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Nucleotide Sequence and Characterization of the *traABCD* Region of IncI1 Plasmid R64

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A 3.6-kb *BglII-SmaI* segment of the transfer region of IncI1 plasmid R64*drd-11* was sequenced and characterized. Analysis of the DNA sequence indicated the presence of four genes, *traA*, *traB*, *traC*, and *traD*, in this region. The expression of the *traB*, *traC*, and *traD* genes was examined by maxicell experiments and that of the *traA* gene was examined by constructing the *traA-lacZ* fusion gene. The introduction of frameshift mutations into the four genes indicated that the *traB* and *traC* genes are essential for conjugal transfer in liquid medium and on a solid surface. Both were also required for the formation of the thin pilus, which is the receptor for phages Iα and PR64FS. Upstream of the *traA* gene, a promoter sequence for σ^{70} of *E. coli* RNA polymerase was identified by S1 nuclease mapping and primer extension experiments.

Bacterial conjugation is a complex process whereby plasmid DNA is transferred from one cell to another by direct cell-to-cell contact. This process is usually mediated by conjugative plasmids such as F and R64 (for reviews, see references 11 and 31).

In the well-characterized conjugation system of the F plasmid, the conjugative transfer of plasmid DNA is mediated by transfer (*tra*) genes on the plasmid. Recently, the entire DNA sequence of the F transfer region was determined, and more than 30 genes were identified within a contiguous DNA region of 33 kb (11). The products of these genes were shown to be required for the following five different functions in conjugation: conjugal DNA metabolism, pilus formation, stabilization of mating pair, surface exclusion, and regulation of transfer gene expression.

IncI1 plasmids such as R64, ColIb, and R144 are transferred by a process similar to that in plasmid F. However, the transfer system of IncI1 plasmids is distinct from that of F in several respects. (i) A 54-kb DNA segment responsible for conjugation in IncI1 plasmid R64 is longer than that of F (13). (ii) IncI1 plasmids form two types of conjugative pili (2), a thick rigid pilus and thin flexible pilus, which are morphologically and antigenically distinct from the IncF pilus. The thick rigid pilus may be essential for the process of conjugation in general, while the thin flexible pilus is essential only for conjugation in liquid medium (2). (iii) IncI1 plasmids carry the *sog* gene responsible for suppressing the *Escherichia coli dnaG* mutation (20). The *sog* gene encodes DNA primase, which may possibly be involved in processing transferred plasmid DNA in recipient cells. (iv) IncI1 plasmids carry a region which undergoes complex DNA rearrangement and is designated shufflon (14–16). R64 shufflon consists of four DNA segments flanked and separated by 19-bp repeat sequences in either direction. Site-specific recombination mediated by the *rci* gene (17) between any two inverted repeats results in inversion of the four DNA segments either separately or in groups. Shufflon may act as a biological switch to select one of seven *pilV* genes in which the N-terminal region is constant while the C-terminal region shows variation in sequence and length (16). The product of the *pilV* gene is required for thin-pilus formation and thus also for conjugal transfer in liquid medium (13).

We previously located the 54-kb transfer region of R64*drd-11* on a physical map by constructing a series of deletion derivatives of R64 (13). The transfer region of R64*drd-11* was separated into two segments, Tra-1 and Tra-2 (see Fig. 1). The 19-kb Tra-1 segment was shown to be responsible for thin-pilus formation, since *E. coli* cells harboring the Tra-1 plasmid, pKK641, were sensitive to IncI1-specific phages, Iα and PR64FS (13). The Tra-2 segment included the *sog* gene (20), surface exclusion (*exc*) gene (10), *oriT* operon (7), and others. The transfer region of the related IncI1 plasmid ColIb-P9 has also been analyzed by transposon mutagenesis (23).

In the present work, an upstream 3.6-kb DNA region of the Tra-1 segment of plasmid R64*drd-11* was sequenced, and four new transfer genes, *traA*, *traB*, *traC*, and *traD*, were found. The introduction of frameshift mutations into the four genes indicated that the *traB* and *traC* genes are required for conjugal transfer in liquid medium and on a solid surface.

MATERIALS AND METHODS

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

Media. Luria-Bertani (LB) and H media were prepared as described by Miller (22). K and Hershey media (26) were used for the maxicell experiment. The agar medium consisted of LB or H broth containing 1.5 or 1.2% agar, respectively. Antibiotics were added to liquid or solid medium 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.

Nucleotide sequence determination. The nucleotide sequence was determined by the dideoxy chain-termination method (27). The sequences of both strands were determined with overlapping fragments.

