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Transfer Region of IncI1 Plasmid R64 and Role of Shufflon in R64 Transfer

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To locate the transfer region of the 122-kiloase plasmid R64*drd-11* belonging to incompatibility group I1, a series of deletion derivatives was constructed by in vitro recombinant DNA techniques followed by double homologous recombination in vivo. A plasmid designated pKK609 and bearing a 56.7-kilobase R64 sequence was the smallest transferable plasmid. A plasmid designated pKK610 and no longer possessing the 44-base-pair sequence from one end of pKK609 essential for the R64 *oriT* function failed to transfer, indicating that the *oriT* sequence of the R64 transfer system is located at one end. The other end of the R64 transfer region comprises a DNA segment of about 19 kilobases responsible for pilus formation. Shufflon, DNA with a novel rearrangement in R64, was found to be involved in pilus formation.

Bacterial conjugation is a complex process in which plasmid DNA is transferred from donor to recipient cells by a mechanism requiring cell-to-cell contact (for reviews, see references 10 and 25). The transfer system of IncF plasmids has been extensively studied. The transfer region of an IncF plasmid consists of more than 25 genes organized in a single contiguous region spanning 33 kilobases (kb), whose products are involved in pilus formation, stabilization of mating pairs, conjugative DNA metabolism, and surface exclusion.

IncI1 plasmids such as R64, ColIb, and R144 also transfer by a mechanism similar to that of IncF plasmids. Despite its general similarity to the IncF conjugation system, however, the IncI transfer system exhibits three distinctive features. (i) IncI plasmids form two types of sex pili, one thick and the other thin, both morphologically and antigenically distinct from the IncF plasmid pilus (1, 2). (ii) IncI plasmids carry the *sog* gene responsible for suppressing *dnaG* mutations in *Escherichia coli* (7, 18). The *sog* gene encodes DNA primase. (iii) An IncI plasmid shows a complex DNA rearrangement mediated by a unique structure designated shufflon (12–14). R64 shufflon consists of four DNA segments flanked and separated by seven 19-base-pair (bp) repeat sequences. The site-specific recombination between any two inverted repeats results in the inversion of DNA segments independently or in groups. The recombination is mediated by the gene *rci*, which is located adjacent to shufflon (17). Shufflon may function as a biological switch for the selection of one of seven genes in which the N-terminal three-quarters are constant and the C-terminal quarter is variable (14). The gene with the changing C-terminal region is tentatively named *pilV* in this paper. The C-terminal portions of the seven genes are called A, A′, B, B′, C, C′, and D (14).

Recently, we cloned, mapped, and sequenced the *oriT* region of the R64*drd-11* plasmid (15). A recombinant plasmid carrying a 141-bp *oriT* sequence of R64 was mobilized with a frequency similar to that of R64 transfer. A 44-bp portion of the 141-bp *oriT* sequence was found indispensable for the R64 *oriT* function. The R64 *oriT* region was located about 55 kb from the *rep* region.

Although many individual functions of the IncI1 transfer system have been clarified, the global organization of IncI1 plasmid transfer genes has yet to be determined in detail. The physical maps of R64, ColIb, and R144 are presently available (7, 9, 22, 24). Recently, the organization of ColIb-P9 transfer genes was analyzed by transposon mutagenesis (22).

In the present work, a series of deletion plasmids of R64*drd-11* was constructed. A plasmid bearing the 56.7-kb R64 sequence was the smallest transferable mini-R64 plasmid. The structure requisite for R64 transfer included the *oriT* sequence at one end, and at the other end of the R64 transfer region, there was a DNA segment of about 19 kb responsible for pilus formation and in which shufflon was found to be involved.

MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. The bacterial strains, bacteriophages, and plasmids used in this study are listed in Table 1.

Media. Luria-Bertani and H media were prepared as described by Miller (19). The agar medium consisted of Luria-Bertani or H broth containing 1.5 or 1.2% agar, respectively. Antibiotics were added to liquid or solid media at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 20 μg/ml; and tetracycline, 12.5 μg/ml.

