibiotic resistant mutant strains SA5017N-37 and SA5017SE-37 were isolated by plating strain SA5017-37 on nutrient agar containing nalidixic acid (N), streptomycin (S) or erythromycin (E). Auxotrophs of the SA6300 series were isolated following nitrosoguanidine mutagenesis (Adelberg *et al.*, 1965) of strain A63. Strain SA6361 was constructed from strain SA6300 by

consecutively isolating single-step mutants resistant to erythromycin, rifampin and nalidixic acid.

Media and chemicals. Nutrient agar, AB minimal medium and AB buffer have been previously described (Hamada et al., 1979). Amino acids and antibiotics, purchased from Sigma, were used in the concentrations described (Pischl and Farrand, 1983; Chapter 4), except that erythromycin was used at 10 µg/ml and nalidixic acid at 100 µg/ml. N-methyl-N'-nitro-N-nitrosoguanidine, obtained from Sigma, was used at 250 µg/ml.

Matings. Filter matings were conducted by the method previously described (Pischl and Farrand, 1983; Chapter 4). Equal volumes of donor and recipient cultures were used. Unless otherwise indicated, matings were terminated after 3 h by vortexing the bacterial suspension for 2 min.

Selection of drug-resistant recombinants. Recombinants for recessive markers such as rifampin resistance (Rif^r) were efficiently recovered by a soft agar overlay method. Briefly, 0.1 ml of the mating mixture or a dilution thereof was suspended in 5 ml of amino acid-supplemented minimal soft agar (0.7% [w/v] agar), and overlaid on a base of 20 ml supplemented minimal agar. Following overnight incubation, an additional overlay of 5 ml supplemented minimal soft agar containing rifampin at 150 µg/ml was added.

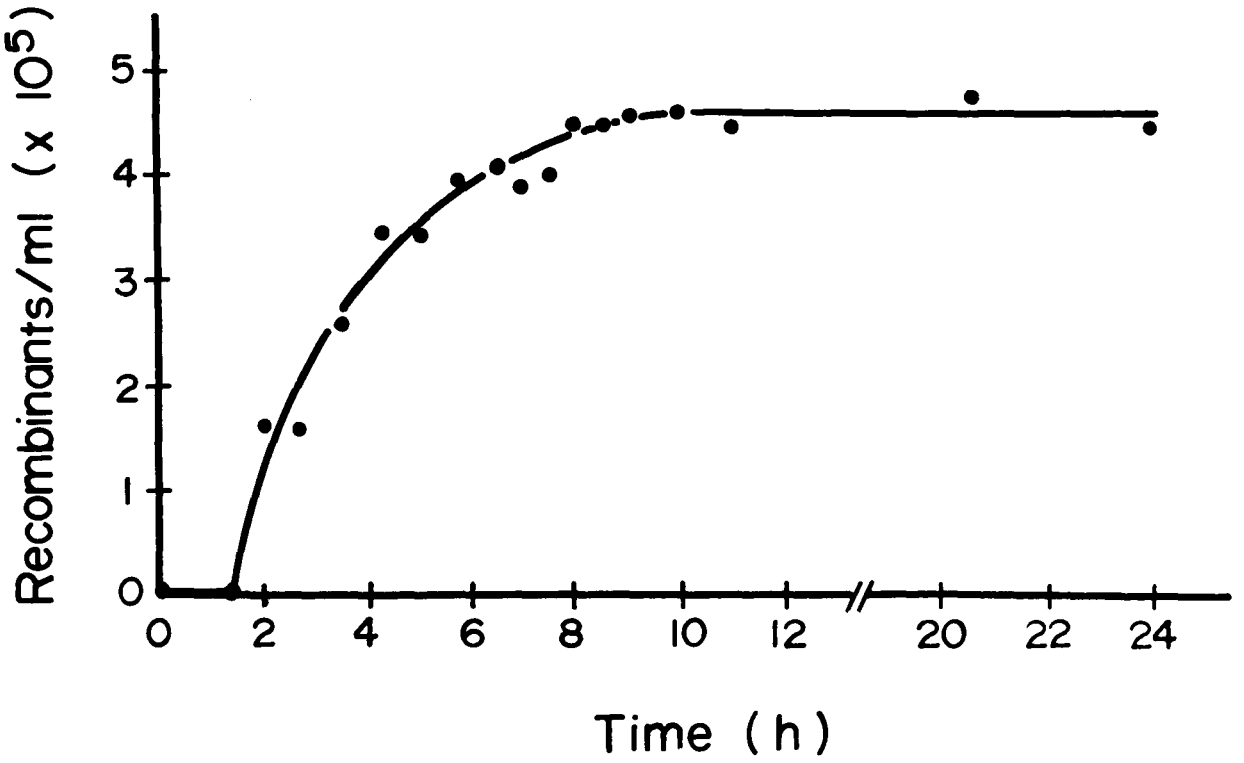
Screen for high-frequency donors. Masses of donor bacteria from single colonies were spread over circular areas on a master plate, incubated 3 days and replica-plated onto a lawn of the recipient spread on medium selective for His^+ Ilv^+ recombinants (Guyer, M. S., 1981). When recombinants appeared on the selective medium, corresponding areas of donor growth on the master plate were subcloned and the procedure repeated.

Results

Selection of drug-resistant recombinants. In a previous study we noted that with short mating periods the efficiency of recovery of rifampin-resistant (Rif^r) recombinants was lower than expected (Pischl and Farrand, 1983; Chapter 4). Since it seemed likely that this effect resulted from a delay in phenotypic expression of the recessive Rif^r mutation (Hayes, W., 1968), we modified our selection protocol by using an agar layer technique (see Materials and Methods). To test whether the recovery of Rif^r recombinants was improved, we determined the kinetics of expression of rifampin resistance. The Rif^r donor strain SA5017-37 was mated with strain SA6300 for 1 h, after which replicate samples were plated in soft agar. At intervals thereafter, duplicate plates were overlaid with soft agar containing rifampin. The results in Fig. 5 show that expression of rifampin resistance in all members of the recombinant population appeared to be complete by 8 to 9 h. After this point the number of Rif^r recombinants remained stable.

FIGURE 5

Kinetics of expression of rifampin resistance. Donor strain SA5017-37 was mated 1 h with strain SA6300. Replicate samples of the mating mixture were plated in soft agar and at various times thereafter overlaid with rifampin medium as described in the text. The yield of rifampin-resistant recombinants was calculated from the average number of colonies from duplicate plates.


Recombinants/ml (x 10^5)
0
1
2
3
4
5
0
2
4
6
8
10
12
20
22
24
Time (h)

Comparison of mobilization by pDP37 and R68.45. The sex factor activity of pDP37 was compared to that of its parent plasmid R68.45 in parallel matings of donor strains SA5017-37 and SA5017-45 with recipient strain SA6300 (Table 6). With pDP37, mobilization frequencies were up to 300-fold higher than those obtained with R68.45. The two plasmids also appeared to differ with respect to chromosomal origin of transfer. With pDP37, recombinants for different markers were recovered with different efficiencies, giving a gradient of decreasing transmission frequencies indicative of a single chromosomal origin of transfer. With R68.45, recombinants for different markers were recovered at roughly similar frequencies, indicating multiple origins. Furthermore, the overall coinheritance patterns (Table 6) showed that R68.45 and pDP37 differed in polarity of chromosome transmission. Recombinants formed with strain SA5017-45 as the donor showed a strong tendency to acquire either all or none of the unselected markers. In contrast, coinheritance values for recombinants formed from matings with strain SA5017-37 varied with the marker selected. Recombinants selected for acquisition of the proximal Ilv^+ marker acquired few unselected markers, whereas recombinants for the distal Met^+ marker coinherited other markers more frequently. Therefore, in a donor strain harboring a chromosomal Tn5 element, the Tn5-containing pDP37 exhibited polarized transmission from a single origin, while R68.45 showed low-frequency nonpolar transmission from multiple origins.

