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F Factor Inhibition of Conjugal Transfer of Broad-Host-Range Plasmid RP4: Requirement for the Protein Product of Pif Operon Regulatory Gene *pifC*

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By the use of deletions, point mutations, and gene fusions, we show that the protein product of the F factor *pifC* gene is responsible for F factor inhibition of plasmid RP4 conjugal transfer. Deletion analysis of *pif* sequences carried by pSC101-F chimeric plasmids demonstrated that removal of all or part of the *pifC* coding sequence greatly decreased or abolished the ability of these plasmids to inhibit RP4 transfer. Amber mutations in the *pifC* gene eliminated inhibition in an Su^- host strain but not in and Su^+ (*supF*) host. Plasmids carrying nonpolar *pifC* mutations did not decrease the efficiency of RP4 transfer when present in *trans*. Whereas $pifC^+$ plasmids inhibited RP4 transfer, the presence of RP4 in the same cell as F' lac increased F'lac Pif activity approximately 1,000-fold. This effect most likely resulted from the binding of the *pifC* product to RP4 DNA and concomitant derepression of the F factor *pif* operon. PifC inhibited *trans* mobilization of pMS204, a nonconjugative plasmid carrying the RP4 *oriT* locus, by the RP1 derivative pUB307. pMS204 had no *trans* effect on pif operon expression, whereas pUB307 increased F'lac Pif expression, as did RP4. Our results suggest that the *pifC* product inhibits expression of one or more RP4 genes, the products of which are required for conjugal transfer of RP4 and are required in *trans* for mobilization of nonconjugal RP4 *oriT* containing plasmids.

The F factor and many of its derivatives inhibit conjugal transfer of the broad-host-range plasmid RP4 by as much as 500-fold (18). F factor DNA sequences located in the vicinity of the *pif* operon have been implicated in this inhibition (18). In contrast, RP4 has no effect on the efficiency of F factor transfer. By analyzing plasmids containing various segments of the F factor, Tanimoto and Iino (18) localized the sequences responsible for inhibition of RP4 transfer to the same region in which we mapped the *pifC* gene (13). In this report we examine whether inhibition of RP4 transfer is due to the *pifC* protein product (PifC), an RNA species transcribed in the *pifC* region, or a closely linked gene that requires the *pif* promoter for expression.

The F factor *pifC* gene is located in the immediate vicinity of *oriV1* (13), the primary origin of mini-F vegetative replication (5, 6). The *pifC* gene product plays several roles: it is involved in the regulation of *pif* gene expression and the initiation of F factor DNA replication (13, 14, 19). We have shown previously that the *pifC* structural gene is contained within the *pif* operon, which includes two distal genes, *pifA* and *pifB*. The protein products of the *pifA* and *pifB* genes inhibit development of bacteriophage T7 (3, 16, 17), and the product of the *pifC* gene functions as an autoregulatory control element which negatively regulates expression of the operon (13). The *pifC* product has been implicated by Tanimoto and Iino (19) as a requirement for replication of an *oriV1*-dependent mini-F replicon. The relationship between autoregulation of *pifC* expression and regulation of the frequency of initiation at *oriV1* remains to be determined.

We have recently described the location and properties of a site, *pifO*, that is required in *cis* for autoregulation of the *pif* operon (14). Mutations in *pifO* result in derepressed expression of the *pif* operon. In addition, the presence of multiple copies of *pifO* (carried on pSC101-derived vectors) in *trans* to the F'lac *pif* operon leads to a 1,000-fold increase in *pifA* and *pifB* product activity. This is a result of titration of the PifC protein by multiple copies of *pifO* and subsequent derepression of *pif* operon expression. This phenomenon therefore provides a functional in vivo assay for the presence of PifC-binding sites.

To determine whether one or more products of the *pif* operon are responsible for inhibition of RP4 transfer, we employed a series of deletions and point mutations that define the structural genes of the *pif* operon and the *pif* regulatory sequences. We find that the *pifC* protein product itself inhibits RP4 transfer. In addition, we have discovered that RP4 exerts a *trans* effect on the F factor pif operon, resulting in increased F'lac *pifA* and *pifB* product activity. This effect is phenotypically similar to the *trans* effect of multiple copies of the F factor *pifO* sequence. Our results suggest that PifC inhibition of RP4 transfer is mediated by RP4 sequences that bind the *pifC* product.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophage, and growth media. *Escherichia coli* K-12 strains are listed in Table 1. Plasmids constructed for this study are listed in Table 2, along with the sources of plasmids obtained from other laboratories. Phage T7 was originally from F. W. Studier. Solid and liquid growth media (ML) have been described previously (13, 15).

DNA manipulations and analysis. Isolation of plasmid DNA, use of restriction endonucleases, DNA ligation, and calcium chloride transformation procedures have been described (13).

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TABLE 1. Bacterial strains

E. coli K-12 strain	Relevant phenotype or genotype	Source or reference
JF50	F⁻ Δ*lac(X74) supF58* Nal^r	J. Felton
JF270	F⁻ Δ*lac(X74) recA rpsL*	J. Felton
RV101	F⁻ Δ*lac(X74) gyrA101*	Derived from strain RV (17)
RV200	F⁻ Δ*lac(X74) rpsL200*	Derived from strain RV (17)
W3110::Tn*7*	F⁻ Tn*7* (Tp^r)	P. T. Barth

T7 efficiency of plating. The plating efficiency of T7 on plasmid-containing strains was determined as previously described (17) and is reported as the ratio of the T7 titer obtained on plasmid-containing cells divided by the T7 titer obtained on isogenic, plasmid-free cells.