Construction of deletion mutants of R64*drd-11*. By the in vivo gene replacement method (11), the deletion derivatives of R64 were constructed (Fig. 1A). Recombinant plasmids, in each of which a part of the R64 sequence was replaced with a DNA fragment for kanamycin resistance from Tn*5* or for chloramphenicol resistance from pACYC184, were constructed on a vector pUC9. After being converted into linear form, plasmid DNA was introduced by transformation into *E. coli* JC7623 (*recBC sbcC*) harboring R64 or its deletion derivative; the recombinant plasmid was selected on Luria-Bertani agar containing kanamycin or chloramphenicol. Insertion mutants of R64 were constructed in a similar manner.

Cloning of R64 Tra-2 segment. The R64 Tra-2 segment was cloned by the in vivo recombination method (Fig. 1B). The 7.1-kb *Hin*dIII-*Bgl*II (map coordinates 21.2 to 28.3 kb in Fig. 2) and 2.55-kb *Bgl*II (map coordinates 54.35 to 56.9 kb) fragments of R64 were successively inserted into the *Hin*dIII and *Bam*HI sites of pHSG576, respectively, to give

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TABLE 1. *E. coli* K-12 strains, phages, and plasmids

Strain, phage, or plasmid	Relevant genotype or phenotype	Source or reference
E. coli		
JM83	Δ(*lac-proAB*) *rpsL thi ara* φ80 *lacZΔM15*	26
TN102	W3110 Nal^r	This study
MC1061	*araD139* Δ(*ara leu*)*7697* Δ*lacX74 galU galK hsr hsm strA*	3
JC7623	*recB21 recC22 sbcB15 thr thi leu his pro arg rpsL*	21
Bacteriophage		
Iα		H. Ackermann (4)
PR64FS		H. Ackermann (5)
P1::Tn*5 lac*	*clr100* Cm^r	D. Kaiser (16)
Plasmid		
R64*drd-11*	Tc^r Sm^r	M. Yoshikawa
ColIb*drd-1*	*cib*	M. Ohki (20)
pUC9	Ap^r *lacZ'* pMB9 replicon	26
pTK219[a]	Km^r *lacZ'* pSC101 replicon	This study
pHSG576	Cm^r *lacZ'* pSC101 replicon	JCRB[b] (23)

[a] Constructed by ligating the 445-bp *Hae*II fragment of pUC19 (26), which contains *lacZ'* with a multicloning site, with the 3.6-kb *Hin*dIII fragment of pTF1 (6), which contains the pSC101 replicon and kanamycin resistance.
[b] Japanese Cancer Research Resource Bank.

pKK661a. pKK661a DNA was digested with *Sal*I, which cuts a unique *Sal*I site between the two cloned fragments, and then introduced into strain JC7623 harboring R64. The recombinant plasmid pKK661 was selected on Luria-Bertani agar containing chloramphenicol.

Conjugal transfer. Donor cells were grown to the log phase and then mixed with recipient cells in the stationary phase. After standing for 90 min at 37°C, the mixture was plated at various dilutions onto selective media. The frequency of transfer was expressed as the ratio (percentage) of the number of transconjugants to that of the donors.

Formation of pilus. The sensitivity of plasmid-carrying cells to phages Iα (4) and PR64FS (5) provided an indication of the synthesis of thin pili. Phage stocks were spotted on plasmid-carrying JM83 cells spread in soft agar. Phages Iα and PR64FS made turbid plaques on JM83 harboring R64*drd-11* or its derivative plasmids but clear plaques on JM83 harboring ColIb*drd-1* (20). In most experiments, phage propagation was also measured, as follows. A stock of Iα or PR64FS phage was mixed with a log-phase culture of JM83 harboring R64 or its derivative plasmids. Following incubation at 37°C for 18 h, the phage titer was measured with JM83 harboring ColIb*drd-1* as indicator cells and compared with the initial titer. An increase in the phage titer on the test strains of more than 100-fold indicated sensitivity to the phages.

Other methods. The transposition of Tn*5 lac* from P1::Tn*5 lac* was carried out as described by Kroos and Kaiser (16). The activity of β-galactosidase was determined as described by Miller (19). The preparation of plasmid DNA, construction of recombinant plasmids, transformation, and other methods have already been described (12–15).