TABLE 6. Comparison of mobilizing abilities of R68.45 and pDP37

Selected marker of donor strain	Mobilization frequency[a]	No. of selected recombinants screened[b]	% coinheritance of the following recipient marker[c]				
			Ilv^+	Rif^r	$Trp\text{-}101^+$	His^+	Met^+
SA5017-37							
Ilv^+	9.0×10^{-5}	200	100.0	58.0	40.5	3.0	0.5
Rif^r	3.0×10^{-5}	199	94.7	100.0	41.7	0.5	0.5
$Trp\text{-}101^+$	4.0×10^{-5}	200	97.5	62.0	100.0	1.5	< 0.5
His^+	6.3×10^{-6}	112	16.1	13.4	20.5	100.0	13.4
Met^+	1.6×10^{-6}	228	38.2	39.0	44.3	69.3	100.0
SA5017-45							
Ilv^+	3.2×10^{-7}	79	100.0	5.1	6.3	6.3	3.8
Rif^r	1.6×10^{-8}	8	62.5	100.0	50.0	50.0	50.0
$Trp\text{-}101^+$	1.7×10^{-7}	48	50.0	8.3	100.0	20.8	6.2
His^+	9.9×10^{-7}	78	14.1	14.1	14.1	100.0	14.1
Met^+	8.2×10^{-7}	83	59.0	59.0	59.0	60.2	100.0

[a]Number of recombinants per input donor.

[b]Recombinants selected for acquisition of a single marker were tested for ability to grow on various media selective for each additional marker.

[c]Number of recombinants showing growth on test medium divided by the number scored and multiplied by 100.

Characterization of additional donor strains. In our first report on the Tn5-facilitated gene transfer system, we made several observations (Pischl and Farrand, 1983; Chapter 4). First, donor strains had acquired the ability to transmit the chromosome in a polarized manner. Second, this oriented transfer could only occur when Tn5 was present on both plasmid and chromosome. Third, donor strains which differed in the site of the chromosomal Tn5 insertion generated different transmission gradients. Finally, two pairs of donor strains each identical with respect to the chromosomal Tn5 element but which harbored either pDP37 or pDP38 (and therefore oppositely oriented plasmid Tn5 elements) appeared to give opposite transmission gradients. These observations suggested that the origin of transfer was determined by the location of Tn5 in the chromosome and that the polarity of transfer depended upon the orientations of both plasmid and chromosomal Tn5 elements. To further investigate these possibilities, we characterized the transmission gradients generated by pDP37 and pDP38 in a number of additional donor strains. The results presented in Table 7 show that pDP37-containing donors could be grouped into two classes. In general Class I strains gave a transmission gradient of $Ilv^+ \rightarrow Rif^r \rightarrow His^+ \rightarrow Met^+$. Class II strains gave a permutation of that gradient: $His^+ \rightarrow Rif^r \rightarrow Ilv^+ \rightarrow Met^+$. We noted however that in several of the crosses where sufficient time was not allowed for full expression of the incoming Rif^r marker (e.g., SA5037-37, SA5018-37 and some pDP38-containing donors), the frequency of Rif^r recombinants was low. The same observation of a reduced yield of Rif^r recombinants was made when the

TABLE 7. Transmission gradients of A. tumefaciens donor strains[a]

Donor strain[b]	Donor genotype	Mating period (h)	Mobilization frequency for phenotype[c]: Ilv$^+$	Rif^{re}	His$^+$	Met$^+$	Transmission gradient[d]
Class I[f]							
SA5000-37	leu/ile::Tn5	1	3.1×10^{-5}	3.2×10^{-6}	7.9×10^{-7}	4.1×10^{-7}	Ilv$^+$→Rif^r→His$^+$→Met$^+$
SA5000-38			1.9×10^{-6}	1.5×10^{-6}	3.4×10^{-6}	5.8×10^{-7}	
SA5000-37	ade::Tn5	1	1.9×10^{-5}	1.2×10^{-6}	6.5×10^{-7}	5.1×10^{-7}	Ilv$^+$→Rif^r→His$^+$→Met$^+$
SA5000-38			2.8×10^{-6}	8.5×10^{-8}	1.1×10^{-6}	5.8×10^{-7}	
SA5000-37	ser/gly::Tn5	3	8.4×10^{-5}	7.2×10^{-6}	3.1×10^{-6}	8.4×10^{-7}	Ilv$^+$→Rif^r→His$^+$→Met$^+$
SA5000-38			2.2×10^{-7}	2.3×10^{-8}	1.1×10^{-7}	4.4×10^{-7}	
SA5000-37	ura::Tn5	16	5.9×10^{-6}	NT[g]	2.1×10^{-6}	6.7×10^{-8}	Ilv$^+$→His$^+$→Met$^+$
SA5000-38			1.0×10^{-6}	NT	9.6×10^{-7}	7.6×10^{-9}	
SA5000-37	leu::Tn5	1	2.9×10^{-6}	1.0×10^{-8}	1.3×10^{-6}	2.4×10^{-7}	Ilv$^+$→[Rif^r]→His$^+$→Met$^+$
SA5000-38			9.0×10^{-9}	1.8×10^{-9}	1.1×10^{-8}	1.8×10^{-9}	
Class II							
SA5000-37	trp::Tn5	18	3.7×10^{-6}	2.0×10^{-6}	1.2×10^{-5}	3.2×10^{-7}	His$^+$→[Rif^r]→Ilv$^+$→Met$^+$
SA5000-38			1.6×10^{-7}	2.4×10^{-7}	7.4×10^{-8}	4.6×10^{-7}	

Table 7 continued on next page

TABLE 7. (cont'd) Transmission gradients of A. tumefaciens donor strains[a]

Donor strain[b]	Donor genotype	Mating period (h)	Mobilization frequency for phenotype[c]: Ilv$^+$	Rif$^{r e}$	His$^+$	Met$^+$	Transmission gradient[d]
Class II							
SA5000-37	leu::Tn5	3	2.6×10^{-6}	6.4×10^{-7}	3.5×10^{-5}	1.3×10^{-7}	His$^+$→[Rif$^+$]→Ilv$^+$→Met$^+$
SA5000-38			3.5×10^{-7}	7.6×10^{-8}	6.6×10^{-7}	1.3×10^{-7}	
SA5000-37	trp::Tn5	3	3.6×10^{-7}	3.1×10^{-6}	2.3×10^{-5}	2.9×10^{-7}	His$^+$→Rif^r→Ilv$^+$→Met$^+$
SA5000-38			1.3×10^{-7}	2.2×10^{-7}	8.9×10^{-6}	2.7×10^{-7}	

[a]Each pair of donor strains was mated in parallel with recipient strain A63 or one of its derivatives (see text). Each experiment was done once. The results of the SA5004 crosses have been previously reported (Pischl and Farrand, 1983; Chapter 4).

[b]Each donor strain contained either pDP37 of pDP38, as indicated in the strain designation by the appropriate numeral following the hyphen.

[c]Recombinants per input donor.

[d]For those crosses in which Rif^r frequencies appeared to be depressed (see text), the tentative order of the Rif^r marker is indicated by brackets. Further data corroborating this marker order are presented in the text and in Table 6.

[e]Except for the strain SA5039 cross, the 1- and 3-h crosses did not allow for full expression of Rif^r before exposure to selective conditions (Fig. 5). In addition, Rif^r frequencies for the 18-h cross may be depressed as a result of overgrowth by Rif^s recombinants (see text).