Qualitative filter matings. RP4 was introduced into suitable recipient strains by mixing 1 ml of log-phase donor cells with 1 ml of recipient cells and filtering the mating mix onto the surface of a Nalgene filter (0.45 μm pore size; Nalge Co.). The filter was placed on the surface of a moist L plate which was then incubated at 37°C for approximately 2.5 h. Following incubation, the bacterial lawn on the surface of the filter was scraped with an inoculating loop and streaked onto appropriate selective media.

Construction of RP4-Tc7. Transposon Tn*7* mutagenesis was used to construct a tetracycline-sensitive (Tc^s) derivative of RP4 (Km^r Ap^r Tc^r) so that selection could be applied for comaintenance of Tc^r pSC101-derived replicons. Tn*7* codes for trimethoprim resistance (1), which is selected by using Mueller-Hinton plates (Difco Laboratories) supplemented with 30 μg of trimethoprim per ml. RP4 was introduced into strain W3110::Tn*7*, and Tc::Tn*7* (Tc^s Tp^r) Km^r Ap^r RP4 derivatives were isolated after mating with recipient cells. One such plasmid, designated RP4-Tc7, was used for further analysis. The transfer and replication properties of RP4-Tc7 are indistinguishable from those of the parental RP4. Tn*7* also codes for low-level resistance to streptomycin (1), which can be overcome by high concentrations (200 μg/ml) of the antibiotic.

Quantitative filter matings. The efficiency of conjugal transfer of RP4 and derivatives RP4-Tc7, pMS204, and pUB307 was determined by a procedure similar to that described by Tanimoto and Iino (18). Donor cells (strain RV101 or JF50 containing the plasmids of interest) and recipient cells (strain RV200) were grown overnight in ML broth, containing antibiotics to select for plasmid maintenance when appropriate. A 0.25-ml portion of an overnight donor culture was diluted with 4.75 ml of ML broth and incubated in a 250-ml flask at 37°C for 5 h without shaking. Overnight recipient cultures were diluted 1:50 in ML broth and incubated at 37°C for 5 h with shaking. After incubation, 0.25 ml of donor culture was gently mixed with 2.5 ml of recipient culture and filtered through a 0.45-μm Nalgene filter. The donor-recipient cell ratio was approximately 1-100. Filters were incubated on moist, prewarmed L plates at 37°C for 2 h. Matings were terminated by placing filters in 4 ml of ice-cold phosphate-buffered saline, followed by vigorous agitation for 10 s.

The number of transconjugants per donor cell was determined by plating dilutions of the mating mixture on L plates containing antibiotics. Strain RV101 and JF50 donor cells containing RP4, RP4-Tc7, or pUB307 were selected with nalidixic acid (50 μg/ml) and kanamycin (50 μg/ml). Strain RV200 recipient cells containing RP4, RP4-Tc7, or pUB307 were selected with streptomycin (200 μg/ml) and kanamycin.

TABLE 2. Plasmids used in this study

Plasmid	Description and relevant genotype	Phenotype[a]	Source or reference
R factors and derivatives			
RP4		Ap^r Km^r Tc^r	4
RP4-Tc7	*tet*::Tn*7*	Ap^r Km^r	This work
pUB307	RP1ΔTn*1*	Km^r Tc^r	E. Lanka (2)
pMS204	RP4 *Sph*I fragment D, containing RP4 *oriT*$^+$, pBR325 replicon	Ap^r Cm^r	E. Lanka (11)
pSC101		Tc^r	S. Cohen
F factors			
F'lac	*pifO*$^+$*C*$^+$*A*$^+$*B*$^+$	Lac$^+$ Pif$^+$ PifC$^+$	Pasteur Institute
F'lac-55	*pifO55 pifC*$^+$*A*$^+$*B*$^+$	Lac$^+$ Pif^{++} PifC$^+$	14
F'C521	*pifO*$^+$ (*pifC-lacZ*)*521* fusion at 41.5 kb in *pifC*Δ*pifAB*	Lac$^+$ Pif$^-$	13
B9	*pifO*$^+$ (*pifC-lacZ*)*B9* Δ*pifAB*	Lac$^-$ Mel$^+$ Pif$^-$	Pasteur Institute (8)
O5	*pifO*$^+$ (*pifC-lacZ*)*O5* Δ*pifAB*	Lac$^-$ Mel$^+$ Pif$^-$	Pasteur Institute (8)
pif plasmids and deletions			
pGS103	*pifO*$^+$*C*$^+$*A*$^+$*B*$^+$; 5.2 kb (f5 through f7, 44.1–38.9 kb), 0.46 kb (f6), 0.1 kb (f3); 9.1-kb pSC101 replicon	Pif^{++} PifC$^+$ Tc^r	17
pGS103Cam5	*pifO*$^+$ *pifC5*(Am) *pifA*$^+$*B*$^+$; derived from pGS103 by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis	Pif$^-$ PifC$^-$ Tc^r	E. Buchert
pGS103C10, -C11, -C12	*pifO*$^+$ *pifC10,11,12 pifA*$^+$*B*$^+$; spontaneous *pifC* mutants derived from pGS103	Pif^{++} PifC$^-$ Tc^r	This work
pRB2	*pifO*$^+$*C*$^+$ Δ*pifAB*; 1.5-kb *Eco*RI deletion of pGS103	Pif$^-$ PifC$^+$ Tc^r	R. Braun
pGS211	*pifO*$^+$*C*$^+$ Δ*pifAB*; 1.9-kb *Hin*dIII deletion of pGS103	Pif$^-$ PifC$^+$ Tc^r	17
pGS221	*pifO*$^+$ Δ*pifCAB*; 2.45-kb *Bgl*II deletion of pGS103	Pif$^-$ PifC$^-$ Tc^r	17
pEB42.1	*pifO*$^+$ Δ*pifCAB*; 4.5-kb *Xho*I deletion of pGS103	Pif$^-$ PifC$^-$ Tc^r	E. Buchert
pVU14	*pifO*$^+$ Δ*pifCAB*; 6.4-kb *Pvu*II deletion of pGS103	Pif$^-$ PifC$^-$ Tc^r	17