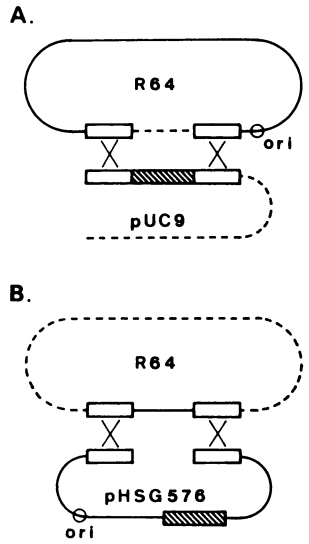

FIG. 1. Construction of deletion derivatives of R64 and cloning of R64 Tra-2 segment by double homologous recombination in vivo. (A) Construction of deletion derivatives of R64. Two DNA segments (▭) of R64 were cloned into two separate restriction sites of pUC9, and a Km^r or Cm^r DNA fragment (▨) was inserted between them. Linearized DNA of pUC9 derivative plasmid was introduced into strain JC7623 harboring R64, causing a DNA segment (– – –) of R64 to be replaced with a Km^r or Cm^r DNA fragment. (B) Cloning of Tra-2 segment of R64. Two DNA segments (▭) of R64 were cloned into the *Bam*HI and *Hin*dIII sites of pHSG576. After being digested with *Sal*I, the pHSG576-derived plasmid DNA was introduced into strain JC7623 harboring R64. As a result, the DNA segment (———) of R64 was cloned into pHSG576. ▨, Cm^r gene for selection. Figure is not to scale.

RESULTS

Mapping of R64*drd-11*. The physical and genetic map of the plasmid R64 was reported in our previous paper (7). The R64 strain F used in that study was a derivative of R64*drd-11* but was found to carry a 2.8-kb insertion. Therefore, a physical and genetic map of R64*drd-11* was constructed (Fig. 2). R64*drd-11* was found to have a molecular size of 121.7 kb.

Construction of deletion plasmids. A detailed comparison of restriction maps of IncI1 plasmids including R64, ColIb, and R144 (Fig. 2) (7, 9, 22, 24) indicated that the DNA regions corresponding to the map coordinates 1.6 to about 78 kb (Fig. 2) of these plasmids are highly homologous, so that possibly the transfer region of these plasmids is present within this region. Consequently, DNA segments were deleted stepwise leftward from the *Eco*RI site at coordinate 0 (set at 121.7 kb).

A series of deletion plasmids was constructed by in vitro recombinant DNA techniques followed by double homologous recombination in vivo as shown in Fig. 1A. A total of 15 deletion derivatives of R64*drd-11* were constructed (Fig. 3). Transfer frequency of the various deletion plasmids was determined by liquid mating. After *E. coli* JM83 donor cells were mated with TN102 recipient cells, pKK601 through