[f]Classified by the type of transmission gradient exhibited by the pDP37-containing donor strain.

[g]NT, Not tested.

mating period was increased to 18 h (SA5004-37 cross and additional experiments not shown). This disparity indicated that transmission gradients as such were not sufficiently precise to identify marker order with respect to rifampin resistance. However, since for most of these crosses we also tested selected recombinants for coinheritance of unselected markers, we were able to obtain data consistent with our assignment of donors to only the two transmission classes. In each case we observed a gradient in the frequency of coinheritance which matched the transmission gradient, except that the Rif^r marker was always located between Ilv^+ and His^+. Table 6, cross 1 shows a representative coinheritance gradient.

In contrast to the pDP37-containing donors, it was not generally possible to classify pDP38-containing donors with respect to transmission gradient (Table 7). Some donors (e.g., SA5018-38 and SA5004-38) exhibited too narrow a range in mobilization frequencies to allow precise identification of a transmission gradient. Others (e. g., SA5037-38) yielded recombinants at frequencies only slightly greater than the rate of spontaneous reversion (10^{-9} to 10^{-8}), so that again no transfer polarity could be identified. Overall, the data in Table 7 suggested that pDP38 was a less efficient chromosome mobilizing plasmid than was pDP37. We suspected that this decreased efficiency most likely resulted from a reduced expression of pDP38 transfer functions. To test this possibility we compared the abilities of these two plasmids to transfer themselves autonomously by measuring the efficiencies with which each transmitted the plasmid-associated tetracycline resis-

tance (Tet^r) marker. In parallel 3-h filter matings strain SA5017-37 yielded transconjugants at a wild-type frequency of 2.1×10^{-1} per input donor, while its counterpart, strain SA5017-38, generated only 7.9×10^{-3} transconjugants per donor. As a control for this experiment, we also monitored the appearance of His^+ chromosomal recombinants in each mating. Donor strain SA5017-37 yielded 5.1×10^{-6} recombinants per input donor while strain SA5017-38 yielded 4.3×10^{-7} recombinants per donor. These results show that, under conditions in which each donor yielded His^+ recombinants with typical efficiencies (compare above data with Table 7, lines 5 and 6), the pDP38-containing donor exhibited a 200-fold reduction in ability to transfer itself autonomously. This reduced transfer efficiency may very likely result from effects exerted by the Tn*5* element itself on expression of pDP38 transfer functions. Our observation that Tn*5* in pDP38 maps to a site which is near the *tra*-3 region of the closely related plasmid RP4 supports this contention (Barth, P. T., 1979; Pischl and Farrand, 1983; Chapter 4). As is the case for many transposable elements, insertion of Tn*5* into a locus can prevent the expression of the target gene as well as genes distal to the insertion (Berg *et al.*, 1980). Thus, we suspect that while Tn*5* enhanced the chromosome mobilizing ability of pDP37, in pDP38 it interfered with expression of plasmid *tra* genes, and for this reason we were unable to confirm that pDP37 and pDP38 give inverse transmission gradients in all donor strains.

Attempts to isolate stable high frequency donors. We undertook to improve the transfer efficiency of strain SA5017-37 by sequentially screening donor subclones for high frequency chromosome mobilizing activity (see Materials and Methods). After 2 cycles of enrichment, we noted that both the mean number and the variance in the mean number of recombinants had increased tenfold. This was consistent with our expectation of a clonal distribution of efficient donors (Jacob and Wollman, 1961) and suggested that the population had indeed been enriched for mobilizing ability. However, after 10 additional cycles there was little further increase in recombinant formation. We then tested whether chromosome transfer frequencies of the enriched donor had in fact been improved. After a 3 h mating recombinants were recovered at the following frequencies: Ilv^+, 9×10^{-5}; Rif^r, 6×10^{-5}; His^+, 6×10^{-6}, and Met^+, 4×10^{-7} per input donor. These frequencies were similar to those of the unenriched donor (Table 7, line 5), indicating that the enrichment technique had not been successful.

Attempts to isolate R-prime plasmids in vivo. In many bacterial genera plasmids into which fragments of chromosomal DNA have been inserted acquire sex factor activity (Beck et al., 1982; Holloway, B. W., 1978; Julliot and Boistard, 1979). We thought that the use of analogous R-prime plasmids might allow us to mobilize the A. tumefaciens chromosome from other origins. In vivo, R-primes can form following recombination between plasmid and chromosome (Holloway, B. W., 1979). Such plasmids can be isolated in matings between the donor with a genetically heterologous recipient by selecting for transfer of

a donor chromosomal gene (Low, K. B., 1972). Because there is insufficient nucleotide sequence homology between the R-prime and the recipient chromosome, the plasmid will not undergo further recombination events, but will be stably maintained. Accordingly, we crossed A. tumefaciens strains containing R68.45 or pDP37 with restrictionless Pseudomonas aeruginosa strains PAO403 and PAO1161, with E. coli strains 1231, A767, B463, and D4314 and with A. tumefaciens strain SA117. In a series of 11 overnight matings we selected for transfer of genes conferring nutritional independence. We observed either that there was no growth on the selective media or that the numbers of colonies were no greater than the numbers on control plates, and therefore pursued these experiments no further.

Construction of a chromosome map. Since all donor and recipient strains are derivatives of strain 15955, the chromosomal organization in each strain should be identical, assuming that no major rearrangements have occurred. Therefore, the distances between genetic markers should be the same for any donor-recipient combination, and linkage data for different crosses should be similar.

Preliminary experiments suggested that the A. tumefaciens chromosome could be mapped using primarily three donor strains. The majority of the linkage data could be obtained with donor strain SA5017-37 and its two derivatives, strains SA5017SE-37 and SA5017N-37. For the Class I donor strain SA5017-37 we have demonstrated a transmission gradient of $Ilv^+ \rightarrow Rif^r \rightarrow His^+ \rightarrow Met^+$ (Pischl and Farrand, 1983; Chapter 4).

This evidence combined with the observed coinheritance pattern (Pischl and Farrand, 1983) suggested that the transmission gradient reflects the order with which donor markers are transmitted to the recipient, (leading marker ilv to distal marker met). Given this type of model, the Class II donor strains SA5018-37 and SA5004-37 (transmission gradients $His^+ \rightarrow Rif^r \rightarrow Ilv^+ \rightarrow Met^+$, Table 7) were also expected to be useful because they should transmit the chromosome from a different origin and with opposite polarity.

Chromosome mapping experiments proceeded in two stages. First, strain SA5017-37 or one of its derivatives was mated with each of the recipient strains. By recombination analysis (Hopwood, D. A., 1972) we then identified the order of markers. Recombinants selected for acquisition of a distal marker were screened for inheritance of unselected donor markers and classified in terms of the number of recombination events required to produce the exhibited phenotype. We assumed that the frequency with which a recombination event occurs between two markers is directly related to the distance between the markers (Hayes, W., 1968). Therefore, the correct marker order could be identified as that which gave the fewest numbers of recombinants in the multiple event (4 or more crossovers) classes. An example of an analysis of Met^+ recombinants derived from the SA5017SE-37 donor is shown in Table 8. Based upon the transmission gradient we tentatively proposed a marker order of ilv → str → rif → trp-101 → his → met. When Met^+ recombinants were classified by number of recombination events, 92% fell into the 2-event class and 8% into the 4-event class. For any other

possible order of markers the proportion of recombinants in the 4- and 6-event classes was much larger. Therefore our proposed marker order must be correct.