[a] Phenotypes: Pif$^+$, inhibition of T7 plating to an extent characteristic of the wild-type F factor; PifC$^+$, inhibition of F'C521 *pifC-lacZ* fusion expression; Pif^{++}, inhibition of T7 plating to an extent that is several orders of magnitude greater than that of F.

Strain RV200 recipients containing pMS204 were selected with streptomycin and ampicillin (50 μg/ml), and transconjugants carrying both pMS204 and pUB307 were selected with streptomycin, ampicillin, and kanamycin.

Isolation and characterization of pGS103 derivatives carrying the *pifC10*, *pifC11*, or *pifC12* mutations.

Mutations carried in pGS103 (Table 2, Fig. 1) that result in a PifC$^-$ phenotype (inability to repress *pif* expression in *trans*) were isolated from strain JF270 containing both F'C521 and pGS103. Repression of F'C521 *pifC-lacZ* fusion expression by the pGS103 *pifC* product results in Lac$^-$ colonies which, after incubation at 37°C for 48 h on MacConkey lactose agar, give rise to Lac$^+$ papillae that result from spontaneous mutations in one or more components of the *pif* regulatory system (14). Spontaneous independent Lac$^+$ mutants were screened for the presence of pGS103 plasmids defective in the ability to supply functional *pifC* product in *trans* by demonstrating that the resident F'C521 plasmids retained sensitivity to wild-type *pifC* product. Three of these isolates were designated pGS103C10, -C11, and -C12 (carrying the *pifC10*, *pifC11*, and *pifC12* mutant alleles, respectively; see below).

Plasmid DNA was prepared and used to transform strain JF270 containing wild-type F'C521. JF270 strains carrying both F'C521 and pGS103C10, -C11, or -C12 were Lac$^+$, confirming that the pGS103 mutant plasmids were *pifC*. The phage T7 efficiency of plating on strain RV101 containing pGS103C10, -C11, or -C12 was approximately 10^{-6}, showing that distal genes (*pifA* and *pifB*) are expressed at high levels. Restriction analysis of pGS103C10, -C11, and -C12 showed no detectable alterations in restriction sites or fragment sizes.

The positions of the pGS103 mutations that resulted in PifC$^-$ phenotypes were determined by recombination with F'lac derivatives F'C521, B9, and O5, which contain overlapping deletions that join the *pifC* structural gene to *lacZ*. F'C521 carries a fusion between *pifC*, at 41.5 kilobases (kb), and the N terminus of *lacZ* and has been previously described in detail (13). O5 carries a *pifC-lacZ* fusion which retains only a small N-terminal portion of the *pifC* coding sequences (M. Malamy, unpublished data) fused to the distal portion of the α region of *lacZ* (8). The deletion endpoint of B9 within *pifC* lies between the endpoints of O5 and F'C521 (M. Malamy, unpublished data), fusing the remaining sequences to the distal portion of the *lacZ* β region.

Mobilization-associated recombination (9, 13) with F'C521 restores PifC$^+$ activity to pGS103C10, -C11, and -C12, as shown by the ability of recombinants to repress expression of a *pifC-lacZ* fusion carried by a lambda prophage (J. Miller, unpublished data). This demonstrates that the *pifC10*, *pifC11*, and *pifC12* mutations are located promoter proximal to the *Bgl*II site at 41.5 kb. B9 can recombine with the *pifC11* allele to restore repressor activity, but cannot recombine with *pifC10* or *pifC12*. None of the *pifC* alleles are restored to wild type by recombination with O5. These results show that the *pifC10*, *pifC11*, and *pifC12* mutations are located within the *pifC* structural gene and that *pifC11* is promoter proximal to *pifC10* and *pifC12*.

RESULTS

F factor *pifC* and *pifO* mutations alter the ability of F'lac to inhibit RP4 conjugal transfer. In agreement with the results of Tanimoto and Iino (18), we found that the presence of F'lac in *trans* to RP4 resulted in an approximately 100-fold decrease in the efficiency of RP4 transfer compared with isogenic cells containing only RP4 (Table 3).

TABLE 3. Effect of F' factors on RP4 conjugal transfer[a]

Donor strain plasmid(s) (relevant genotype or phenotype)[b]	No. of RP4 transconjugants/donor cell[c]	Relative frequency of transfer[d]
RP4 (Ap^r Tc^r Km^r)	28.2 ± 16.1	1.0
RP4 and F'lac (*pifO*$^+$*C*$^+$*A*$^+$*B*$^+$)	0.27 ± 0.02	9.6×10^{-3}
RP4 and F'lac-55 (*pifO55 pifC*$^+$*A*$^+$*B*$^+$)	0.06 ± 0.01	2.1×10^{-3}
RP4 and F' C521 (*pifO*$^+$ *pifC-lacZ* Δ*pifAB*)	39.4 ± 15.6	1.4

[a] All matings were performed with RV101 as the donor strain (containing the plasmids indicated) and RV200 as the recipient strain.