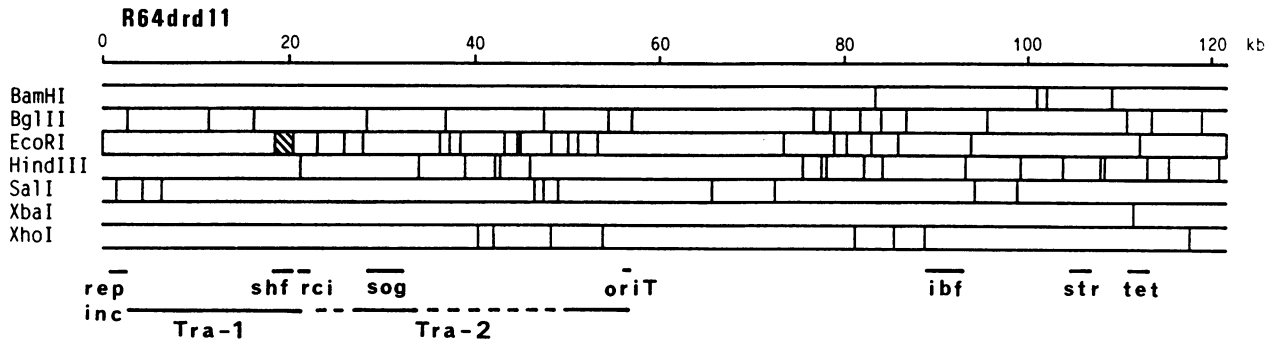

FIG. 2. Physical and genetic map of R64*drd-11*. This map is based on the previous one (7). The map shows R64 DNA arbitrarily opened at the *Eco*RI site to the left of the *rep* region, where the kilobase coordinate 0 is defined, and indicates the extent of movement of the *Eco*RI site due to DNA rearrangement of shufflon (▨). The Tra-1 and Tra-2 regions are indicated. rep, Replication functions; inc, incompatibility; shf, shufflon; rci, recombinase for shufflon; sog, suppression of *dnaG*; oriT, origin of transfer; ibf, growth inhibition of phage BF23; str, streptomycin resistance; tet, tetracycline resistance. The DNA sequence was determined for coordinates 2.7 to 21.8 kb (14, 17; S. R. Kim and T. Komano, unpublished results) and 53.2 to 56.7 kb (15; N. Furuya and T. Komano, unpublished results).

pKK609 transferred at essentially the same frequency as that observed for R64*drd-11* transfer (Fig. 3). However, there was no transfer at all on the part of pKK610, pKK613, pKK617, pKK618, pKK620, or pKK621, indicating that pKK609 was the smallest mini-R64 plasmid capable of successful transfer. pKK610 was smaller than pKK609 by only 44 bp. The 44-bp sequence lacking in the pKK610 plasmid has been shown to be essential for the R64 *oriT* function as described above. From the results described above, it is evident that transfer region of R64 bears the *oriT* sequence at one end, as was also found for IncF plasmids (10, 25). *E. coli* cells harboring pKK601 to pKK621 were found to be sensitive to phages Iα and PR64FS, indicating that pKK601 to pKK621 carry the region for thin pilus formation.