In many of the crosses we observed that certain recombinant phenotypes were under-represented (Table 8, lines 4 and 5), while others were over-represented (Table 8, lines 23 and 25). This may have resulted either from a preferential exclusion of the donor Rif^r marker, or from an increased frequency of recombination between the rif and trp-101 markers. The observation that the rif-101 marker is preferentially excluded from recombinants constitutes one exception to the general rule that an incoming chromosomal segment is incorporated as an intact unit; that is, that all markers located between the distal selected marker and a proximal coinherited marker are also coinherited (Barrett et al., 1982; Beringer et al., 1978; Hooykaas et al., 1982; Meade and Signer, 1977).

Once we knew the order of the markers, we determined the distance between each based upon the frequency with which recombinants acquired the unselected marker adjacent and proximal to the selected marker. In general, each marker pair was studied in at least two crosses. Whenever possible each marker pair was also examined using a donor strain of each transmission class. Using the linkage data summarized in Table 9 we then constructed a map of the A. tumefaciens strain 15955 chromosome (Fig. 6).

TABLE 8. Analysis of Met^+ recombinants[a]

Phenotype						Recombination events in intervals[b]	No. of recombination events	No. of recombinants
Ilv^+	Str^r	Rif^r	$Trp\text{-}101^+$	His^+	Met^+			
−	−	−	−	−	+	5,y	2	63
−	−	−	−	+	+	4,y		54
−	−	−	+	+	+	3,y		12
−	−	+	+	+	+	2,y		4
−	+	+	+	+	+	1,y		1
+	+	+	+	+	+	x,y		76
+	−	−	−	−	+	x,1,5,y	4	1
−	+	−	−	−	+	1,2,5,y		0
+	+	−	−	−	+	x,2,5,y		0
−	−	+	−	−	+	2,3,5,y		1
−	+	+	−	−	+	1,3,5,y		1
+	+	+	−	−	+	x,3,5,y		0
−	−	−	+	−	+	3,4,5,y		1
−	−	+	+	−	+	2,4,5,y		1
−	+	+	+	−	+	1,4,5,y		1
+	+	+	+	−	+	x,4,5,y		1
+	−	−	−	+	+	x,1,4,y		0
−	+	−	−	+	+	1,2,4,y		0
+	+	−	−	+	+	x,2,4,y		1
−	−	+	−	+	+	2,3,4,y		2
−	+	+	−	+	+	1,3,4,y		0
+	+	+	−	+	+	x,3,4,y		1
+	−	−	+	+	+	x,1,3,y		4
−	+	−	+	+	+	1,2,3,y		0
+	+	−	+	+	+	x,2,3,y		3
+	−	+	+	+	+	x,1,2,y		0

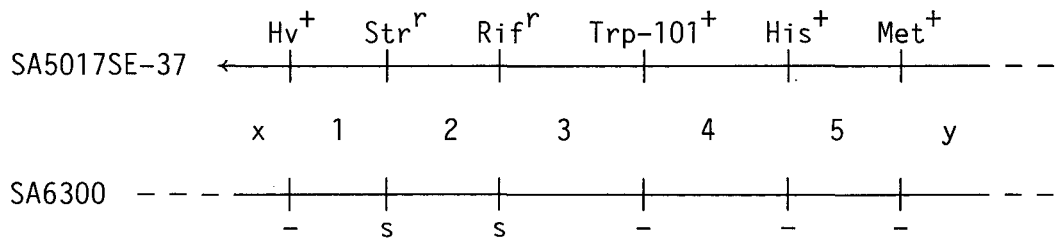
Table 8 continued on next page

TABLE 8. (cont'd) Analysis of Met^+ recombinants

Phenotype						Recombination events in intervals[b]	No. of recombination events	No. of recombinants
Ilv^+	Str^r	Rif^r	$Trp\text{-}101^+$	His^+	Met^+			
+	−	+	−	−	+	x,1,2,3,5,y	6	0
+	−	−	+	−	+	x,1,3,4,5,y		0
−	+	−	+	−	+	1,2,3,4,5,y		0
+	+	−	+	−	+	x,2,3,4,5,y		0
+	−	+	+	−	+	x,1,2,4,5,y		0
+	−	+	−	+	+	x,1,2,3,4,y		0

[a]Met^+ recombinants formed in a 3-h mating between donor strain SA5017SE-37 and recipient strain SA6300 were screened for coinheritance of unselected donor markers and then assigned to the appropriate phenotypic class.

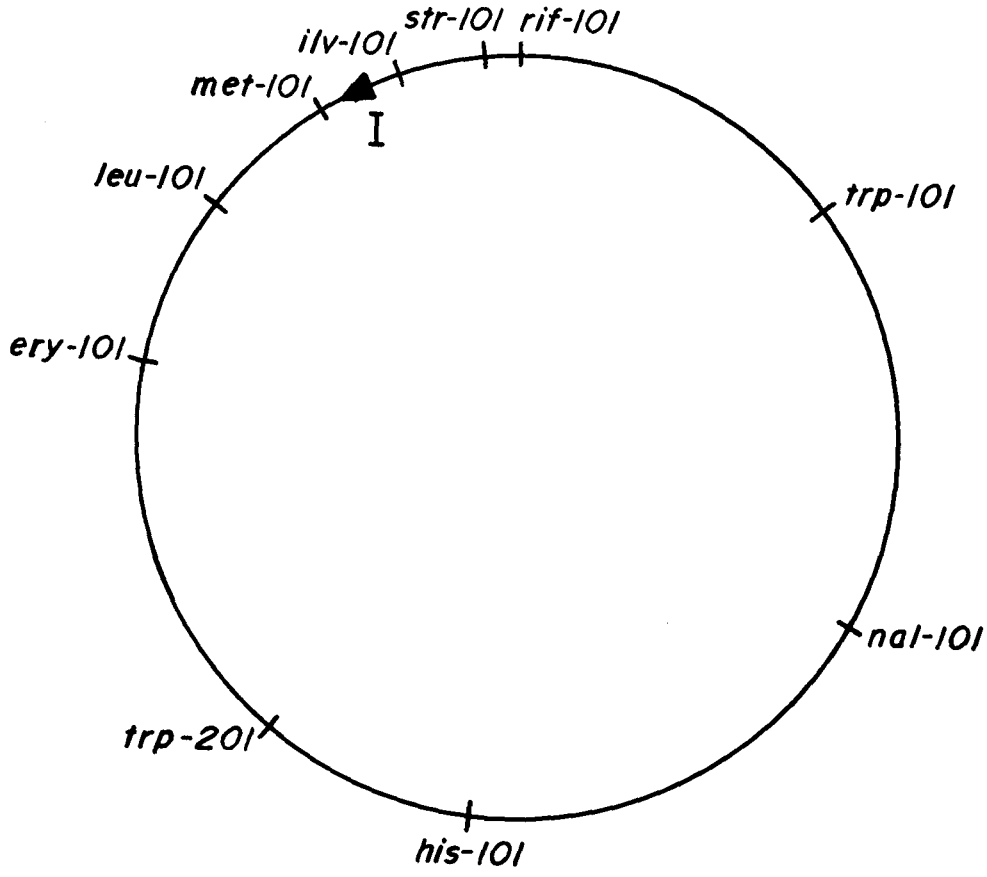
[b]Pertinent segments of donor and recipient chromosomes and the inter-marker intervals (x,1-5,y) in which recombination events must have occurred are as follows:



[c]Minimum number of events detectable.