[b] *pif* genotypes have been described (13, 14).

[c] The number of RP4 transconjugants per donor cell was determined as described in Materials and Methods. Values reported are averages of at least two independent determinations.

[d] The average frequency of transfer of RP4 in the absence of other plasmids in *trans* is given a value of 1.0, and all other determinations are normalized to this value.

F'lac-55 is an F'lac derivative carrying the *cis*-dominant *pifO55* mutation which results in decreased sensitivity to PifC and a concomitant increase in *pif* operon expression (14). F'lac-55 inhibited RP4 transfer to a greater extent than did wild-type F'lac (Table 3). This demonstrates that the F function that inhibits RP4 transfer is part of the *pif* operon. The F'lac derivative F'C521 had no effect on RP4 transfer. F'C521 (13) carries a *pifC-lacZ* fusion at the *Bgl*II site in *pifC* (41.5 kb; F coordinates have been described [13, 14]) and a deletion of F'lac sequences lying between the *Bgl*II site in *pifC* and codon 9 of *lacZ*. The lack of inhibition by F'C521 shows that the required function(s) either overlaps the *Bgl*II site at 41.5 kb or is located in a distal portion of the *pif* operon.

Deletions of the *pif* operon localize the F sequences required for inhibition of RP4-Tc7 transfer. To delimit the functions required for inhibition of RP4 transfer, we employed pGS103 (Fig. 1), a pSC101 derivative which carries the F factor *pif* region, and several pGS103 deletion derivatives and examined their effect on the efficiency of transfer of the RP4 derivative RP4-Tc7. RP4-Tc7 contains a Tn*7* insertion in the tetracycline resistance determinant (*tet*::Tn*7*; the construction of RP4-Tc7 is described in detail in Materials and Methods). RP4-Tc7 is Tc^s and resistant to trimethoprim (Tp^r), an antibiotic resistance expressed by Tn*7*. Since pGS103 and its derivatives are Tc^r, the Tc^s phenotype of RP4-Tc7 allows for selection for the presence of RP4-Tc7 and the pSC101 replicons.

The addition of pGS103 in *trans* to RP4-Tc7 resulted in a dramatic inhibition of RP4-Tc7 transfer, by a factor of about 7,000 (Table 4). A similar effect was seen with deletion plasmids pRB2 and pGS211. Both of these plasmids contain the entire *pifC* gene (Fig. 1). pRB2 lacks all *pifB* sequences and approximately 40% of the promoter-distal region of *pifA*, and pGS211 carries a large deletion of *pifA* and *pifB*. These deletions therefore locate the inhibitory function to the sequences between the *Hin*dIII site in *pifA* (41.1 kb) and the *Pst*I site at 43.6 kb.

pEB42.1 and pVU14, both of which carry deletions removing most of the *pifC* coding sequences, had no effect on RP4-Tc7 transfer. This result shows that the sequences from 42.1 to 43.6 kb are not sufficient to inhibit RP4-Tc7 transfer and that *pifC* sequences are required. pGS221 showed an intermediate effect, resulting in much less inhibition than seen with pGS103. pGS221 contains a fusion of most of the