Construction of insertion or deletion mutants of pKK641. To introduce mutations into various genes of pKK641, appropriate restriction sites of pKK641 were modified in vitro. pKK641 DNA was partially digested with *Hae*III or *Ssp*I, ligated with a 1.45-kb DNA cassette for tetracycline

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TABLE 1. Bacterial strains, phages, and plasmids

Strain, phage, or plasmid	Relevant genotype or phenotype	Source or reference
E. coli K-12 strains		
JM83	Δ (*lac-proAB*) *rpsL thi ara* φ80 d*lacZ*Δ*M15*	32
JM109	*recA1* Δ(*lac-proAB*) *endA1 gyrA96 thi hsdR17 supE44 relA1*/F' *traD36 proAB lacI*^{q}*Z*Δ*M15*	32
CSR603	*uvrA6 recA1 phr thr leuB6 proA2 thi argE3 lacY1 galK2 ara xyl mtl rpsL31 tsx supE33*	26
GM33	*dam-3*	19
TN102	W3110 Nal^r	13
Phages		
Iα		4
PR64FS		5
M13mp18	*lacZ'*	32
M13mp19	*lacZ'*	32
Plasmids		
pKK641	Km^r; R64*drd-11* derivative carrying Tra-1 segment and *rep* sequence	13
pKK661	Cm^r; pHSG576 (29) derivative carrying the Tra-2 segment from R64*drd-11*	13
pMC1403	Ap^r; *lacZ'*	3
pUC9	Ap^r; *lacZ'*	32
pUC19	Ap^r; *lacZ'*	32
pUC7Tc[a]	Ap^r; *lacZ'*; Tc^r cassette	This study

[a] pUC7Tc was constructed by inserting the 1.4-kb *Eco*RI-*Bal*I fragment expressing resistance to tetracycline of pBR322 into the *Pst*I site of pUC7.

resistance from pUC7Tc, and used to transform strain JM83. The DNA fragment for tetracycline resistance was removed from the resultant plasmid DNA by *Bam*HI digestion of DNA followed by self-ligation. A 22-bp DNA sequence, AATTCCCCGGATCCGGGGAATT, remaining at the restriction site, gave rise to a frameshift mutation in the specific gene.

pKK641 derivatives with 2- or 4-bp deletions were constructed in a similar manner. pKK641 DNA was partially digested with *Pvu*I or *Nsp*7524I, nibbled at the 3'-staggered ends with the Klenow fragment of DNA polymerase I, self-ligated, and transformed into strain JM83. The locations of insertion or deletion sites were determined by restriction enzyme analysis.

Construction of *traA-lacZ* and *traB-lacZ* fusion genes. To construct the *traA-lacZ* fusion gene, a 760-bp *Bgl*II-*Ava*I fragment of pKK671 was inserted into the *lacZ* fusion vector pMC1403 to generate pKK674.

To construct a series of *traB-lacZ* fusion genes with upstream control sequences of various lengths, various *Sau*3AI sites of pKK671 DNA were converted to *Cla*I sites. pKK671 DNA was partially digested with *Sau*3AI, filled in with the Klenow fragment of DNA polymerase I, self-ligated, and transformed into *E. coli dam* mutant GM33. The locations of *Cla*I sites were determined by restriction enzyme analysis. *Cla*I-*Nae*I fragments of resultant plasmid DNAs were inserted into the *Eco*RI-*Sma*I site of pMC1403 to generate pKK676 (R64 segments; positions 44 to 1488 [see Fig. 2]), pKK677 (positions 138 to 1488), pKK678 (positions 146 to 1488), pKK679 (positions 341 to 1488), pKK681 (positions 454 to 1488), pKK682 (positions 573 to 1488), pKK684 (positions 1050 to 1488), and pKK685 (positions 1403 to 1488). The *Bgl*II-*Nae*I, *Hin*cII-*Nae*I, and *Ava*I-*Nae*I fragments of pKK671 were also inserted into pMC1403 to generate pKK675 (positions 1 to 1488), pKK680 (positions 404 to 1488), and pKK683 (positions 761 to 1488), respectively.

Conjugal transfer. The donor strain JM83, harboring pKK641 or its derivative plasmids along with pKK661, was grown to the log phase and then mixed with an overnight culture of recipient strain TN102. For liquid mating (13), the mixture was incubated with standing for 90 min at 37°C and plated at various dilutions onto selective media.

For surface mating (33), the mating mixture was filtered through a nitrocellulose membrane (pore size, 0.45 μm). The membrane was then incubated on an LB agar plate for 90 min at 37°C. After resuspension of the cells from the nitrocellulose membrane in saline, the mating mixture was plated on selective medium in a manner similar to that used for liquid mating. Transfer frequencies were expressed as the ratios (percentages) of the numbers of transconjugants to those of donor cells.

Phage sensitivity. Thin-pilus formation was evaluated on the basis of the sensitivity of plasmid-carrying cells to phages Iα (4) and PR64FS (5), as already described (13).

Identification of protein products. pKK671 and its derivative plasmids were introduced into strain CSR603 by transformation, and the protein products were labeled with [^{35}S]methionine according to the maxicell procedure (26). The labeled proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18) and detected by fluorography. Molecular weight standard proteins were stained with Coomassie brilliant blue.

Determination of the transcription initiation site. Total RNA was prepared from plasmid-carrying JM83 cells by the hot-phenol method (1). DNA was end labeled by using T4 polynucleotide kinase with [γ-^{32}P]ATP (25).

S1 nuclease mapping was carried out as described by Sambrook et al. (25). The end-labeled DNA probe was hybridized with about 50 μg of RNA and treated with 60 U of S1 nuclease. DNA fragments protected from S1 nuclease digestion were electrophoresed on a 6% polyacrylamide gel containing 7 M urea and were visualized by autoradiography.