FIGURE 6

Chromosomal linkage map for A. tumefaciens 15955 constructed from the data presented in Table 9. Recombinants selected for acquisition of a distal marker were screened for coinheritance of the adjacent marker proximal to the selected marker. The distance between loci was assumed to be directly proportional to the frequency of recombination (100% - % coinheritance) between markers (Hayes, W., 1968). The location of the Class I origin of transfer, symbolized by an arrow, is approximate. The Class II origin is located between the trp-201 and the met-101 markers. Abbreviations are as presented in Table 1.


str-101
rif-101
ilv-101
met-101
I
leu-101
trp-101
ery-101
nal-101
trp-201
his-101

After the preceding chromosome mapping experiments had been completed, Putnoky et al. (Putnoky et al., 1983) reported data which suggested that Tn5 itself encodes high-level streptomycin resistance in Rhizobium meliloti and in A. tumefaciens. Since our streptomycin-resistant (Str^r) A. tumefaciens strain SA5017SE-37 was constructed from a parent which harbored plasmid as well as chromosomal copies of Tn5, this raised the possibility that our str-101 marker was actually encoded by Tn5, rather than constituting a unique chromosomal locus, as we had deduced from the mapping data. To determine whether str-101 was indeed a chromosomal locus, we designed an experiment to study the effect of placing the chromosomal streptomycin resistance marker in the recipient. We therefore isolated from the auxotrophic strain SA6300 a spontaneous mutant resistant to streptomycin. This Str^r Rif^s strain SA6361 was then mated with the Rif^r Str^s donor strain SA5017-37 and Rif^r recombinants of the recipient were selected. When screened for acquisition of the unselected Str^s donor marker, 119 of 289 (41.2%) of the recombinants were streptomycin sensitive, indicating that the donor markers Rif^r and Str^s are linked. Since the recipient parent lacked Tn5 and was chromosomally Str^r, this demonstration of linkage between the wild-type Str^s allele and rif-101 is entirely consistent with our placement of the str-101 marker on the A. tumefaciens chromosome (Fig. 6).

Discussion

In gene transfer systems such as the E. coli Hfr the origin of chromosomal transfer is determined by the site and polarity of integration of the F plasmid, which varies from strain to strain. This variation in donor origins is reflected in their respective transmission gradients, whose orders are unique but circular permutations of one another (Hayes, W., 1968). Since we had purposely constructed A. tumefaciens donor strains containing Tn5 at different sites in the chromosome (Pischl and Farrand, 1983; Chapter 4), we asked whether different donors exhibited similar circularly permuted transmission gradients. Characterization of a number of independently constructed pDP37-containing donors revealed, however, only two types of gradients. Five strains transmitted markers in the order $Ilv^+ \rightarrow Rif^r \rightarrow His^+ \rightarrow Met^+$ and three strains gave a permutation of that gradient: $His^+ \rightarrow Rif^r \rightarrow Ilv^+ \rightarrow Met^+$ (Table 7). As described in Results we did note some ambiguity with respect to the position of the Rif^r marker in these gradients. This phenomenon occurred with both brief and lengthy matings plated directly on selective media. The results of Fig. 5 indicate that in the first case yields of recombinants were reduced because insufficient time was allowed for expression of the recessive Rif^r gene product. In the second case the extended incubation on non-selective medium may have promoted overgrowth of the Rif^r recombinants by isolates bearing the wild-type Rif^s marker. Our findings that the donor Rif^r marker was preferentially excluded from recombinants (Table 8, lines 4, 5, 23, and 25; additional data not shown) suggest that the sites of individual crossover events may also affect yields of recom-

binants. For these reasons we concluded that those Tn5-facilitated donors tested exhibited only two classes of transmission gradients.

With our Tn5-facilitated mobilization system, the various classes of selected recombinants show a gradient in acquisition of unselected markers. That this coinheritance gradient matches the donor transmission gradient strongly suggests that a single origin and only that origin is used by any given donor (Tables 7 and 8 and additional data not shown). However with the R68.45 mobilization system, donors exhibited nonpolar transmission from multiple origins (Table 6). While these findings emphasize the importance of the plasmid Tn5 element in pDP37 mobilization, previous experiments showed that the chromosomal Tn5 element also makes an essential contribution (Pischl and Farrand, 1983; Chapter 4). One possible explanation for these characteristics is that Tn5 may only be able to insert itself in two regions of the A. tumefaciens chromosome. This seems unlikely because we observed such a wide variety of auxotrophic mutations (Pischl and Farrand, 1983; Chapter 4), each of which most probably originated from insertion of Tn5 into a unique structural gene (Beringer et al ., 1978). An alternative explanation is that interaction of chromosome and plasmid can only occur in two regions of the chromosome. Such interaction may be governed by plasmid Tn5 sequences or by nucleotide sequences on the plasmid modified by removal of the HindIII recognition site (Pischl and Farrand, 1983; Chapter 4). Given this limitation to two regions, it would appear unlikely that it is the chromosomal Tn5 element which determines the site of interaction. This suggests that

stable integration of the plasmid does not occur in this mobilizing system, which may explain our inability to isolate R-prime plasmids or Hfr-like donors. Such a phenomenon is by no means unique to our gene transfer system. The plasmid Col I, for example, shows chromosome mobilizing activity but gives no evidence of stable integration (Clowes and Moody, 1966; Moody and Runge, 1972).

We expected that derivatives of R68.45 which carried a portion of the A. tumefaciens 15955 chromosome would very likely demonstrate sex factor activity in strain 15955. Therefore we looked for R-prime plasmids formed in vivo by selecting for transfer of an A. tumefaciens chromosomal gene to any of a variety of recipient strains (see Results). Despite our use of restrictionless strains, we were unable to isolate R-primes using E. coli or P. aeruginosa recipients. This failure may have resulted from an inability of the recipient to express genes of A. tumefaciens origin (Holloway, B. W., 1979). However, we were also unable to isolate Leu^+ or Rif^r recombinants in A. tumefaciens SA117, a strain C58 derivative less likely to exhibit an expression barrier (although restriction or recombination may have limited the recovery of recombinants).

Our transposon-facilitated chromosomal gene transfer system has two advantages over R68.45 in strain 15955, namely polarity and improved mobilization frequencies (Bryan et al., 1982; Hamada et al., 1979; Pischl and Farrand, 1983; Chapter 4). Polarized gene transfer systems are particularly useful because, among recombinants selected

for acquisition of a distal marker, coinheritance of proximal markers depends only on recombination events. Therefore, no correction for prezygotic exclusion (Hamada et al., 1979) need be made. Thus, the chromosome mobilizing properties of pDP37 allowed us to construct a map from the frequency of recombination between a distal selected marker and an unselected marker adjacent and proximal to the selected marker. The use of donors from each polarized transmission class allowed us to show linkage between ilv-101 and met-101 as well as between his-101 and met-101 (Table 9). Thus, all chromosomal loci mapped form a single circular linkage group (Fig. 6).

Careful study of coinheritance of the donor Str^s marker by recombinants selected for acquisition of the rif-101 marker wholly supports our assignment of the str-101 marker as a chromosomal locus (see Results and Fig. 6). Since the recipient parent was chromosomally streptomycin-resistant, the only way recombinants could have acquired a streptomycin-sensitive phenotype at such a high frequency was by inheriting the rif-101-linked Str^s marker from the donor chromosome. This indicates that the Str^r allele in our Tn5-containing donor is of chromosomal rather than transposon origin.

