Proc. Natl. Acad. Sci. USA
Vol. 75, No. 12, pp. 5836–5840, December 1978
Biochemistry

Fertility repression of F-like conjugative plasmids: Physical mapping of the R6-5 *finO* and *finP* cistrons and identification of the *finO* protein

(gene cloning/replication region/conjugation/minicells/*in vitro* mutagenesis)

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Communicated by Dale Kaiser, September 8, 1978

ABSTRACT **The locations of the fertility inhibition genes *finO* and *finP* of the F-like conjugative multiple antibiotic-resistance plasmid R6-5 have been determined. As found previously for that of the fertility plasmid F, the *finP* gene of R6-5 is located close to the origin of DNA transfer, *oriT*, and to the promoter-proximal segment of the *tra* operon. Thus, *finP* is close to the site of action of the FinOP fertility inhibition system. In contrast, the *finO* gene is located on the other side of the *tra* operon, greater than 35 kilobases from the *finP* gene; *finO* is very close to the origin of vegetative replication, *oriV*, and to cistrons encoding functions involved in autonomous plasmid replication and plasmid incompatibility. A 4.5-kilobase fragment of R6-5 DNA containing the *finO* gene has been cloned on the high-copy amplifiable vector plasmid pBR322. This hybrid plasmid, designated pKTO31, causes severe repression of conjugal transfer of plasmid F, indicating the production of high cellular levels of finO protein. Two independent *finO* mutant derivatives were obtained after mutagenesis of the pKTO31 plasmid. Comparison of proteins synthesized by minicells carrying *finO*$^-$ mutant plasmids with those carrying various *finO*$^+$ plasmids enables the *finO* gene product to be tentatively identified as a 22,000-dalton protein.**

Plasmids are extrachromosomal autonomous genetic elements that code for various functions such as resistance to antibiotics and the ability to degrade deleterious organic compounds (for review, see ref. 1). Many of the larger plasmids are able to transmit themselves (and, in certain cases, other genetic elements that are concurrently present in the same cell) from one bacterial host to another by a mechanism that involves cell-to-cell contact and that requires the synthesis of sex pili by donor bacteria (e.g., ref. 2). The ability to synthesize sex pili and to transfer DNA by conjugation is known to require, in the case of the transfer plasmid F, the activity of at least 13 plasmid-coded transfer (tra) proteins (products of *tra M,J,A,-L,E,K,B,C,F,H,G,D,* and *I* genes). The majority of these *tra* genes are located in a single operon whose expression is under positive control of the *traJ* gene protein (3, 4).

In the case of F, this operon is expressed constitutively such that, in a culture of exponentially growing F-carrying bacteria, every cell is capable of DNA transfer. However, the majority of F-like plasmids isolated from nature express only a small fraction of their full potential for conjugal DNA transfer (5). In many cases, this reduced expression of transfer genes is due to the activity of the plasmid-determined FinOP fertility inhibition system which exerts negative control of the expression of *traJ* and consequently of the *tra* operon (6, 7). It seems likely that the FinOP fertility inhibition system has evolved in response to the ubiquity of male-specific bacteriophages and the need to reduce unnecessary protein synthesis. However, when a population of cells carrying a FinOP$^+$ plasmid encounters a suitable population of recipient cells, the few plasmids that are initially transferred from the primary donor cells are then fully capable of highly efficient transfer from the primary recipient cells to the remainder of the recipient population ("epidemic spread"; see ref. 8).

The FinOP system is thought to consist of three components: *finO*, *finP*, and *traO* (9). The *finO* product, which is not plasmid-specific, and the plasmid-specific *finP* product (6) are both required for the repression of transcription of *traJ* (7). *Cis*-dominant mutations in *traO*, which are located close to *traJ* (10), prevent this repression (7). It has been suggested that *traO* is the *traJ* operator as well as being the site of action of *finO* and *finP* (10). However, accurate mapping of *traO* as well as the identification and isolation of the *finO* and *finP* gene products will be required before the interactions between the *finO*/*finP* gene products and the *traO* site can be analyzed *in vitro*.

The *finP* gene of F has been mapped and is located close to *traJ* (10). F is a naturally occurring *finO*$^-$ mutant and hence could not be used to map the *finO* cistron. Plasmids R6-5 and R100 (11) are F-like FinOP$^+$ conjugative antibiotic-resistance plasmids. During the course of investigations of their structure and functions, the majority of the *Eco*RI- and *Hin*dIII-generated DNA fragments of R6-5 have been cloned on high-copy-number vector plasmids (12) and the resulting hybrid plasmids have been used to identify various plasmid functions such as those involved in replication (ref. 12; unpublished data), incompatibility (13), conjugal transfer (14), and antibiotic resistance (12) (see Fig. 1). We have now utilized these small plasmids to determine the map location of the *finO* and *finP* genes and to identify tentatively the *finO* protein.