Primer extension was carried out as described by Geliebter et al. (8) with slight modification. The end-labeled DNA primer was hybridized with about 50 μg of RNA and treated with 5 U of avian myeloblastosis virus reverse transcriptase in the presence of dATP, dCTP, dGTP, and dTTP. The reaction products were electrophoresed as described above.

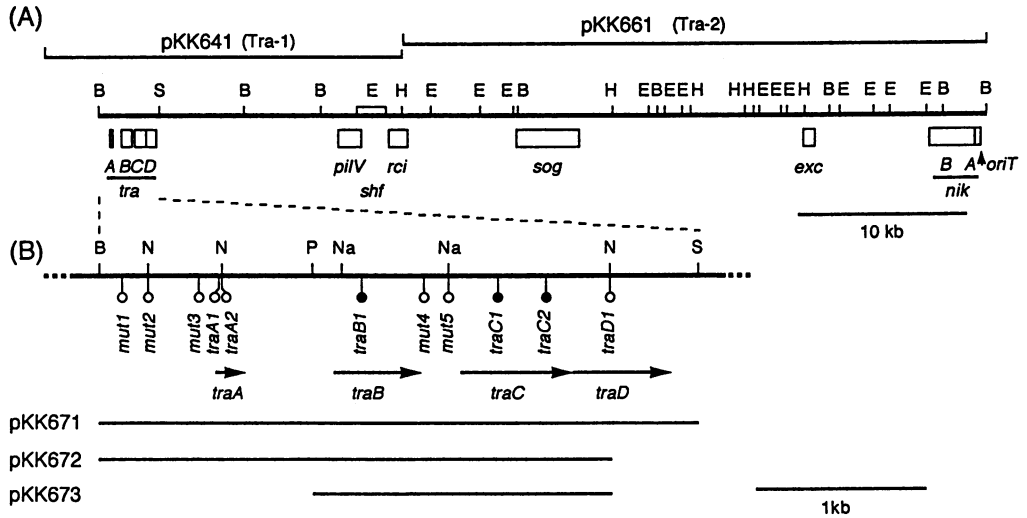

FIG. 1. (A) Structure of the 54-kb R64 transfer region. Horizontal bold line represents a restriction map. B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sma*I. Only the relevant *Sma*I site is indicated. The open bar on the map shows how far the *Eco*RI site has moved because of DNA rearrangement of shufflon. The open bars below the map represent the open reading frames that have so far been determined. *tra*, transfer; *pil*, thin-pilus formation; *shf*, shufflon; *rci*, shufflon-specific recombinase; *sog*, suppression of *dnaG*; *exc*, surface exclusion, *nik*, *oriT*-specific nickase; *oriT*, origin of transfer. All genes except *nikAB* are transcribed rightward, while *nikAB* genes are translated leftward. DNA regions of pKK641 and pKK661, which contain Tra-1 and Tra-2 segments, respectively, are indicated above the map. (B) Gene organization of the *traABCD* region. At the top, a restriction map of the 3.6-kb *Bgl*II-*Sma*I segment is indicated. B, *Bgl*II; N, *Nsp*7524I; Na, *Nae*I; P, *Pst*I; S, *Sma*I. Locations of insertion or deletion mutations and transfer capacity as determined from Table 2 are indicated (open circles, transfer proficient; filled circles, transfer deficient). Arrows indicate the four *tra* genes deduced from the nucleotide sequence in Fig. 2. Solid lines below the map show DNA portions present in various plasmids.

Other methods. β-Galactosidase activity was assayed as described by Miller (22). Construction of recombinant plasmid, preparation of plasmid DNA, transformation, and other methods have already been described elsewhere (25).

Materials. Restriction enzymes, sequencing kits, and other materials for recombinant DNA experiments were commercially obtained and used as recommended by the manufacturers. [α-^{32}P]dCTP (800 Ci/mmol), [γ-^{32}P]ATP (3,000 Ci/mmol), and [^{35}S]methionine (1,000 Ci/mmol) were from Du Pont, NEN Research Products and ICN Biomedicals.

Nucleotide sequence accession number. The nucleotide sequence reported here will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases under the accession number D14607.

RESULTS

Location of the DNA region required for surface mating of R64*drd-11*. In our previous study, the 54-kb transfer region of R64*drd-11* was separated into two segments, Tra-1 and Tra-2 (13). pKK641 and pKK661 carry the Tra-1 and Tra-2 segments, respectively (Fig. 1). When donor cells harboring both pKK641 and pKK661 were mated with recipient cells, pKK661 DNA carrying the *oriT* sequence was transferred to recipient cells at a frequency of 3.0% in liquid medium and 4.3% on a solid surface (Table 2). With donor cells harboring pKK661 alone, pKK661 failed to be transferred either in the liquid medium or on the solid surface (data not shown).