The order of markers in our strain 15955 map is similar to that for A. tumefaciens strain C58 presented by Hooykaas (Hooykaas et al., 1982), although the relative order of rif and str appears to be reversed. Both strains show tight linkage of rif and str alleles, as

TABLE 9. Linkage data used to construct chromosome map

Donor strain	Distal selected marker	Proximal unselected marker	No. of matings	No. of selected recombinates	Linkage (%)[b]
SA5017-37[a]	rif-101	str-101	4	717	94.3
	trp-101	rif-101	7	968	52.0
	nal-101	trp-101	2	350	41.4
	his-101	nal-101	2	614	41.5
	trp-201	his-101	2	362	69.6
	ery-101	trp-201	2	132	45.4
	leu-101	ery-101	2	419	76.8
	met-101	leu-101	2	360	82.2
SA5018-37	his-101	trp-201	1	100	78.0
	trp-101	his-101	2	219	40.2
	rif-101	trp-101	2	205	53.6
	ilv-101	rif-101	3	344	65.1
	met-101	ilv-101	3	155	80.0
SA5004-37	met-101	ilv-101	4	121	97.5
	ilv-101	rif-101	4	131	80.9

[a]For convenience, strains SA5017-37, SA5017N-37, and SA5017SE-37 are considered together since they differ only at the nal-101, str-101, or ery-101 locus (Table 1).

[b]Number of selected recombinats acquiring the unselected marker divided by the number tested and multiplied by 100.

well as dispersal of *trp* loci (Bryan *et al.*, 1982; Hooykaas *et al.*, 1982; see above). This is also the case for the closely related bacteria *Rhizobium meliloti* and *Rhizobium leguminosarum*. In the Hooykaas map of strain C58, each of four *his* mutants maps to a single site, suggestive of an operon-like organization. Our strain 15955 *his* mutant maps to a position analogous to that of the *his* loci in strain C58 (Fig. 6) (Hooykaas *et al.*, 1982). Despite the apparent similarity in genetic organization between these two strains we were unable to obtain Leu^+ or Rif^r recombinants in strain SA117, a derivative of strain C58. Although this result may be due to the small number of markers tested or the restriction of incoming DNA, it may on the other hand reflect the degree to which the two strains have diverged. The latter hypothesis is supported by the results of solution hybridization experiments which show that strain 15955 shares only 43% nucleotide sequence homology with strain NT1, a plasmid-cured derivative of strain C58 (Currier and Nester, 1980). Thus it may be that while the overall genetic organization of these strains is similar, nucleotide sequence divergence may have raised an expression or recombination barrier between the two.

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APPENDIX A

OPTIMUM MATING PERIOD AND KINETICS OF RECOMBINANT FORMATION FOR THE A. TUMEFACIENS Tn5-FACILITATED CHROMOSOME MOBILIZATION SYSTEM

Chapter 4 describes the construction of an effective chromosomal gene transfer system in A. tumefaciens 15955. These donor strains harboring Tn5 in both plasmid and chromosome transmitted different chromosomal markers at various frequencies, such that a reproducible gradient of decreasing marker transmission frequencies could be described for each. Recombinants generated by these donors coinherited unselected markers at detectible frequencies. These findings suggested that the mobilization system could indeed be useful for mapping chromosomal loci. Therefore it was judged appropriate to characterize the system in further detail. This appendix describes the results of experiments designed to study two particular parameters: (1) the optimum ratio of donors to recipients and (2) the kinetics of recombinant formation.

Materials and Methods

Agrobacterium tumefaciens strains SA5017-37, A63 and other materials are described in in detail in Chapter 3.

Results and Discussion

Optimum donor-recipient ratio. Although the Tn5-facilitated donors appeared to transfer some chromosomal markers more efficiently

than did strains carrying R68.45 (Chapter 3), transfer frequencies for the distal Met^+ marker were still somewhat limiting. Since the efficiency of chromosome mobilization is in some systems markedly reduced in the presence of an excess of donor cells (Ou, 1980), we asked whether the efficiency of our system also depended upon the donor-recipient cell ratio. Table 10 shows the yields of Ilv^+ recombinants from a series of parallel filter matings in which varying numbers of donor cells were mixed with a constant number of recipients. The yield of recombinants increased by as much as 100-fold at low donor-recipient ratios. These results were similar to those previously obtained by Hamada et al. (1979) with the R68.45-based A. tumefaciens mobilization system. The basis of this phenomenon may be that the cell membrane is damaged in the presence of excess donor, as occurs with E. coli Hfr donors (Ou, 1980).

Kinetics of recombinant formation. To accurately map chromosomal loci by recombination analysis, we wanted to ensure that we were scoring a representative population of recombinants, so that coinheritance frequencies would accurately reflect the distance between loci. Since it seemed very possible that recombinants acquiring more of the prototrophic markers might have a growth advantage over others, we thought it advisable to minimize the time between initiation of mating and plating on selective medium. To identify the minimum mating period that would yield sufficient numbers of recombinants, we assessed recombinant yields from parallel filter matings terminated after various periods of time. We observed that the total number of

Table 10. Effect of donor-recipient ratio on formation of recombinants[a]

Ratio[b] (donor-recipient)	Donor titer	Recipient titer	Recombinants per donor
0.012	8.5×10^6	6.8×10^8	4.5×10^{-3}
0.12	8.5×10^7	"	2.7×10^{-3}
1.25	8.5×10^8	"	6.3×10^{-4}
12.5	8.5×10^9	"	4.7×10^{-5}

[a]Donor strain SA5017-37 was mated 4 h with strain A63; Ilv^+ recombinants were selected.

[b]Ratio of donor titer to recipient titer.

Met^+ recombinants increased over a period of 16 h and then began to decrease. We expected that the rate of formation of recombinants would be affected by two factors: (1) the rate of formation of de novo recombinants, which should be particularly significant early in the mating, and (2) the rate of formation of recombinant progeny, which should be more significant at later times. Therefore, our observation that the recombinant yield increased most rapidly during the first four hours of mating suggested that this was the time interval most significant for the de novo formation of recombinants. Partly to further reduce the contribution of progeny formation and partly for convenience, we subsequently adopted as standard conditions a mating period of 3 h (Chapters 4 and 5).

In both E. coli and in Pseudomonas aeruginosa interrupted matings have proven very useful for mapping chromosomal markers (Hayes, 1968; Stanisich, 1969). We therefore asked whether such methods could be effectively used with our donors. Since mating pair formation with IncP1 plasmids is very inefficient in broth, we initiated matings between strains SA5017-37 and A63 by immobilizing the cells on filters for 5 min. We then resuspended the cells in broth and incubated the mixture at 29°C., and periodically removed samples to determine the titers of various classes of selected recombinants over a period of 2-3 h. Each class of recombinants appeared to rise in titer at 15 min, decrease at 30 min and then rise again in a linear fashion. It therefore appeared likely that an error had been made with the 15-minute sample (Such errors could have been avoided

by titering the number of recombinants in terms of the donor titer at each time point, but the increase in labor made this approach impractical). The yield of recombinants for the various markers appeared to increase in a linear fashion. For the proximal Ilv^+ marker the recombinant titer reached a plateau at approximately 60 min. Recombinants for the His^+ and the Met^+ markers did not reach a plateau within two h. The time of entry of each marker was determined by extrapolation from experimental results (data not shown). The proximal Ilv^+ marker appeared to enter at 0 min, possibly because some chromosome transfer had occurred during the 5 min allowed for mating pair formation. The His^+ marker appeared to enter at 16 min and the distal Met^+ marker at 23 min. These conclusions are limited in certainty by the relatively low level of confidence, inherent to their extrapolation. The experimental results did appear to be consistent with the transmission gradient exhibited by this donor strain and to provide further evidence that chromosome transfer is polarized. On the other hand, the relatively short time required for the entry of the distal Met^+ marker did not support a model of Hfr-like oriented linear transfer of the chromosome requiring as long as 90 min for completion in E. coli. The usefulness of this mapping method was further limited by the relative difficulty of initiating and terminating mating pair formation, the shortage of suitable chromosomal markers and by the large number of technical manipulations required of a single investigator. Therefore another mapping strategy (recombination analysis) was pursued.