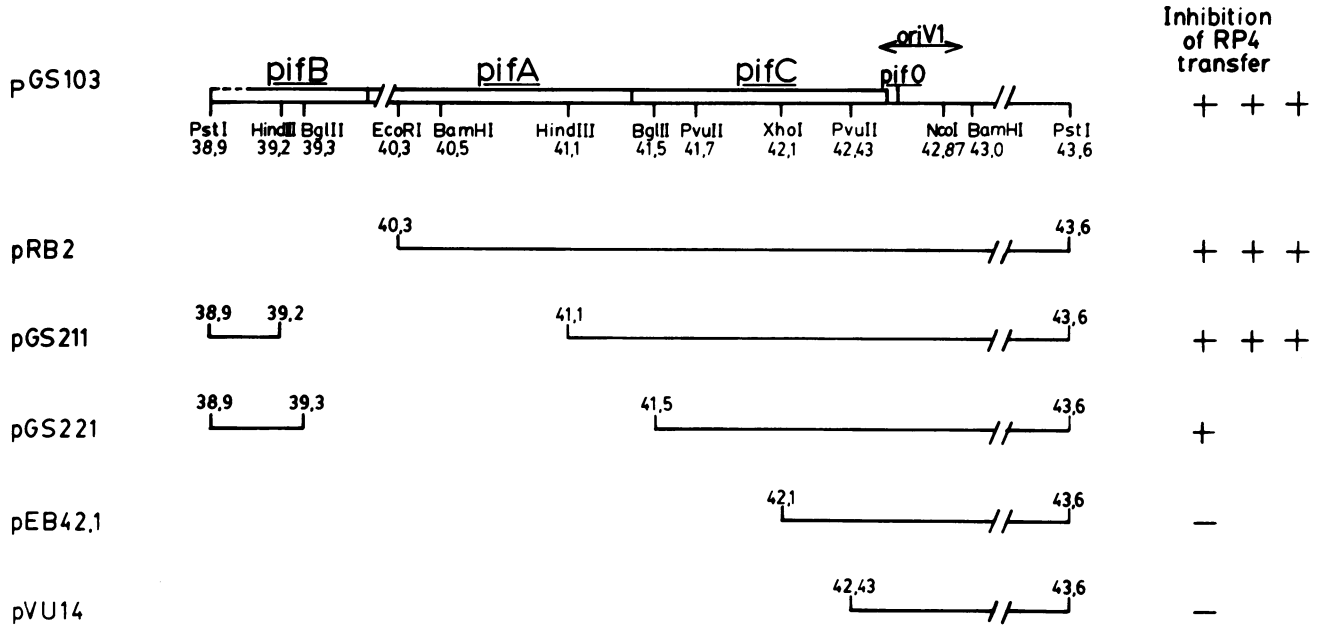

FIG. 1. Structure of pSC101 derivatives carrying sequences from the F factor *pif* operon. The effect of these plasmids on the efficiency of RP4 transfer is indicated (also see Table 4). Restriction sites have been assigned F factor kilobase coordinates as previously described (13, 14). This figure is drawn to scale, and interruptions are indicated by slash lines. The top line represents the DNA content of pGS103 (17), the Pif$^+$ plasmid used to construct the deletion derivatives shown. The *pif* sequences retained by the deletion derivatives are indicated by lines, with the kilobase coordinates of the deletion endpoints indicated. The coding sequences for the *pifC*, *pifA*, and *pifB* protein products are indicated by open rectangles on the pGS103 map. The N terminus of PifC, at 42.616 kb, was deduced from nucleotide sequence information (10). The approximate boundaries of the PifA and PifB coding sequences were determined from the size of PifA-LacZ and PifB-LacZ fusion proteins (13). *pifO* is the *cis*-acting regulatory sequence required for autoregulation by PifC (14) and has been localized at 42.647 to 42.663 kb (Miller and Malamy, manuscript in preparation). *oriV1* (5, 6) is the primary origin of mini-F replication. Symbols: -, no inhibition of RP4 transfer; +, slight inhibition; +++, greatly increased inhibition.

TABLE 4. Efficiency of RP4-Tc7 conjugal transfer in the presence of pSC101 replicons carrying the *pifC* gene and *pifC* deletions[a]

Donor strain plasmid(s) (relevant genotype or phenotype)[b]	No. of RP4-Tc7 transconjugants/donor cell[c]	Relative frequency of transfer[d]
RP4-Tc7 (Ap^r Km^r *tet*::Tn*7*)	80.1 ± 8.9	1.0
RP4-Tc7 and pGS103 (*pifO*$^+$*C*$^+$*A*$^+$*B*$^+$)	0.011 ± 0.004	1.4×10^{-4}
RP4-Tc7 and pRB2 (*pifO*$^+$*C*$^+$ Δ*pifAB*)	0.027 ± 0.004	3.4×10^{-4}
RP4-Tc7 and pGS211 (*pifO*$^+$ *C*$^+$ Δ*pifAB*)	0.020 ± 0.009	2.5×10^{-4}
RP4-Tc7 and PGS221 (*pifO*$^+$ Δ*pifCAB*)	1.21 ± 0.11	1.5×10^{-2}
RP4-Tc7 and pEB42.1 (*pifO*$^+$ Δ*pifCAB*)	99.2 ± 4.8	1.2
RP4-Tc7 and pVU14 (*pifO*$^+$ Δ*pifCAB*)	89.7 ± 5.3	1.1
RP4-Tc7 and pSC101 (Tc^r vector)	84.8 ± 9.8	1.1

[a] All matings were performed with RV101 as the donor strain (containing the plasmids indicated) and RV200 as the recipient strain.

[b] *pif* genotypes have been described (13, 14). The structures of the pSC101 plasmids carrying *pif* sequences are shown in Fig. 1.

[c] The number of RP4-Tc7 transconjugants per donor cell was determined as described in Materials and Methods. Values reported are averages of at least two independent determinations.

[d] The average frequency of transfer of RP4-Tc7 in the absence of other plasmids in *trans* is given a value of 1.0, and all other determinations are normalized to this value.

pifC gene to *pifB* (see Discussion). The vector alone, pSC101, had no effect on RP4-Tc7 transfer efficiency.

Amber mutations and nonpolar point mutations in *pifC* do not inhibit RP4-Tc7 transfer. To determine whether *pif* inhibition of RP4 transfer is due to a protein product, the effect of a suppressible nonsense mutation in *pifC* on the ability of pGS103 to inhibit RP4-Tc7 transfer was tested (Table 5). pGS103Cam5 carries the *supF*-suppressible *pifC5*(Am) amber mutation, located between 41.7 and 42.1 kb, which results in a PifC$^-$ phenotype (inability to repress expression of F'C521 *pifC-lacZ* fusion protein) as well as a Pif$^-$ phenotype (inability to inhibit T7 plating) due to a strong polar effect on *pifA* and *pifB* expression (E. Buchert and M. Malamy, manuscript in preparation). pGS103Cam5 had no effect on RP4-Tc7 transfer from the Su$^-$ donor strain RV101. The *supF* allele of strain JF50, however, restored the ability of pGS103Cam5 to inhibit RP4-Tc7 transfer. Suppression resulted in a degree of inhibition that was about 10-fold less than that of the parental pGS103 in the strain JF50 background (pGS103 inhibited RP4-Tc7 transfer from strain JF50 to an extent similar to that seen in strain RV101). These results show that the inhibitor of RP4-Tc7 transfer responds to amber suppression and is therefore a protein product of the *pif* operon.