To determine the DNA segment required for surface mating, a 3.6-kb *Bgl*II-*Sma*I fragment of pKK641 was subcloned into the *Bam*HI-*Sma*I site of pUC9 to generate pKK671 (Fig. 1B). When donor cells harboring pKK661 and pKK671 were mated with recipient cells on the solid surface, pKK661 was transferred to recipient cells at essentially the

TABLE 2. Effects of mutation on frequency of transfer and sensitivity to phages

Plasmid	Mutation[a]		Frequency of transfer (%)[b]		Sensitivity to phages[c]	
	Location	Length (bp)	Liquid	Surface	Iα	PR64
pKK641			3.0	4.3	+	+
pKK641 *mut-1*	147	−2	2.4	2.0	+	+
pKK641 *mut-2*	304	−4	1.9	3.3	+	+
pKK641 *mut-3*	600	+22	1.3	1.9	+	+
pKK641 *traA1*	734	+22	1.6	1.2	+	+
pKK641 *traA2*	753	−4	1.3	3.4	+	+
pKK641 *traB1*	1608	+22	—	—	−	−
pKK641 *mut-4*	1978	+22	2.2	2.1	+	+
pKK641 *mut-5*	2144	+22	2.0	2.4	+	+
pKK641 *traC1*	2429	+22	0.002	0.001	−	−
pKK641 *traC2*	2743	+22	0.002	0.001	−	−
pKK641 *traD1*	3119	−4	3.1	3.7	+	+
pKK671			0.005	2.5		
pKK671 *traA1*			NT	1.4		
pKK671 *traA2*			NT	3.2		
pKK671 *traB1*			NT	—		
pKK671 *traC1*			NT	—		
pKK671 *traC2*			NT	—		
pKK672			NT	2.1		
pKK673			NT	8.0		

[a] Locations (positions in Fig. 2) and lengths (+, insertion; −, deletion) of the mutations are indicated.

[b] Frequency of transfer of pKK661 from donor cells harboring the indicated plasmids as well as pKK661 in liquid or surface mating is indicated as percent of transconjugants to donor cells. —, less than 10^{-4}%; NT, not tested.

[c] Sensitivity to phages Iα and PR64 of *E. coli* cells harboring the indicated plasmid, (+, sensitive; −, resistant).