APPENDIX B

CONSTRUCTION OF A CHROMOSOMAL MAP

Use of the Tn*5*-facilitated mobilization system to obtain linkage data for 10 chromosomal loci has been described (Chapter 5, especially Table 9). From that data the frequency of recombination between adjacent markers (1.0 - coinheritance frequency; Hayes, 1968) was calculated. When coinheritance frequencies from both classes of donor strains was available, the average frequency of recombination between two markers was determined from the following equation:

$$\text{ave RF} = \frac{(RF_1)(RS_1) + (RF_2)(RS_2)}{\text{total RS}}$$

where RF is the recombination frequency and RS is the number of selected recombinants screened for coinheritance. The frequency of recombination between adjacent markers was assumed to be directly proportional to the distance between the markers (Hayes, 1968; Hop wood, 1972). For a circular map, the distances between markers were determined by calculating the fraction (f) of total recombination occurring in each interval between markers:

$$f = \frac{(RF_i)(C)}{\Sigma\ RF_i}$$

where RF_i is the average recombination frequency for the interval of interest, C is the circumference of the circle to be drawn and $\Sigma\ RF_i$ is the sum of all the average recombination frequencies.

APPENDIX C

Tn5- AND R-PRIME- FACILITATED CHROMOSOME MOBILIZATION: DETERMINATION CHROMOSOMAL TRANSFER ORIGIN AND ORIENTATION

The properties of two related Agrobacterium tumefaciens chromosomal gene transfer systems were further characterized by studying chromosome mobilization by an R-prime plasmid both in strains containing transposon Tn5 inserted in oposite orientations in the chromosome and in a strain lacking the transposon. The chromosomal element was found to play a role in specifying both transfer origin and orientation. In addition, plasmid sequences were found to contribute to the selection of transfer origin and orientation.

Chapters 4 and 5 describe a method of chromosomal gene transfer for the plant pathogenic Agrobacterium tumefaciens. The method involves brief filter matings between auxotrophic recipient strains and donor strains which contain an insertion of transposon Tn5 in both plasmid and chromosome. Efficient mobilizing activity occurs only in donor strains that harbor Tn5 on both replicons (Pischl et al., 1983). Each Tn5-facilitated donor mobilizes chromosomal markers at frequencies which vary with the selected marker and with the donor strain, such that a characteristic transmission gradient can be described for each donor (Pischl et al., 1984). All donors surveyed fall into one of two classes with respect to transmission gradient. Class I strains show a gradient of $Ilv^+ \rightarrow Rif \rightarrow Trp^+ \rightarrow His^+ \rightarrow Met^+$,

while class II donors give a permutation of that gradient: $His^+ \rightarrow Trp^+ \rightarrow Rif \rightarrow His^+ \rightarrow Met^+$ (Pischl et al., 1984). The transmission gradient matches the gradient in frequencies of coinheritance for the various classes of selected recombinants, indicating that Tn5-facilitated donors mobilize the chromosome in a polarized manner (Pischl et al., 1984).

The sequence of events leading to chromosome mobilization in Tn5-facilitated donors is not known, but the observation of only two classes of selected recombinants suggests that it may differ from mobilization in the E. coli Hfr. We have recently developed a second mobilization system using R-prime plasmids constructed by in vitro ligation of a chromosomal DNA fragment into the same plasmid used as the parent for the Tn5-facilitated plasmid (D. L. Pischl, unpublished results). A donor strain containing a chromosomal insertion of Tn5 and harboring R-prime plasmid pDDP24 exhibited a transmission gradient of $His^+ \rightarrow Trp^+ \rightarrow Rif \rightarrow Ilv^+ \rightarrow Met^+$, and recombinants showed a coinheritance gradient which matched the transmission gradient (D. L. Pischl, unpublished results). Since a donor with the same chromosomal background but using a Tn5-facilitated plasmid mobilized the chromosome from a different origin and in the opposite direction (Pischl et al., 1984, D. L. Pischl, unpublished results), we suspected that these two components of transfer were determined by plasmid sequences homologous to chromosomal sequences. However, the observation that the Tn5-facilitated plasmid when placed in diffrent chromosomal backgrounds gave only two different classes of transmission gradients

(Pischl et al., 1984) suggested that chromosomal sequences might also determine origin and transfer orientation. This report describes the results of two experiments designed to further study the contributions of chromosomal and plasmid sequences to mobilization.

Results

Role of chromosomal Tn5 element in determining orientation of transfer. The two classes of Tn5-facilitated donors show opposite directions of chromosome transfer (Pischl et al., 1984). Since these donors differ only with respect to the chromosomal transposon, this element appears to specify the direction of chromosome mobilization. We wondered whether the transfer orientation would also be regulated by the chromosomal Tn5 in donors that harbored an R-prime plasmid. To begin to answer this question we compared the mobilizing properties of R-prime plasmids in strains thought to harbor Tn5 in opposite orientations. The plasmid pDDP24 has previously been shown to mobilize the chromosome of strain SA5017 in the following direction: $His^+ \rightarrow Trp^+ \rightarrow Rif \rightarrow Ilv^+ \rightarrow Met^+$. We therefore undertook to identify the transfer orientation of pDDP24 in strain SA5018. Accordingly, pDDP24 was introduced into strain SA5018 by cross-streak mating with strain SA5017-24. This method does not permit chromosome mobilization at detectible frequecies (D. L. Pischl, unpublished results) and was therefore unlikely to have altered the organization of the strain SA5018 chromosome. Furthermore, it ensured that the R-prime plasmids in the two test donors were as closely identical as possible. The test donor strain was then mated with an appropriate recipient and

recombinants were screened for acquisition of unselected markers. As predicted, this donor gave a coinheritance gradient which was the inverse of the strain SA5017-24 gradient (Table 11). Confirmation of the marker transmission order for strain SA5018-24 was then obtained by recombination analysis (Pischl et al., 1984). Since the absolute order of markers on the A. tumefaciens chromosome was already known (Pischl et al., 1984), the donor transmission order could be determined by identifying those two markers between which recombination was enhanced as a result of breakage at the trasfer origin. This analysis yielded only three possible transmission orders, two of which could be ruled out by the mobilization frequencies of Table 11. The remaining marker (Table 12) was identical to the predicted order, namely $Met^+ \rightarrow Ilv^+ \rightarrow Rif \rightarrow Trp^+ \rightarrow His^+$.

Properties of a donor which lacks Tn5. The role of Tn5 was further evaluated by comparing mobilization by R-prime plasmid pDDP24 in strains either with or without the chromosomal Tn5 element. Accordingly, a donor strain was constructed by introduction of pDDP24 into strain SA12203, the leucine auxotrophy of which permitted its subsequent counterselection. The mobilization properties of this donor were determined by analyzing selected recombinants for coinherited markers. As shown in Table 13, a clearly defined coinheritance gradient aligning with the transmission gradient was not observed. Instead there appeared to be a discrepancy between the coinheritance pattern for the His^+ marker and its position in the transmission gradient. The former was typical of a proximal marker

TABLE 11. Mobilization and coinheritance data for strain SA5018-24[a]

Selected marker[b]	Mobilization frequency	No. coinheriting unselected marker[c] (%)				
		Met$^+$	Ilv$^+$	Rif	Trp$^+$	His$^+$
Met$^+$	1.6×10^{-5}	220(100.0)	220(100.0)	220(100.0)	105(47.7)	8(3.6)
Ilv$^+$	2.9×10^{-6}	132(60.3)	219(100.0)	210(95.9)	73(333)	42(19.2)
Rif	3.3×10^{-7}	138(62.7)	211(95.9)	220(100.0)	77(35.0)	0(≤0.4)
Trp$^+$	2.9×10^{-7}	130(56.8)	223(97.4)	215(93.9)	229(100.0)	27(11.8)
His$^+$	3.4×10^{-8}	35(28.4)	94(76.4)	91(74.0)	80(65.0)	123(100.0)

[a]Recombinants produced from a mating between strains SA5018-24 and SA6300.