Construction of deletion and insertion plasmids at the other

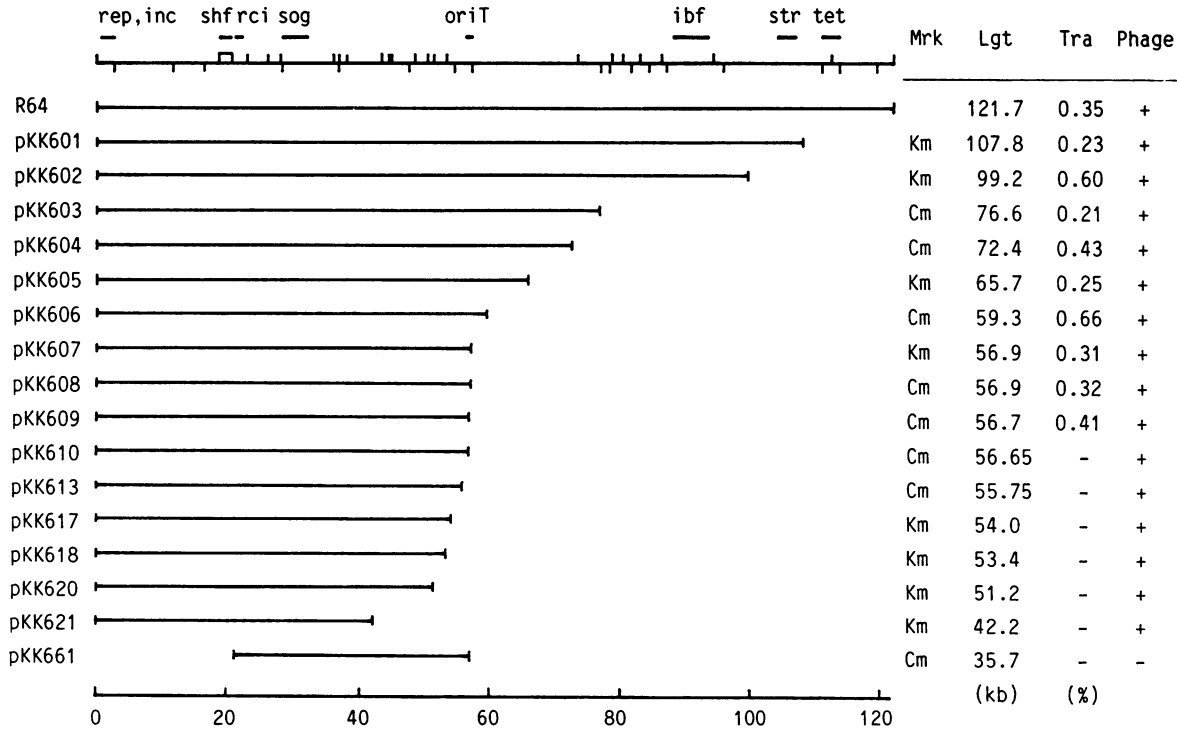

FIG. 3. Transfer frequency of various deletion derivatives of R64. At the top, a physical and genetic map of R64 is shown. The vertical lines above and below the map represent the *Eco*RI and *Bgl*II sites, respectively. The open bar above the map indicates the extent of movement of the *Eco*RI site in shufflon. At the bottom, kilobase coordinates are indicated. Various deletion plasmids (pKK601 to pKK621) were constructed as described in the legend to Fig. 1A. pKK661 was constructed as described in the legend to Fig. 1B. Horizontal lines represent DNA portions present in various plasmids. The left ends of pKK601 through pKK621 are at the *Eco*RI site at coordinate 0 (set at 121.7 kb). The right ends of pKK601 through pKK621 are, from top to bottom, at the *Hin*dIII, *Hin*dIII, *Bgl*II, *Xho*I, *Sal*I, *Sma*I, *Bgl*II, *Bgl*II, *Hae*III, *Fnu*DII, *Hpa*I, *Xho*I, *Hpa*I, *Hpa*I, and *Hin*dIII sites. The left and right ends of pKK661 are the *Hin*dIII (21.2 kb) and *Bgl*II (56.9 kb) sites, respectively. Mrk, Km^r or Cm^r DNA fragment used to construct deletion plasmids; Lgt, length of sequence; Tra, frequency of transfer (percent to donor cells; −, less than 10^{-5}%); Phage, sensitivity of plasmid-carrying cells to phages Iα and PR64FS (+, sensitive; −, resistant).

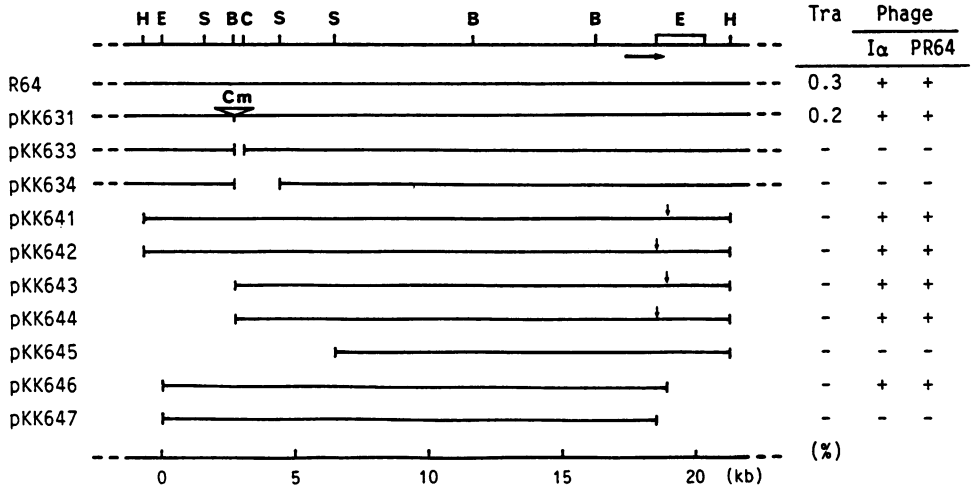

FIG. 4. Deletion and insertion plasmids at the other end of transfer region of R64 and cloning of the *pil* region. At the top, a restriction map is shown. B, *Bgl*II; C, *Hin*cII; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I (*Hin*cII). Only a relevant *Hin*cII site is shown. Symbols: □, extent of movement of the *Eco*RI site in shufflon; →, location of the *pilV* gene. ↓, locations of the *Eco*RI site in fixed shufflon. For the construction of various plasmids, see text. pKK631 carries the insertion of a Cm^r DNA fragment, and pKK633 and pKK634 carry replacements with the Km^r DNA fragment. Tra, Frequency of transfer (percent to donor cells; −, less than 10^{-5}%); Phage, sensitivity of plasmid-carrying cells to Iα and PR64FS (+, sensitive; −, resistant).