MATERIALS AND METHODS

Bacteria and plasmids used in this study are described in the text. Plasmids were introduced into host bacteria by conjugation or transformation (15).

Mutagenesis. pKTO31 DNA (0.5 ml containing 60 μg of DNA per ml) was treated with hydroxylamine as described by Humphreys *et al.* (16) for 1 hr at 65°C. The DNA was then dialyzed against 10 mM $CaCl_2$ and used to transform M3509 with selection for tetracycline-resistant clones. M3509 is a trp_{amber} SuIII A2(P)$_{ts}$ (17) derivative of JC3272 (18), obtained by P1 transduction, which suppresses amber mutations efficiently at 30°C but not at 42°C. F*lac* was introduced into 2000 of the tetracycline-resistant transformants by replica-plate mating, and the clones were screened for sensitivity to M12 bacteriophage by replica-plating. Two such clones, carrying plasmids that have been designated pKTO59 and pKTO60, were found. Because these clones are *finO*$^-$ at 30, 37, and

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Abbreviation: kb, kilobase(s).

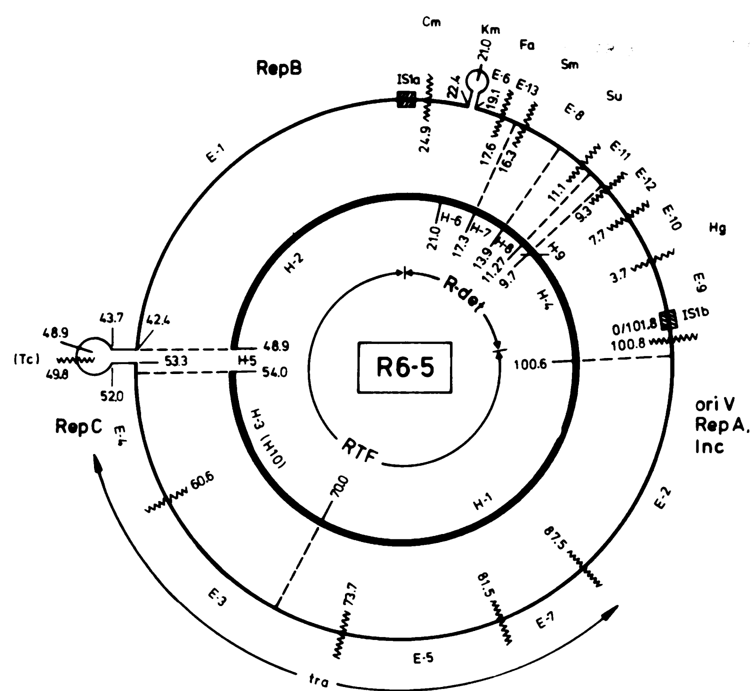


FIG. 1. Endonuclease cleavage maps of the R6-5 plasmid [taken from Timmis et al. (12)]. The wavy lines through the outer circle represent EcoRI cleavage sites; the straight lines through the inner circle represent HindIII cleavage sites.

42°C, the *finO* mutations are probably not amber mutations.

Other Procedures. The purification of plasmid DNA (12), the analysis of restriction endonuclease-generated DNA fragments by electrophoresis through agarose gels (19), and the *in vitro* construction of hybrid plasmids (unpublished data) by using the pBR322 cloning vector (20) followed by identification of recombinant molecules by the insertional inactivation procedure (12, 21) all have been described in detail elsewhere.

Table 1. Mapping of *finP* cistron: Influence of R6-5 hybrid plasmids on transfer efficiency of R100 mutants derepressed for transfer

	Transfer efficiency (%) of mutant R100 derivatives*					
R100 mutant	Flac† —	pKT003 H-3	pFC003. E-4	pFC005 E-3	pFC007 E-5	pFC011 E-7
UCR122	0.2	2	0.3	26	259	91
UCR126	0.2	0.05	0.04	187	193	286
UCR123	0.2	106	58	90	311	307
R100-1	54	107	73	11	142	100

All donor cells were derivatives of the *F*lac⁻ strain JC3272 (18). DNA transfer was assayed by using M1354, a nalidixic acid-resistant mutant of JC3272, as recipient and selecting lactose-fermenting, nalidixic acid-resistant (for *F*lac) or chloramphenicol- and nalidixic acid-resistant (for the R factors) clones on selective minimal media. The conditions used for DNA transfer involved 40-min matings at 37°C with 10-fold excesses of recipients and total cell concentrations of approximately 2×10^8/ml. The pFC series of hybrid plasmids consists of *Eco*RI fragments of R6-5 linked to ColE1; pKT003 consists of R6-5 *Hin*dIII fragment H-3 linked to pML21.