Table 5 also shows the effects of three spontaneous *pifC* mutations on RP4-Tc7 transfer. pGS103C10, -C11, and -C12 carry the *pifC10*, *pifC11*, and *pifC12* mutations, respectively. These plasmids are phenotypically PifC$^-$ and Pif^{++} and carry mutations that map within the *pifC* structural gene that are nonpolar on *pifA* and *pifB* expression (see Materials and Methods). pGS103C10, -C11, and -C12 had little effect on the

TABLE 5. Effect of *pifC* amber and missense mutations on RP4-Tc7 transfer[a]

Donor strain plasmid(s) (relevant genotype or phenotype)[b]	JF50 (Su$^+$) donor		RV101 (Su$^-$) donor	
	No. of RP4-Tc7 transconjugants/ donor cell[c]	Relative frequency of transfer[d]	No. of RP4-Tc7 transconjugants/ donor cell	Relative frequency of transfer
RP4-Tc7 (Ap^r Km^r *tet*::Tn*7*)	81.3 ± 14.7	1.0	80.1 ± 8.9	1.0
RP4-Tc7 + pGS103 (*pifO*$^+$*C*$^+$*A*$^+$*B*$^+$)	0.07 ± 0.02	8.6×10^{-4}	0.011 ± 0.004	1.4×10^{-4}
RP4-Tc7 + pGS103Cam5 (*pifO*$^+$ *pifCam5 pifA*$^+$*B*$^+$)	0.55 ± 0.25	6.8×10^{-3}	100.8 ± 22.1	1.3
RP4-Tc7 + pGS103C10 (*pifO*$^+$ *pifC10 pifA*$^+$*B*$^+$)	ND[e]	ND	50.4 ± 10.4	0.6
RP4-Tc7 + pGS103C11 (*pifO*$^+$ *pifC11 pifA*$^+$*B*$^+$)	ND	ND	85.8 ± 9.8	1.1
RP4-Tc7 + pGS103C12 (*pifO*$^+$ *pifC12 pifA*$^+$*B*$^+$)	ND	ND	41.9 ± 5.2	0.8

[a] All matings were performed with the indicated donor strain (JF50 or RV101) and RV200 as the recipient strain.
[b] See Table 2 and references 13 and 14.
[c] See Table 4, footnote *c*.
[d] See Table 4, footnote *d*.
[e] ND, Not determined.

number of RP4-Tc7 transconjugants per donor cell. The lack of inhibition by these plasmids must therefore result from defects in the *pifC* structural gene.

PifC inhibits the ability of an RP1 derivative to mobilize, in *trans*, a nonconjugal plasmid containing the RP4 *oriT* sequence. pMS204 (11) (Table 2, Fig. 2) is a nonconjugal pBR325 derivative carrying the 7.03-kb RP4 *Sph*I-D fragment. Lanka et al. (11) have demonstrated that pMS204 can be mobilized efficiently in *trans* by RP4, showing that pMS204 contains a functional RP4 origin of transfer (*oriT*).

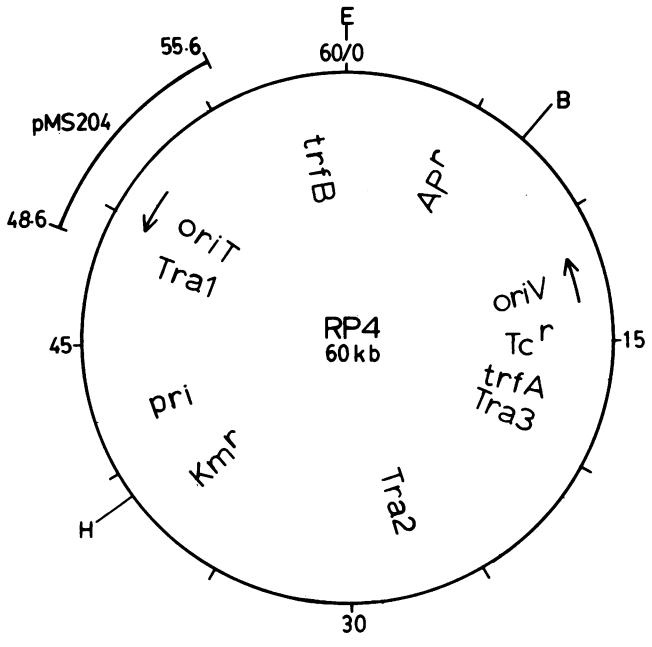

FIG. 2. Physical and genetic map of RP4 and pMS204. Map coordinates are in kilobases, and the location of genetic loci and phenotypic markers are from Thomas (20) and Lanka et al. (11). pMS204 (11) is a pBR325 derivative carrying the 7.03-kb RP4 *Sph*I fragment D, which contains the functional RP4 origin of transfer, *oriT*. Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance; *oriV*, unidirectional origin of vegetative plasmid DNA replication; *trfA*,*B*, *trans*-acting replication or maintenance functions; Tra1, Tra2, Tra3, regions required for conjugal transfer; *pri*, overlapping coding sequences for two related proteins with DNA primase activity (12); E, B, and H, unique *Eco*RI, *Bam*HI, and *Hin*dIII sites, respectively.

Guiney and Yakobson (7) have further localized the RK2 *oriT* sequence to a 112-base-pair *Hpa*II fragment which is entirely contained within the *Sph*I-D fragment. pMS204 was also mobilized by the RP1 derivative pUB307 (2) (Table 6) pUB307 was isolated as a spontaneous Tn*1* deletion of RP1, resulting in the loss of ampicillin resistance (RP1, RP4, and RK2 have homologous physical and genetic properties [20]). The efficiency of transfer of pMS204, pUB307, and both pMS204 and pUB307 was approximately equal. The addition of F'lac decreased the number of Ap^r (pMS204), Km^r (pUB307), and Ap^r Km^r (pMS204 and pUB307) transconjugants to about the same level. F'lac-55 (*pifO55*) also inhibited transfer of both plasmids equally, and as observed with RP4 the level of inhibition was greater than that seen with F'lac. The absolute level of inhibition of pUB307 and pMS204 by F'lac and F'lac-55 was less than that of RP4. The interaction between pMS204, pUB307, and the *pif* regulatory system is further investigated below.