end of the transfer region. Furuichi et al. (7) reported that the *rep* region of R64 exists on the *Eco*RI-*Bgl*II fragment (map coordinates 0 to 2.7 kb in Fig. 2). In this study, a chloramphenicol-resistant DNA fragment was initially inserted into the *Bgl*II site at 2.7 kb (Fig. 4) and the resultant plasmid pKK631 was Tra^+. However, when a 0.4-kb *Bgl*II-*Hin*cII fragment (map coordinates 2.7 to 3.1 kb) was replaced by a kanamycin-resistant DNA fragment, the plasmid pKK633 thus obtained was Tra^-, as was pKK634, which lacked the 1.7-kb *Bgl*II-*Sal*I fragment (map coordinates 2.7 to 4.4 kb). *E. coli* cells harboring pKK633 or pKK634 were resistant to phages Iα and PR64FS. The other end of the transfer region of R64 thus appears to be present between map coordinates 2.7 and 3.1 kb, and *pil* genes appear to be situated in the vicinity of this end.

Cloning of the *pil* region. For more precise mapping of the *pil* region, the *pil* genes of R64 were cloned. By ligating the *Hin*dIII digest of R64 DNA with the kanamycin-resistant DNA fragment, the 21.9-kb *Hin*dIII fragment (map coordinates 121.0 to 21.2 kb) bearing the R64 *rep* region was cloned (Fig. 4). Many types of plasmids with different restriction maps were obtained since they carried the shufflon region but lacked the *rci* gene. Further analysis of two plasmids, pKK641 and pKK642, was made. Restriction enzyme analysis indicated that the C-terminal regions of *pilV* in pKK641 and pKK642 are A′ and A (14), respectively. Since *E. coli* cells harboring these plasmids were sensitive to the phages Iα and PR64FS, the *pil* region of R64 appears to be located within the 21.9-kb *Hin*dIII fragment. From this and the results presented above, the *pil* region of R64 apparently extends from the *Bgl*II site to the *Hin*dIII site (map coordinates 2.7 to 21.2 kb). This was actually confirmed by the finding that *E. coli* cells harboring pKK643 or pKK644 were sensitive to phages that were constructed by cloning the 18.5-kb *Bgl*II-*Hin*dIII region of pKK641 or pKK642, respectively, into vector pTK219 carrying the pSC101 replicon. However, cells harboring pKK645 containing the 14.7-kb *Sal*I-*Hin*dIII region were resistant to phages. When the 18.9- or 18.5-kb *Eco*RI fragment liberated from pKK641 or pKK642 was ligated with a tetracycline-resistant DNA fragment from pBR322, the plasmid pKK646 or pKK647, respectively, was obtained. pKK646 carried the complete *pilV* gene whose C-terminal region is A′, while pKK647 carried an incomplete *pilV* gene lacking the C-terminal region. Cells harboring pKK646 were sensitive to phages, while those harboring pKK647 resisted them. It thus follows that the region for pilus formation is located within the 18.5-kb *Bgl*II-*Hin*dIII region and the *pilV* gene, in whose C-terminal region shufflon undergoes a DNA rearrangement, may be requisite to the formation of a thin pilus.