* Transfer efficiency is the number of transconjugants per 100 donor cells. First entry in each column heading is second plasmid; second entry is restriction fragment.

† Transfer efficiency in this case refers to *F*lac transfer; all other transfer efficiencies refer to R100 transfer. It should be noted that pFC005 caused a modest reduction in the transfer efficiencies of all R100 derivatives. The reason for this is not known but the reduction may result from high cellular levels of one or more *tra* gene products specified by the high-copy-number pFC005 plasmid causing reduced transfer of R100 by inhibiting expression of some *tra* genes or by interfering with one or more transfer-dependent functions.

RESULTS

Location of *finP* on R6-5. The current *Eco*RI and *Hin*dIII restriction endonuclease cleavage maps of R6-5 (12) are shown in Fig. 1. Results obtained with amplifiable high-copy-number hybrid plasmids containing restriction endonuclease fragments of R6-5 (12) have shown that *Eco*RI restriction fragments E-3, E-4, E-5, and E-7 contain all of the *tra* cistrons tested (14). The *traA* and *traL* cistrons which are situated at the beginning of the *tra* operon of F (3) are located on *Eco*RI fragment E-4 and on *Hin*dIII fragment H-3. Because $finP_F$ is located on an *Eco*RI fragment of the F factor that also carries *traA* and *traL* (22), *finP* was expected to be located on fragments E-4 and H-3 of R6-5.

Fertility inhibition by the FinOP system is manifested only when the gene products of *finO* and *finP* are synthesized in cells carrying a susceptible $traO^+$ sex factor (9). Thus, a $finO^+$ $traO^+$ $finP^-$ mutant of the R6-5 specificity group was needed to test whether hybrid plasmids carrying R6-5 fragment E-4 or H-3 express *finP*. Hoar described derepressed mutants of a tetracycline-sensitive mutant of R100 (23), some of which were recessive (24). Three of these mutants—UCR122, UCR123, and UCR126—were examined for their defect in transfer repression. In contrast to the $finO^-$ R100-1 plasmid, all three of these plasmids inhibited transfer of *F*lac and hence must be $finO^+$ (Table 1). Such mutant plasmids might be derepressed for transfer because of a lesion in *finP*, which would be recessive, or because of a lesion in *traO*, which would be *cis*-dominant. If any of these derepressed R-plasmids were $finP^-$ and $traO^+$, its transfer should be repressed by a hybrid plasmid carrying the R6-5 *finP* cistron. This was indeed found to be the case for UCR122 and UCR126 (but not for UCR123) in bacteria concurrently carrying hybrid plasmids containing either R6-5 E-4 or H-3 (Table 1, lines 1–3). Thus, UCR122 and UCR126 are $finP^-$ mutants and the *finP* cistron is therefore located on the R6-5 DNA sequence delineated by the *Hin*dIII site at 54.0 kilobases (kb) and the *Eco*RI site at 60.6 kb. This position is similar to that of *finP* on F. Furthermore, we can conclude that R6-5 and R100 share the same *finP* specificity. Neither the *finP* gene product of F nor that of R6-5 has yet been identified.

Table 2. Mapping of the *finO* cistron in cells carrying F*lac* plus an R factor derived plasmid

First plasmid			Second plasmid			
			F*lac*		F*lac finP*⁻	
Designation†	Original source	Composition‡	M12 response§	Transfer efficiency¶	M12 response‖	Transfer efficiency‖
R6-5			R	0.5	S	ND
pML21			S	134	ND	ND
pKT001	R6-5	H-1 + H-2	R	0.9	S	85
pKT002	R6-5	pML21 + H-2	S	220	S	244
pSC102	R6-5	E-2 + E-6 + E-8*	R	25	S	207
pBR322			S	187	ND	ND
pKTO81	pSC102	pBR322 + S-2	R	1	S	137
pKTO31	pSC102	pBR322 + P-2	R	0.05	S	123
pKTO58	pSC102	pBR322 + P-2	R	0.05	ND	ND
pKTO59	pKT031	pBR322 + P-2 (H.A.)	S	25	ND	ND
pKTO60	pKT031	pBR322 + P-2 (H.A.)	S	36	ND	ND
pSM1	R100	82.7–87.4, 88.6–89.3 kb	R	1.3	S	22.5