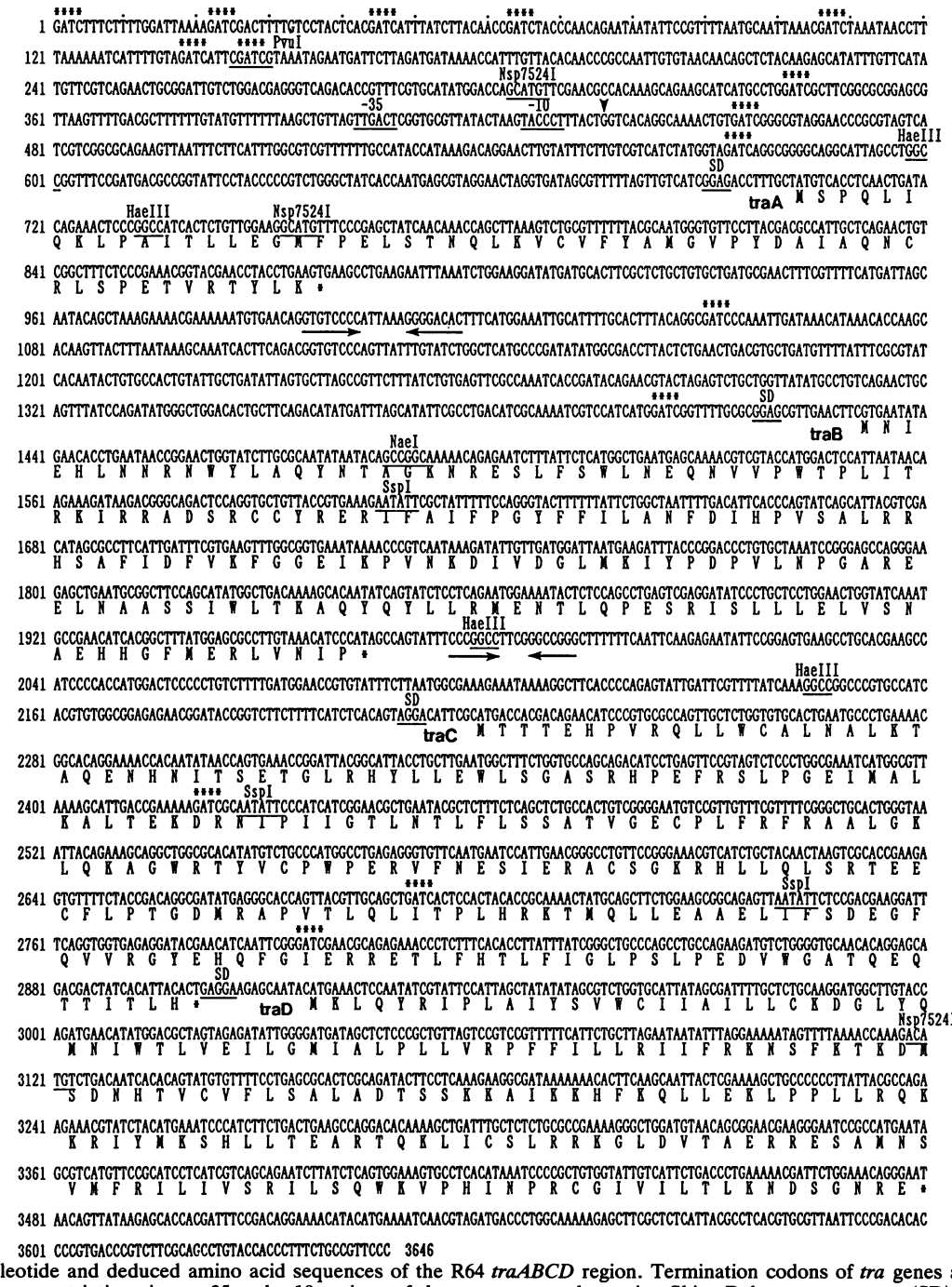

FIG. 2. Nucleotide and deduced amino acid sequences of the R64 *traABCD* region. Termination codons of *tra* genes are indicated by asterisks. Relevant restriction sites, −35 and −10 regions of the promoter, and putative Shine-Dalgarno sequences (SD) are underlined. Downward arrowhead indicates the transcription initiation site shown in Fig. 5. Inverted repeat sequences are indicated by opposing arrows. Asterisks above the nucleotide sequence indicate *dam* methylation sites.

same frequency as that in the case of donor cells harboring pKK661 and pKK641 (Table 2). In contrast, in the case of liquid mating, pKK661 was transferred at very low frequency. The genes required for surface mating are thus shown to be located within the 3.6-kb *Bgl*II-*Sma*I segment and 35.7-kb Tra-2 segment of R64*drd-11*.

Nucleotide sequence of the *traABCD* region of R64*drd-11*. For clarification of the gene organization in the 3.6-kb *Bgl*II-*Sma*I fragment of R64*drd-11*, the nucleotide sequence of this region was determined by the dideoxy chain termination method. Figure 2 shows the nucleotide sequence of the 3,646-bp *Bgl*II-*Sma*I fragment. The G+C content of this sequence was 45%.

By examination of the sequence, four open reading frames were identified and tentatively designated the *traA* (positions 703 to 876), *traB* (positions 1432 to 1962), *traC* (positions

2219 to 2899), and *traD* (positions 2916 to 3479) genes. These started with ATG or GTG initiation codons preceded by reasonable Shine-Dalgarno sequences (Fig. 2). Analysis of codon usage indicated that the *traABCD* genes tend to use codons that are rarely used in *E. coli*. Gene organization, as determined from the nucleotide sequence in this region, is schematically shown in Fig. 1B. Translation of the four genes, *traA*, *traB*, *traC*, and *traD*, led to the production of proteins possessing 58, 177, 227, and 188 amino acids with calculated molecular weights of 6,532, 20,695, 25,892, and 21,892, respectively. The calculated isoelectric points of the TraA, TraB, TraC, and TraD proteins were 8.2, 9.2, 6.9, and 10.8, respectively. The four proteins seemed to be hydrophilic.

Between the coding regions of *traA* and *traB* and between *traB* and *traC*, long intergenic spaces consisting of 555 and 256 nucleotides (nt), respectively, were found. Within intergenic spaces between *traA* and *traB* and between *traB* and *traC*, there were 8-bp and 7-bp GC-rich inverted repeat sequences, respectively, which possibly may function as ρ-independent transcription terminators (opposed arrows in Fig. 2).

Construction of frameshift mutations in the *traABCD* genes of R64. To determine the roles of the four genes in the conjugation of plasmid R64, insertion or deletion mutations were introduced into various restriction enzyme sites of the 3.6-kb *Bgl*II-*Sma*I region of pKK641. All mutations were constructed so that frameshift of the coding sequences would occur. The introduction of insertions and deletions was limited to a small number of nucleotides to minimize the effects of mutations on downstream gene expression. Thus, 11 mutations were introduced into pKK641. Five mutations (*mut-1* through *mut-5*) were outside the protein-coding region, and the other six (*traA1*, *traA2*, *traB1*, *traC1*, *traC2*, and *traD1*) were within the coding region (Fig. 1B; Table 2). pKK671 derivatives carrying the *traA1*, *traA2*, *traB1*, *traC1*, and *traC2* mutations were constructed from pKK641 derivatives carrying the corresponding mutations (Table 2).

The effects of various mutations in pKK641 on both liquid and surface mating were examined (Table 2). By using donor cells harboring pKK661 as well as pKK641 derivatives with *mut-1* through *mut-5*, *traA*, and *traD* mutations in the conjugation experiments, pKK661 was transferred at a frequency that was basically the same as that of pKK641 in liquid and surface mating. However, with *traB* and *traC* derivatives of pKK641, pKK661 was transferred at very low frequency. It thus follows that both *traB* and *traC* are required for the transfer of R64 in liquid medium and on a solid surface. That the *mut-1* through *mut-5* mutations had no effect on R64 transfer confirms the validity of assignment of the four genes in this region.