Analysis of Tn*5* *lac*-insertion derivatives of pKK646. To determine the boundaries of the *pil* region and the direction of transcription within this region, Tn*5* *lac* (16) was transposed into pKK646. Tn*5* *lac* has a promoterless *trp-lacZ* fusion gene in IS*50*L of Tn*5* and, by measuring β-galactosidase activity, can be used to assay the in vivo promoter activity of the DNA segment into which Tn*5* *lac* is transposed. Thirteen Tn*5* *lac* insertion derivatives of pKK646 were obtained. The locations and orientations of Tn*5* *lac* insertions were established by restriction enzyme analysis (Fig. 5). Four mutants had the Tn*5* *lac* insertion in a rightward direction, with the direction being leftward in the other nine mutants. Since all *E. coli* cells harboring these insertion derivatives were resistant to phages, there is the possibility that the entire DNA region into which Tn*5* *lac* was inserted participates in pilus formation. These plasmids were introduced into *E. coli* MC1061, and their β-galactosidase activities were measured (Table 2). *E. coli* cells harboring pKK646::Tn*5* *lac* Ω1 through Ω4 showed approximately two times the activity of cells harboring the remaining

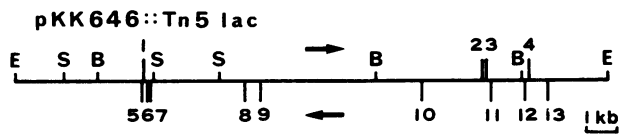

FIG. 5. Locations of Tn*5* *lac* insertions in pKK646. B, *Bgl*II; E, *Eco*RI; S, *Sal*I. Vertical lines above and below the map indicate rightward and leftward insertions, respectively.

TABLE 2. β-Galactosidase expression in strain MC1061 from Tn*5* *lac* insertions in pKK646

Plasmid	β-Galactosidase activity[a]
pKK646::Tn*5* *lac* Ω1	629
pKK646::Tn*5* *lac* Ω2	497
pKK646::Tn*5* *lac* Ω3	468
pKK646::Tn*5* *lac* Ω4	477
pKK646::Tn*5* *lac* Ω5	282
pKK646::Tn*5* *lac* Ω6	253
pKK646::Tn*5* *lac* Ω7	220
pKK646::Tn*5* *lac* Ω8	219
pKK646::Tn*5* *lac* Ω9	185
pKK646::Tn*5* *lac* Ω10	235
pKK646::Tn*5* *lac* Ω11	271
pKK646::Tn*5* *lac* Ω12	321
pKK646::Tn*5* *lac* Ω13	255

[a] Expressed in Miller units (19). All cells carrying insertion plasmids were resistant to phages Iα and PR64FS.

insertion derivatives, indicating that the *pil* region of R64 is transcribed rightward, which is the case for *pilV* and *rci* genes.

Effect of shufflon region deletion on R64 transfer. To examine the biological significance of shufflon, five insertion and deletion mutants in the vicinity of the shufflon region of R64*drd-11* were constructed (Fig. 6). pKK651 had the insertion of the kanamycin-resistant DNA fragment in the *rci* gene, and consequently, no DNA rearrangement of shufflon was noted. It was confirmed by restriction enzyme analysis that the *pilV* gene of pKK651 carried A′ of the C-terminal sequence. pKK652 lacked a part of the shufflon region and the *rci* gene but carried the entire *pilV* gene whose C-terminal region was A′. pKK651 and pKK652 transferred at a frequency similar to that of R64, indicating that the *rci* gene and DNA segments of shufflon, but not the C-terminal segment of the *pilV* gene, are not directly required for the transfer of R64. pKK653, which lacked the C-terminal half of the *pilV* gene, transferred at a frequency 1/10 of that of R64. pKK654 and pKK655, both lacking a longer region in the *pil* region, transferred less frequently. pKK651 and pKK652 were sensitive to phages Iα and PR64FS, while pKK653, pKK654, and pKK655 resisted them. It is thus evident that the *pilV* gene participates in pilus formation and therefore also in the conjugal transfer of R64.

Deletion of the 3′ portion of the *pilV* gene resulted in phage resistance in plasmid-carrying *E. coli* cells, while plasmids carrying deletions in the 3′ end of the *pilV* genes could still transfer, but less frequently. This apparent discrepancy may be explained as follows. The plasmid with the 3′-deleted *pilV* gene directs the formation of an immature pilus which is not functional as a receptor for phages Iα and PR64FS but is partially so for making a mating pair in conjugation.