Bacterial strains carrying either the F*lac* plasmid JCFLO (18) or its *finP301* mutant *EDFL51* (9) plus one of the plasmids listed were tested. Note that the high-copy-number pSM1 plasmid caused a modest reduction of transfer of the F*lac finP*⁻ plasmid. This effect is superficially similar to the slight inhibition of transfer of R100 derivatives by pFC005 (Table 1) and may have a similar, although not identical molecular basis (because pFC005 and pSM1 contain different sequences of R6-5/R100).

† pKTO58 and pKTO31 are identical except that the orientations of the cloned pSC102 P-2 fragments within the pBR322 vector are opposite.

‡ Restriction endonuclease-generated DNA fragments obtained by cleavage with *Hin*dIII, *Eco*RI, *Sal* I, and *Pst* I are indicated by H, E, S, and P, respectively. For map positions see Figs. 1, 2, and 3. H.A. indicates hydroxylamine-induced mutant plasmids.

§ The response to M12 male-specific bacteriophage was determined by replica-plating as described (22).

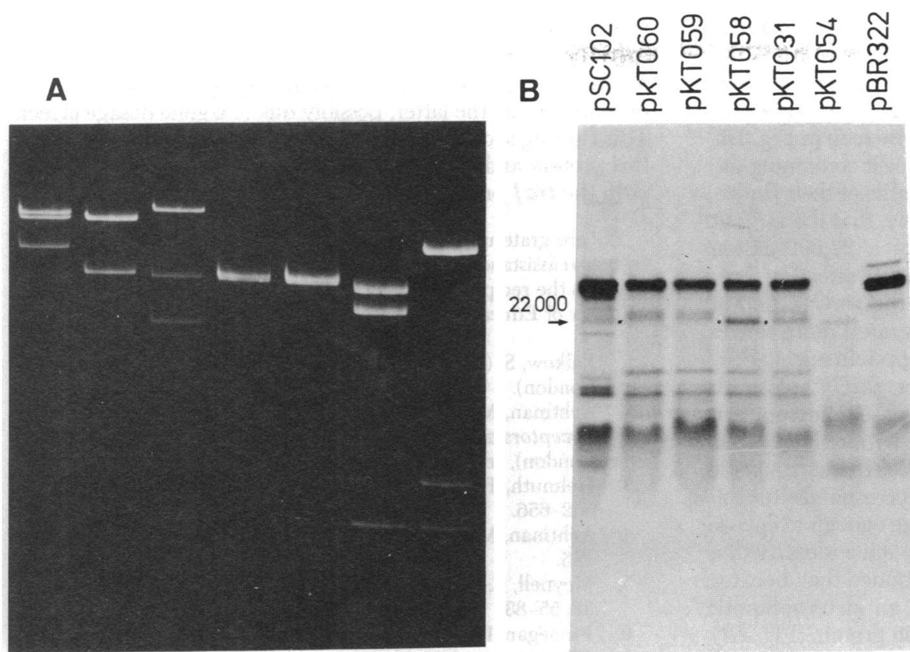
¶ DNA transfer was measured as described in Table 1.

‖ ND, not done.