The effects of various mutations on thin-pilus formation were assessed on the basis of the sensitivity of *E. coli* cells harboring pKK641 derivatives toward phages Iα and PR64FS, both of which infect cells expressing thin pili of IncI1 plasmids such as R64 or ColIb (4, 5). *E. coli* cells harboring *traB* or *traC* derivatives of pKK641 were resistant to phages Iα and PR64FS, while those harboring pKK641 with *mut-1* through *mut-5* or *traA* or *traD* mutations were sensitive (Table 2). The *traB* and *traC* genes are thus also required for thin-pilus formation.

The effects of the *traABCD* mutations in pKK671 on surface mating were also examined. pKK672, carrying a 3.1-kb *Bgl*II-*Nsp*7524I fragment in pUC9 and lacking the C-terminal half of the *traD* gene, conferred the Tra^+ phenotype on pKK661 in surface mating, indicating that *traD* is not essential for R64 transfer (Fig. 1B; Table 2). A *traA* derivative of pKK671 was also Tra^+. However, when donor cells harbored pKK661 as well as pKK671 derivatives with mutations in the *traB* or *traC* gene, pKK661 failed to be transferred. Thus, only two genes, *traB* and *traC*, are required for surface mating in addition to the Tra-2 segment. To confirm this, plasmid pKK673, which carried a 1.8-kb *Pst*I-*Nsp*7524I fragment and had only *traB* and *traC*, was constructed (Fig. 1B). pKK673 was Tra^+ on solid surfaces (Table 2).

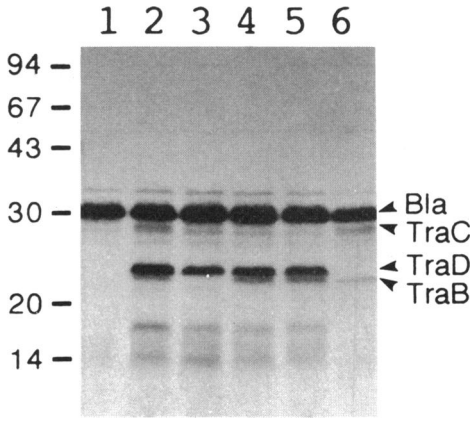

FIG. 3. Detection of *traBCD* gene products by the maxicell procedure. *E. coli* CSR603 cells harboring each plasmid were labeled with [^{35}S]methionine by the maxicell procedure, and each sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then by autoradiography. Lanes: 1, pUC9; 2, pKK671; 3, pKK671 *traB1*; 4, pKK671 *traC1*; 5, pKK671 *traC2*; 6, pKK672. Identified protein bands are indicated on the right side. Numbers on the left show the sizes (in kilodaltons) of marker proteins.

Analysis of the protein products of the four *tra* genes. To identify the protein products of these genes, pKK671 or its derivative plasmids with mutations were introduced into *E. coli* CSR603 by transformation, and the protein products were analyzed by the maxicell procedure (Fig. 3). Maxicells harboring pKK671 produced three polypeptides with apparent molecular masses of 27, 23, and 22 kDa, in addition to β-lactamase (Fig. 3, lane 2). However, maxicells harboring pKK671 *traB1* produced only one polypeptide with a molecular mass of 23 kDa in addition to β-lactamase (Fig. 3, lane 3). With pKK671 *traC1* and pKK671 *traC2*, the 27-kDa polypeptide directed by pKK671 was not detected (Fig. 3, lanes 4 and 5). pKK672 lacking the C-terminal half of the *traD* gene did not produce the 23-kDa polypeptide (Fig. 3, lane 6). It thus follows that three polypeptides with molecular masses of 27, 23, and 22 kDa correspond to the products of the *traC*, *traD*, and *traB* genes, respectively. The observed molecular masses of the *traB*, *traC*, and *traD* gene products were consistent with the calculated molecular weights. It would thus also appear that the *traB* mutation affects *traC* gene expression. However, under the conditions used, it was not possible to detect the product of the *traA* gene with a calculated molecular mass of 6.5 kDa.

Construction of the *traA-lacZ* fusion gene. The protein product of the *traA* gene could not be detected in the maxicell experiments. To show that the *traA* gene is translated into a polypeptide, we constructed a *traA-lacZ* fusion gene. In pKK674 carrying a 760-bp *Bgl*II-*Ava*I fragment in pMC1403, the N-terminal 20 codons of the *traA* gene were

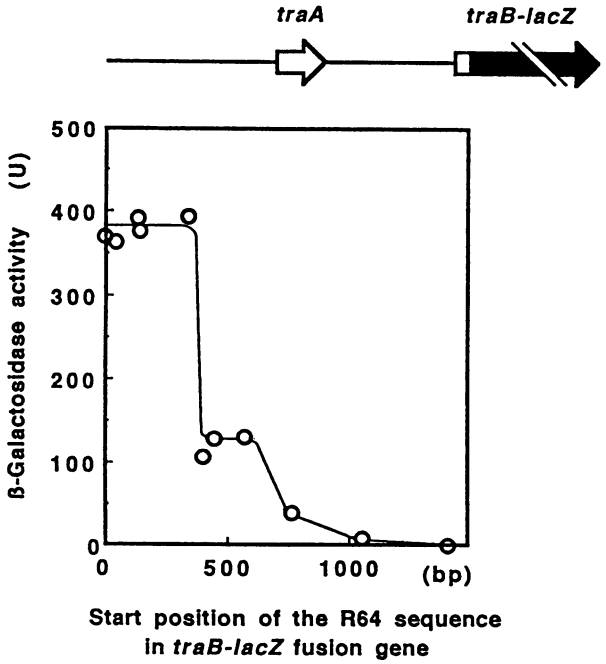

FIG. 4. β-Galactosidase expression of *traB-lacZ* fusion genes carrying upstream control sequences of various lengths. β-Galactosidase activities (in Miller units) of *E. coli* cells harboring pKK675 through pKK685 were plotted against the start nucleotide of the R64 sequence in each plasmid. Above the figure, the structure of the *traB-lacZ* fusion gene in pKK675 is shown.