[b]Abbreviations are as given in Table 1.

[c]Recombinants for the selected marker were scored for coinheritance of unselected markers.

TABLE 12. Analysis of Met^+ recombinants

Phenotype Met	Ilv	Rif	Trp	His	No. recombinants	No. recomb. events
−	−	−	−	+	28	
−	−	−	+	+	1	
−	−	+	+	+	0	2
−	+	+	+	+	47	
+	+	+	+	+	29	
−	−	+	−	+	0	
−	+	−	−	+	1	
−	+	−	+	+	2	
−	+	+	−	+	9	4
+	−	−	−	+	0	
+	−	−	+	+	0	
+	−	+	+	+	0	
+	+	−	−	+	0	
+	+	−	+	+	0	
+	+	+	−	+	0	
+	−	+	−	+	0	6

Recombination analysis for the cross of strains SA5018-24 and SA6300 described in Table 11. Recombinants selected for the selected distal marker were classified by the number of recombination events required to produce the exhibited phenotype.

TABLE 13. Mobilization and coinheritance data for strain SA12203-24[a]

Selected marker[b]	Mobilization frequency	Coinheritance of unselected marker[c](%)				
		Trp^+	Rif	Ilv^+	Met^+	His^+
Trp^+	7.5×10^{-5}	203(100.0)	12(5.9)	26(12.8)	11(5.4)	0(≤0.5)
Rif	1.5×10^{-6}	15(16.1)	93(100.0)	86(92.5)	9(9.7)	6(6.4)
Ilv^+	4.4×10^{-7}	34(21.9)	37(21.9)	155(100.0)	29(18.7)	15(9.7)
Met^+	1.0×10^{-7}	37(23.3)	25(15.7)	55(34.6)	159(100.0)	61(38.4)
His^+	2.8×10^{-8}	13(7.1)	2(1.1)	5(2.7)	8(4.4)	183(100.0)

[a]Recombinants from a mating between strains SA12203-24 and SA6300.

[b]Abbreviations as in Table 1.

[c]Recombinants inheriting the selected marker were scored for acquisition of other donor markers.

while the latter indicated a more distal position. This discrepancy was thought to be an artifact resulting from the counterselection against inheritance of the donor Leu^- marker, which appeared to be positioned between Met^+ and His^+. At least one other *leu* allele has previously been mapped to this interval (Pischl *et al.*, 1984). The counterselection may also have reduced the efficiency of recovery of recombinants (Table 11). Although unclear with respect to the His^+ marker, the data of Table 11 did however strongly suggest that the Trp^+ marker was transmitted as a proximal marker and Met^+ as a relatively distal marker.

By recombination analysis the transmission order of strain SA5018-24 was then narrowed down to the three possibilities given in Table 14. Since two of these potential orders were not consistent with the overall data of Table 13, the transmission order was therefore tentatively identified as $Trp^+ \rightarrow Rif \rightarrow Ilv^+ \rightarrow Met^+ \rightarrow Leu^- \rightarrow His^+$.

Discussion

Plasmid pDP37 mobilizes the chromosomes of strains SA5017-37 and SA5018-37 in opposite orientations (Pischl *et al.*, 1984). Since these two strains differ only with respect to the chromosomal Tn*5* insertion, this observation suggests that this element regulates the orientation of transfer and that Tn*5* is oppositely oriented in the two strains. The accuracy of these conclusions can be tested by comparing the mobilization properties of other plasmids in these chromosomal backgrounds. The R-prime plasmids are ideal for such purposes since they mobilize effectively but do not contain Tn*5*. The results reported in

TABLE 14. Analysis of selected recombinants[a].

Recombinant phenotype[b]	No. recombinants for marker order[c]						No. recomb. events
	TRIMH		MIRTH		HMIRT		
- - - - +	162		162		171		
- - - + +	5		9		0		
- - + + +	0	-(91.2%)	0	-(94.5%)	10	-(90.1%)	2
- + + + +	0		2		2		
+ + + + +	0		0		0		
- - + - +	3		0		11		
- + - - +	0		3		6		
- + - + +	0		0		0		
- + + - +	0		0		3		
+ - - - +	9		5		0		
+ - - + +	2	-(8.7%)	2	-(5.5%)	0	-(9.8%)	4
+ - + + +	0		0		0		
+ + - - +	0		0		0		
+ + - + +	0		0		0		
+ + + - +	2		0		0		
+ - + - +	0		0		0		6

[a]Recombinants from mating of strains SA12203-24 and SA6300.

[b]Recombinants selected for acquisition of distal donor marker were scored for unselected markers. (+) = donor marker inherited, (-) = donor marker not inherited.

[c]Abbreviations as in Table 1 and: T, *trp*-101; R, *rif*-101; I, *ilv*-101; M, *met*-101; H, *his*-101. The percent of total recombinants in the 2-event and 4-event classes is given in parentheses.

this study show that plasmid pDDP24 in its respective chromosomal backgrounds does indeed effect mobilization in opposite orientations (Tables 11 and 12; D. L. Pischl, unpublished results). However, as previously noted, donor strains SA5017-37 and SA5017-24 differing only in plasmid content also mobilize in opposite directions (Tables 11 and 12; D. L. Pischl, unpublished results). Therefore it can be concluded that both the chromosomal Tn5 element and sequences on the plasmid (either Tn5 or the chromosomal insertion) regulate the orientation of transfer.

Analysis of paired matings shows that the chromosomal Tn5 element also governs selection of the origin of chromosome transfer. For example, strains SA5017-37 and SA5018-37 transfer the chromosome from different origins (Pischl et al., 1984). However, other evidence indicates that it is the plasmid which determines the transfer origin. Strains SA5017-37 and SA5017-24 differing only in mobilizing plasmid utilize different transfer origins (D. L. Pischl, unpublished results). These findings suggest that the transfer origin is selected by an interaction of both plasmid sequences and the chromosomal Tn5 element. Such an argument is further supported by the observation of different chromosomal transfer origins for strains SA5017-24 and SA1223-24. The origin in the latter strain, which lacks any Tn5 element, may be determined by the location of sequence homology between plasmid and chromosome.

The conclusions given above await confirmation by a series of additional matings. It should also be noted that Tn*5*-facilitated and R-prime-containing donors may differ with respect to the contribution of the chromosomal Tn*5* element. The lengthy experiments required to confirm or reject these ideas are beyond the scope of this study, the primary focus of which is the development and practical application of chromosomal mapping methods.

APPROVAL SHEET

The dissertation submitted by Diane L. Pischl has been read and approved by the following committee:

Dr. Stephen K. Farrand, Ph.D., Director
Associate Professor, Microbiology, Loyola

Dr. Tadayo Hashimoto, Ph.D.
Professor, Microbiology, Loyola

Dr. Robert E. Malone, Ph.D.
Assistant Professor, Microbiology, Loyola

Dr. John McNulty, Ph.D.
Associate Professor, Anatomy, Loyola

Dr. Philip Matsumura, Ph.D.
Assistant Professor, Biology, University of Illinois at Chicago

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

21 May 1985
Date

Stephen K. Farrand
Director's Signature