Location of *finO* on R6-5 and R100. R100-1 is a derepressed mutant of R100 and is mutated in the *finO* cistron (6). If *finO* were located on one of the R6-5 restriction endonuclease fragments described above, then one of the hybrid plasmids tested should inhibit the transfer of R100-1. In fact, none did (Table 1, line 4). Furthermore, none inhibited the transfer of F*lac* (14). Thus, *finO* apparently does not map near *finP*. We then examined additional hybrid plasmids that contain restriction endonuclease fragments of R6-5 other than those from the *tra* region. Furthermore, mini R6-5 or R100 deletion derivatives formed *in vitro* (25, 26) or *in vivo* (27) were tested. Cells carrying a *finO*⁺ hybrid plasmid plus F*lac* should not express F's fertility functions. Such cells should be resistant to male-specific bacteriophages and should transfer F*lac* with greatly reduced efficiency. *finO*⁻ hybrid plasmids should not have these effects. Furthermore, no effects should be seen when a *finP*⁻ mutant of F is present together with a *finO*⁺ plasmid. pKT001, a mini R6-5 plasmid that contains R6-5 *Hin*dIII fragments H-1 and H-2 (12), is clearly *finO*⁺ whereas the hybrid plasmid pKT002, which carries R6-5 H-2 (12), is *finO*⁻ (Table 2, lines 3 and 4). Other hybrid plasmids carrying *Hin*dIII fragment H-3, H-4, H-5, H-8, H-9, or H-11 also were *finO*⁻. The results obtained by using hybrid plasmids carrying *Eco*RI fragments of R6-5 were less dramatic. The mini R6-5 plasmids pSC102 (25) and pSC135 (26) that carry *Eco*RI fragment E-2 did inhibit male-specific bacteriophage sensitivity and DNA transfer although they did it less strongly than pKT001 (Table 2, line 5). Hybrid plasmids carrying E-1, E-3, E-4, E-5, E-6, E-7, E-8, E-11, or E-13 had no effect at all. All but 0.2 kb of R6-5 *Eco*RI fragment E-2 is contained within *Hin*dIII fragment H-1 (12, 26). These results therefore place the *finO* cistron on R6-5 fragments E-2 and H-1.

We have confirmed this provisional mapping by cloning subfragments of the low-copy-number mini R6-5 plasmid pSC102 (25) which contains R6-5 fragment E-2. For this purpose we inserted *Sal* I and *Pst* I-generated fragments of pSC102 into the multicopy vector pBR322 (20) (Fig. 2). Whereas neither pBR322 nor pBR322 hybrid plasmids carrying *Pst* I fragment P-1, P-3, P-4, P-5, P-6, P-7, P-8, P-9, or P-10 affected the fertility of F*lac*, hybrid plasmids carrying the *Sal* I S-2 fragment (pKTO81) or the *Pst* I P-2 fragment of pSC102 (pKTO31, pKTO58) strongly inhibited male-specific bacteriophage sensitivity and DNA transfer (Table 2, lines 6–9). *Pst* I fragment P-2 is contained entirely within *Sal* I fragment S-2 (Fig. 2*C*; unpublished data). P-2 has been inserted into the pBR322 vector molecule in both possible orientations with respect to the vector (Fig. 2); both types of hybrid plasmid are *finO*⁺ (Table 2, lines 8 and 9). We thus conclude that the *finO* gene is contained within the P-2 fragment of R6-5 whose termini have coordinates 92.3 and 96.8 kb.

A series of deletion mutants of a copy mutant of R100 have recently been described (27). These mini plasmids contain only very little of the original R factor DNA but are capable of autonomous replication (29). Because they encompass part of the region carried by fragment P-2 of R6-5 (29), the mini R100 plasmids were tested to see whether or not they express *finO*. The four plasmids tested, pSM1, pSM2, pSM3, and pSM4, all inhibited DNA transfer of the F*lac* element (see Table 2, line 12, for results with pSM1). pSM1 carries two R100 DNA segments having R100 coordinates of 82.7–87.4 and 88.6–89.3 kb (29). R100 coordinate 82.7 kb corresponds to R6-5 coordinate 96.3 kb (12). Thus, the DNA on pSM1 overlaps the P-2 fragment of R6-5 (coordinates 92.3–96.8 kb) only by approximately 0.5 kb. This region of overlap is sufficient to encode a protein of 18,000 daltons, although imprecision in the mapping data would allow the region of overlap to encode a somewhat larger protein. These results place *finO* very close to the replication origins of R6-5 [coordinate 98.6 kb (unpublished data)] and R100 [99.1 kb (30) expressed as an R6-5 coordinate]. *finO* is thus located >35 kb from the *finP* cistron (Fig. 3) and, surprisingly,