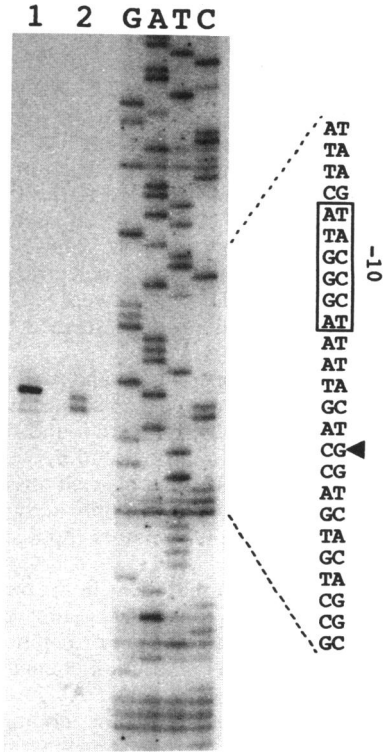

FIG. 5. Transcription initiation site of *traA* mRNA. Total RNA was prepared from *E. coli* JM83 cells harboring pKK641. Lanes: 1, the products of S1 nuclease mapping with the 598-nt *Hpa*II-*Bgl*II fragment as a probe; 2, the products of primer extension with the 115-nt *Hpa*II-*Hha*I fragment as the primer; G, A, T, and C, the sequence ladder prepared by the dideoxy chain termination method with the 115-nt *Hpa*II-*Hha*I fragment used as the primer. DNA sequence around the transcription initiation site is indicated on the right. An arrowhead indicates the proposed transcription initiation site. The −10 region of the putative promoter is boxed.

connected in frame with the *E. coli lacZ* gene. *E. coli* JM83 harboring pKK674 expressed about 1,500 U of β-galactosidase activity, indicating that the *traA* gene is translated into a polypeptide in vivo and that pKK674 carries a promoter sequence for the *traA* gene.

In vivo promoter activity in the *traAB* region. The DNA sequence in the *traABCD* region suggested a rightward direction of transcription in this region (Fig. 2). To locate a promoter sequence, a series of *traB-lacZ* fusion genes with upstream control sequences of various lengths were constructed. Plasmids pKK675 through pKK685 carried a *traB-lacZ* fusion gene in which the N-terminal 19 codons of the *traB* gene were connected in frame to the *lacZ* gene. The β-galactosidase activities of *E. coli* JM83 harboring pKK675 through pKK685 were determined and plotted against the start positions of R64 sequences in *traB-lacZ* fusions (Fig. 4). *E. coli* cells harboring five plasmids with *traB-lacZ* fusions in which the R64 sequence started before position 341 showed about 380 U of β-galactosidase activity, while those harboring three plasmids with fusions in which the R64 sequence started between positions 404 and 573 expressed about 120 U of β-galactosidase activity. In the case of three *traB-lacZ* fusions in which the R64 sequence started after position 761, β-galactosidase activity was low (Fig. 4). These results indicate the presence of two promoters in this region: a major promoter between positions 341 and 404 and a minor promoter between positions 573 and 761.

Transcription initiation site of the *traA* gene. To determine the precise initiation site of transcription in the *traAB* region, S1 nuclease mapping and primer extension experiments were carried out with total RNA prepared from pKK641-carrying *E. coli* JM83 cells. For S1 nuclease mapping analysis, the 598-nt *Hpa*II-*Bgl*II probe (positions 602 to 5) labeled at the *Hpa*II site was hybridized with about 50 μg of RNA, and the mixture was treated with S1 nuclease. For primer extension analysis, the end-labeled 115-nt *Hpa*II-*Hha*I primer (positions 602 to 488) complementary to the *traA* sequence was annealed with about 50 μg of RNA and extended along the RNA sequence by reverse transcriptase. DNA fragments protected from S1 nuclease treatment and extended by reverse transcriptase were electrophoresed together with the sequencing ladder by using the 115-nt *Hpa*II-*Hha*I primer (Fig. 5). S1 nuclease mapping indicated an intense band among a cluster of faint bands (Fig. 5, lane 1). The correlation of this band with the sequence ladder showed that the most intense band protected from S1 nuclease treatment corresponds to the transcript starting from guanine at position 436 (downward arrowhead in Fig. 2). This was further confirmed by a primer extension experiment (Fig. 5, lane 2). Two bands with the same density were detected on the gel, one of which moved at the same position as the fragment protected by S1 nuclease treatment. Considered together, these results indicated that the transcription initiation site is the guanine residue at position 436. Upstream from this initiation site were found sequences TTGACT (positions 402 to 407) and TACCCT (positions 425 to 430), homologous to the consensus −35 and −10 promoter sequences for σ^{70} of *E. coli* RNA polymerase (9). This

promoter corresponds to the major promoter identified by in vivo experiments (Fig. 4).