Presence of RP4 in *trans* to F'lac results in increased F'lac Pif activity. The ability of multiple copies of *pifO* to increase F'lac *pif* expression when present in *trans* is a result of the autoregulation of *pif* operon expression by PifC (13, 14). Since the *pifC* product is responsible for inhibition of RP4 transfer, we tested the effect of RP4 on F'lac Pif expression.

The presence of RP4 alone had no effect on the efficiency of plating of bacteriophage T7 on strain RV101 (Table 7). The presence of F'lac alone, however, resulted in a 100-fold decrease in T7 plating and a corresponding alteration in plaque morphology. Both of these effects are due to the products of the F'lac *pifA* and *pifB* loci (3, 16), and the amount of inhibition results in part from the regulation of *pif* operon expression by PifC (13, 14).

The addition of RP4 (and RP4-Tc7) to an F'lac-containing cell enhanced the magnitude of T7 inhibition, as shown by a 1,000-fold decrease in T7 plating efficiency and a marked alteration in plaque morphology (Table 7). A similar increase in F'lac Pif activity resulted from the presence in *trans* of pSC101 replicons which contained *pifO* and deletions removing all or part of *pifC* (pSC101 replicons carrying both *pifO* and *pifC* decrease F'lac *pif* expression [13, 14]).

As discussed above, F'lac inhibits *trans* mobilization of the nonconjugal *oriT*^{RP4}-containing plasmid pMS204 by the conjugal RP1 derivative pUB307. pMS204 had no effect on F'lac inhibition of T7 plating, whereas the presence of pUB307 increased Pif activity to the same level as did intact RP4 (Table 7). Both plasmids in *trans* to F'lac gave the same result as pUB307.

TABLE 6. Effect of F'lac and F'lac-55 on mobilization of pMS204, containing a functional RP4 *oriT* sequence, by RP1 derivative pUB307[a]

Donor strain plasmid(s) (relevant genotype)[b]	Plasmid content (relevant genotype or phenotype) of transconjugants					
	pMS204 ($oriT^{RP4+}$ Ap^r)		pUB307 (RP1ΔTn*1* Tc^r Km^r)		pMS204 and pUB307	
	No. of transconjugants/ donor cell[c]	Relative frequency of transfer[d]	No. of transconjugants/ donor cell	Relative frequency of transfer	No. of transconjugants/ donor cell	Relative frequency of transfer
pMS204 and pUB307	46.8 ± 0.8	1.0	54.6 ± 0.4	1.0	36.0 ± 9.0	1.0
pMS204 and pUB307 and F'lac ($pifO^+C^+A^+B^+$)	3.6 ± 0.5	7.7×10^{-2}	4.8 ± 0.9	8.8×10^{-2}	2.1 ± 0.4	5.8×10^{-2}
pMS204 and pUB307 and F'lac-55 ($pifO55$ $pifC^+A^+B^+$)	0.54 ± 0.42	1.2×10^{-2}	0.64 ± 0.48	1.2×10^{-2}	0.36 ± 0.25	1.0×10^{-2}

[a] All matings were performed with RV101 as the donor strain (containing the plasmids indicated) and RV200 as the recipient strain. Numbers refer to the plasmid content of recipients as determined by counting Ap^r colonies (containing pMS204), Km^r colonies (containing pUB703), and Ap^r Km^r colonies (containing both pMS204 and pUB307).

[b] See Table 3, footnote *b*.

[c] The number of pMS204 or pUB307 transconjugants per donor cell was determined as described in Materials and Methods. Values reported are averages of at least two independent determinations.

[d] The average frequency of transfer of pMS204 or pUB307 in the absence of other plasmids in *trans* is given a value of 1.0, and all other determinations are normalized to this value.

The level of β-galactosidase activity expressed by the *pifC-lacZ* fusion product of F'C521 was not affected by the presence of RP4 in *trans* (data not shown). This observation and the detection of increased F'lac Pif activity resulting from the presence of RP4 in *trans* indicate that RP4 does not code for a product capable of decreasing *pif* operon expression.

DISCUSSION

F factor inhibition of RP4 conjugal transfer has recently been described by Tanimoto and Iino (18), and the sequences responsible were narrowed to the *Bam*HI fragment at coordinates 40.5 to 43.0 kb. We have previously shown that the *pifC* gene is contained within this region (13). The results of the present study show that the F factor *pifC* protein product is responsible for inhibition of RP4 transfer. Several lines of evidence support this conclusion. Deletions that affected the *pifC* coding sequence decreased or eliminated inhibition of transfer. Our deletion analysis is in agreement with that of Tanimoto and Iino (18) and in addition indicates that removal of a small portion of the *pifC* C-terminal coding sequence has an intermediate effect. pGS221, which carries a C-terminal deletion at the 41.5-kb *Bgl*II site (Fig. 1), inhibited RP4 transfer by about 70-fold, compared with the 4,000-fold inhibition seen with the intact *pifC* locus on pSC101 replicons (Table 4). The observation that pGS221 had no measurable effect on expression of F'C521, which carries a *pifC-lacZ* protein fusion (13), suggests that the amino acids and protein structure required for repression of *pif* expression may not be identical to those that are involved in the inhibition of RP4 transfer.