Dissection of the R64 transfer system. pKK631 carried the genes for pilus information. Hence, we cloned the remaining portion of the transfer region of R64*drd-11*. pKK661 (Fig. 3), which carried the DNA segment of map coordinates 21.2 to 56.9 kb, was constructed by the in vivo recombination method (Fig. 1B). Following conjugation, neither pKK631 nor pKK661 alone transferred at all. When the donor cells harbored both pKK631 and pKK661, however, only pKK661, which carried the *oriT* sequence, transferred at a frequency of 0.3%. Thus, the R64 transfer region consists of at least two blocks, which the authors tentatively designated as Tra-1 and Tra-2 (Fig. 2).

DISCUSSION

The transfer region of many plasmids has been located by transposon mutagenesis. In the present work, however, it was located by a different means for R64*drd-11*. A series of deletion plasmids was construced, and it was found that pKK609 (bearing the 56.7-kb R64 sequence) was the smallest transferable mini-R64 plasmid, while pKK610 (which lacks only the 44-bp *oriT* sequence from pKK609) was incapable of transferring. It is thus clear that the *oriT* sequence determined from the mobilization experiments (15) is the point from which transfer actually starts in the parental R64 plasmid. The present methods for locating the transfer region of R64 are unique in that construction of deletion plasmids per se implies cloning of the transfer region.

The 2.7-kb *Eco*RI-*Bgl*II region of pKK609 is the *rep* region (7). Thus, all or certain portions of the remaining 54.0-kb region may be attributed to the transfer region of R64. The transfer region was separated into two blocks, Tra-1 and Tra-2, which complement each other (Fig. 2). Tra-1 started in the vicinity of the *Bgl*II site at coordinate 2.7 kb, proceeded rightward, and ended at a point beyond the *pilV* gene. Tra-1 was responsible for formation of a thin pilus. DNA sequencing (S.-R. Kim and T. Komano, unpublished results) indicated that the Tra-1 region contains at least 16 open reading frames, including the *pilV* gene, and that the 16 open reading frames are organized into three clusters. All of these open reading frames are oriented rightward, in agree-

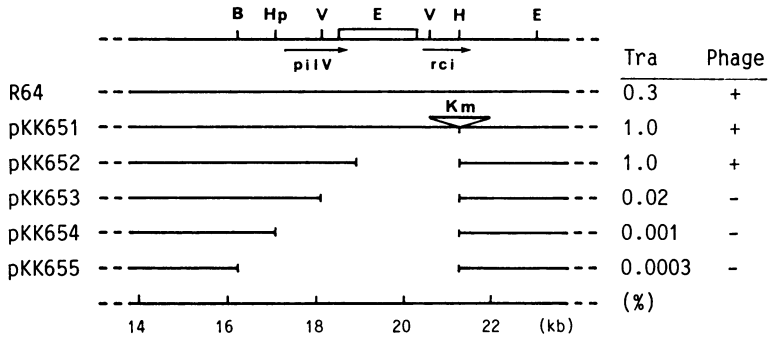

FIG. 6. Deletion and insertion plasmids around the shufflon region of R64. At the top, a restriction map is shown. B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; V, *Eco*RV. The open bar above the map indicates the extent of movement of the *Eco*RI site in shufflon. For the construction of deletion and insertion plasmids, see text. Tra, Frequency of transfer (percent to donor cells); Phage, sensitivity to Iα and PR64FS (+, sensitive; −, resistant).

ment with the results of Tn*5* *lac* insertions. The *pilV* gene thus lies at the downstream end of the operon responsible for formation of a thin pilus. It is quite likely that DNA rearrangement of shufflon, which selects one of the seven C-terminal segments of the *pilV* gene, modifies the structure and function of a thin pilus.

The *rci* gene itself was not directly required for pilus formation or the conjugal transfer of R64. However, it may indirectly participate in conjugal transfer by switching the C-terminal segments of the *pilV* gene.

There is the question of whether the entire Tra-2 region participates in the transfer of R64. In this region, the *sog* and *exc* genes are known to be present (7, 8, 18, 22). In addition, the IncI transfer system may contain many genes, including those for conjugal DNA metabolism, the formation of a thick pilus, and others. Thus, most of the Tra-2 region may participate in the transfer of R64. Research for confirmation of this point is presently being conducted at our laboratory.

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