DISCUSSION

In the present study, we identified and characterized four genes, *traA*, *traB*, *traC*, and *traD*, within the upstream 3.6-kb region of the Tra-1 segment of R64*drd-11*. The protein products of *traB*, *traC*, and *traD* were identified in maxicell experiments, and the expression of *traA* was confirmed by constructing the *traA-lacZ* fusion gene. The introduction of frameshift mutations into the four genes indicated that *traB* and *traC* are required for the conjugal transfer of R64 in liquid medium and on a solid surface as well as for thin-pilus formation, while *traA* and *traD* are not required under the experimental conditions of the present study. That *traB* and *traC* are required for several transfer functions suggests a possibility that the products of *traB* and *traC* function as positive regulators of the expression of other transfer genes. This is supported by the recent finding that the *traB* and *traC* genes are also required for *sog* gene expression (12).

Computer-aided analysis indicated that the predicted amino acid sequences of the four genes are not similar to that of any transfer or virulence gene of the F, RP4, and Ti plasmids. Furthermore, the deduced amino acid sequences of the four genes were compared with those of known genes registered in a data base; however, no significant similarity could be found.

The results of S1 mapping and primer extension experiments (Fig. 5) indicated that a major promoter (the *traA* promoter) is present 267 bp upstream the *traA* gene (Fig. 2). In experiments with the *traB-lacZ* fusion gene, the *traA* promoter was active in vivo. *E. coli* cells harboring pKK679 and carrying the *traB-lacZ* fusion lacking nucleotides up to position 340 expressed 393 U of β-galactosidase activity. Those harboring pKK680 and carrying the *traB-lacZ* fusion and whose deletion extended to position 403 showed only 107 U. In pKK680, the −35 region (TTGACT) of the *traA* promoter lacked the first two nucleotides (TT). In consideration of the method used for constructing pKK680, the nucleotide sequence of the −35 region of the *traA* promoter in pKK680 should have changed to TCGACT instead of TTGACT. TCGACT may be weak as a −35 region of the *E. coli* promoter, since the three nucleotides, TTG, are well conserved as the sequence of −35 region (9). The R64 derivative plasmid pKK633, in which the 403-bp *Bgl*II-*Hin*cII segment of the R64 *traA* upstream region is replaced by a kanamycin-resistant DNA fragment, was previously shown not to transfer at all, and *E. coli* cells harboring pKK633 were resistant to phages Iα and PR64FS (13). In pKK633, the −35 region of the *traA* promoter also changed to TCGACT.

The results of *traB-lacZ* fusion indicate that a minor promoter is possibly present within the *traAB* region; however, the minor promoter has not yet been identified. Nevertheless, the results for pKK633 indicate that the major promoter is required for the expression of R64 transfer functions. In plasmid F, many transfer genes are organized in a long transcription unit, the *traYX* operon, although weak internal promoters were found for the expression of several *tra* genes (11).

traJ gene of the F plasmid, which encodes a positive regulator for the *traYX* operon, has a long (105-bp) 5′-untranslated sequence (30). In this region, the *finP* gene, a negative regulator of *traJ* expression, is situated in the opposite direction. The product of this gene is a short (ca. 78-nt) RNA molecule complementary to *traJ* mRNA that acts as an antisense RNA together with the product of the *finO* gene (6).

Between the transcription initiation and translation initiation sites of R64 *traA*, there was a long 5′-untranslated sequence of 267 nt. The largest open reading frame within this region consists of 39 codons and starts with the GTG codon at position 666. However, whether this open reading frame is actually translated remains to be clarified. The secondary structure of the long untranslated sequence is not clear. The insertion of various DNA segments of the *traABCD* region into a promoter-proving vector, pTK224, gave results indicating that there is possibly no promoter activity in the opposite direction (12). Hence, the 5′-untranslated sequence is unlikely to encode antisense RNA. However, the long 5′-untranslated sequence upstream of *traA* may be involved in regulation of transfer gene expression.

It should be pointed out that there are many GATC *dam* methylation sites in the *traABCD* region (asterisks in Fig. 2). The methylation of GATC sites by *dam* methylase affects many *E. coli* functions such as the initiation of DNA replication at *oriC* (21, 28) and transposition of Tn*10* (24). A comparison of the frequencies of conjugal transfer from *dam*$^+$ and *dam* mutant donor cells harboring R64 failed to indicate any differences of frequencies in this regard for *dam*$^+$ and *dam* mutant donor cells (12).

The functions of the products of the *traABCD* genes have not yet been precisely determined, although it was suggested that the *traBC* genes act as positive regulators of R64 transfer gene expression. The organization and sequences of other R64 transfer genes should be determined for clarification of these functions. This is presently being done at our laboratory.

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