pGS103Cam5, which carries an amber mutation in *pifC*, did not inhibit RP4 transfer in an Su^- host but did decrease the efficiency of RP4 transfer in an Su^+ (*supF*) host. Transfer inhibition is therefore due to the activity of a protein rather than to an RNA species, unless a strong polarity effect prevented transcription of an inhibitory RNA transcript located downstream from the *pifC5*(Am) mutation. Since nonpolar *pifC* alleles greatly decreased or eliminated inhibition (pGS103C10, -C11, and -C12, Table 5), we conclude that the PifC protein is responsible for decreasing the efficiency of RP4 transfer. The observation that F'lac-55 inhibited RP4 transfer to a greater extent than did F'lac (Table 3) is explained by increased expression of the *pif* operon resulting from the *pifO55* regulatory mutation. The increased inhibition of RP4 transfer caused by pGS103 compared with that caused by F'lac may be a result of the higher copy number of pGS103 and perhaps of vector promotion of *pif* expression.

The plasmid pGS221 decreased RP4 transfer to an intermediate level, whereas F'C521 had no measurable effect. Although both plasmids contain the same *pifC* sequences,

TABLE 7. RP4 and certain derivative plasmids elevate F'lac Pif activity when present in *trans*[a]

F' factor (*pif* genotype)[b]	RP4 plasmid or derivative(s)	Relevant genotype of plasmid	T7 efficiency of plating	Plaque morphology[c]
None	None		1.0	Pif^-
	RP4		1.0	Pif^-
F'lac ($pifO^+C^+A^+B^+$)	None		0.01	Pif^+
	RP4		$\leq 10^{-5}$	Pif^{++}
	RP4-Tc7	*tet*::Tn*7*	$\leq 10^{-5}$	Pif^{++}
	pMS204	$oriT^{RP4+}$	0.01	Pif^+
	pUB307	RP1ΔTn*1*	$\leq 10^{-5}$	Pif^{++}
	pMS204 and pUB307	$oriT^{RP4+}$ and RP1ΔTn*1*	$\leq 10^{-5}$	Pif^{++}

[a] All plasmids were carried by strain RV101. T7 efficiency of plating was determined as detailed in the text. T7 efficiency of plating on the F^- strain RV101 is assigned a value of 1.0, and all other determinations are normalized to this value.

[b] *pif* genotypes have been described (13, 14).

[c] The wild-type Pif^- (F^-) plaque is characterized by a large center surrounded by a large halo. Pif^+ plaques are pinpoint. Pif^{++} plaques are irregular and barely visible.

the resulting protein products are quite different. F'C521 produces a PifC-LacZ fusion protein (13), whereas pGS221 probably codes for a PifC-PifB fusion protein (17). In addition, the copy number of pGS221 (a pSC101 replicon) is higher than that of F'C521. It is therefore possible that the difference in gene products, copy number, or both accounts for the above observation.

The marked elevation in F'lac Pif activity that resulted from the presence of RP4 in *trans* (Table 7) was phenotypically similar to the effect of multiple copies of *pifO* in *trans* to F'lac (14). We therefore suggest that the *pifC* product binds to RP4 sequences and that this interaction results in both inhibition of RP4 conjugal transfer and increased F'lac *pif* expression. It is also possible, however, that RP4 encodes a product that is capable of increasing *pif* expression or enhancing the effect of the *pifA* and *pifB* gene products. Since elevated F'lac Pif activity is seen with $pifO^+C^-$ plasmids but not $pifO^+C^+$ plasmids in *trans* (13, 14), and since RP4 has no observable effect on F'C521 *pifC-lacZ* expression, RP4 does not code for a product that efficiently inhibits *pif* operon expression.

The *pifC* product inhibited *trans* mobilization of pMS204, which contains the RP4 *oriT* sequence, to the same extent that it inhibited transfer of the complementing RP1 derivative pUB307 (Table 6). This result demonstrates that PifC affects either a *cis*-acting transfer element common to both plasmids (*oriT* for example), or production or activity of a *trans*-acting factor required for transfer of both plasmids, or both. pUB307 in *trans* increased F'lac Pif activity to the same extent as did RP4, but pMS204 had no such effect (Table 7). This suggests that pUB307 binds PifC and that these binding sequences are absent in pMS204. We therefore conclude that PifC inhibition of pMS204 mobilization is indirect and that PifC inhibition of RP4 transfer involves a *trans*-acting RP4 (and pUB307)-encoded transfer factor. A proposed mechanism for this inhibition is that PifC binding to RP4 or pUB307 DNA decreases expression of a *trans*-acting factor required in donor cells for conjugal transfer. An alternative explanation for these observations is that the *pifC* protein binds to an RP4 gene product, thereby inhibiting RP4 transfer and titrating out PifC repressor activity. F'Lac and F'lac-55 inhibition of RP4 transfer was greater than the inhibition of pMS204-plus-pUB307 transfer seen with these F factors (Tables 3 and 6). The reason for this difference is not yet clear.

Strains containing both RP4 and F still expressed RP4-mediated surface exclusion and retained sensitivity to PRR1, a male-specific phage that adsorbs to IncP-group plasmid sex pili (18). Since adsorption of a pilus-specific phage does not necessarily indicate the presence of a functional transfer apparatus, PifC could affect one or more gene products that are involved in the formation of a transfer-proficient pilus but are not required for PRR1 adsorption. Alternatively, as suggested by Taniimoto and Iino (18), donor cell conjugal DNA metabolism by RP4 could be the target of F inhibition. We are currently localizing the RP4 sequences that are capable of increasing F *pif* expression in *trans* in an effort to determine the mechanism and consequences of PifC inhibition of RP4 transfer.

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