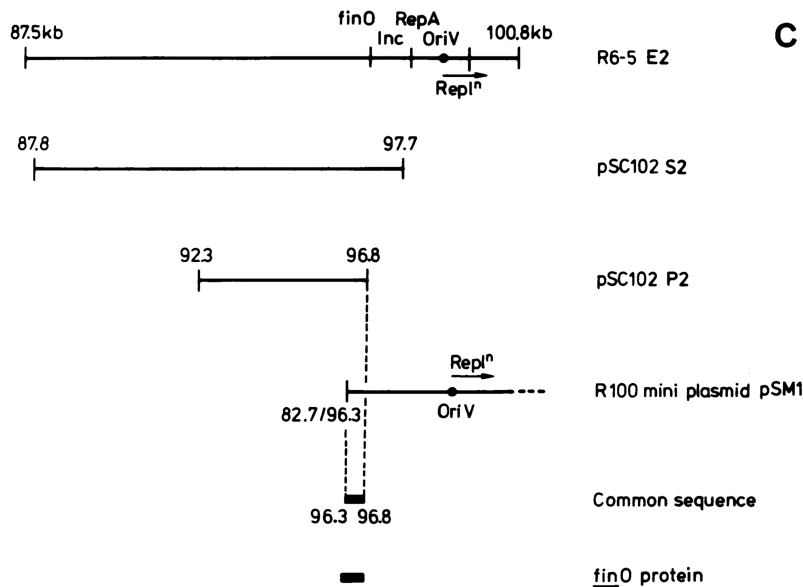


FIG. 2. $finO^+$ plasmids and their proteins. (*A*) Agarose gel electrophoresis of $finO^+$ plasmids. Supercoiled plasmid DNA was prepared (12), digested with the indicated restriction endonuclease, and subjected to electrophoresis through a Tris borate-buffered 0.8% agarose slab gel (19). After electrophoresis the gel was soaked in a solution of ethidium bromide and subsequently photographed with shortwave UV illumination. From left to right: pSC102/*Sal* I (S-1, S-2, S-3); pKT081/*Sal* I (S-2); pSC102/*Pst* I (P-1 – P-11); pKT031/*Pst* I (P-2); pKT058/*Pst* I (P-2); pKT031/*Hin*cII (P-2); pKT058/*Hin*cII (P-2). The last two tracks demonstrate that the orientation of P-2 with respect to the vector molecule in pKT031 is opposite to that of P-2 in pKT058. (*B*) Minicell proteins. Minicells containing the indicated plasmids were prepared (28) and incubated with ^{14}C-labeled casein hydrolysate (Amersham/Buchler). Proteins were then extracted and subjected to electrophoresis through a 15–25% sodium dodecyl sulfate/polyacrylamide gradient gel (28). The gel was subsequently dried and a fluorogram was made. (*C*) The *finO* cistron-containing region of R6-5 and R100. The lines represent the segments of *Eco*RI fragment 2 (E2) of R6-5/R100/pSC102 that are contained in the various $finO^+$ plasmids. The R6-5 coordinates are from Timmis *et al.* (12) and unpublished data; the pSM1 and R100 coordinates are from Mickel *et al.* (29). Ohtsubo *et al.* (30) have assigned the IS1b-proximal *Eco*RI terminus of R100 *Eco*RI fragment 2 to the R100 coordinate 87.2 kb whereas Timmis *et al.* (12) have given the corresponding location on R6-5 the R6-5 coordinate 100.8 kb. Thus, interconversion of R100 and R6-5 coordinates within *Eco*RI fragment 2 is accomplished by the addition or subtraction of 13.6 kb. R100 coordinate 82.7 kb therefore corresponds to R6-5 coordinate 96.3 kb.

maps very near plasmid replication and incompatibility functions (ref. 14; unpublished data).

The *finO* Protein. Examination of proteins synthesized by plasmid-containing minicells has been shown to be a useful method for the identification of plasmid-specific proteins. Two independent $finO^-$ plasmid mutants, pKTO59 and pKTO60, were isolated after *in vitro* hydroxylamine mutagenesis of pKTO31 DNA (Table 2, lines 10 and 11). Although neither mutant carried an amber mutation, it seemed possible that they might produce altered *finO* proteins that would migrate at rates different from the rate of the wild-type protein during electrophoresis through sodium dodecyl sulfate/polyacrylamide

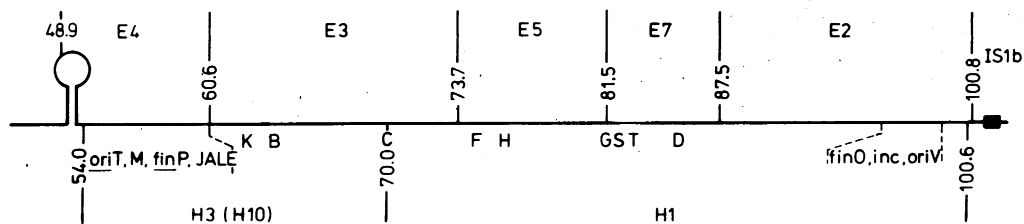


FIG. 3. Genetic and physical map of the *tra* and *RepA* region of R6-5/R100. The coordinates of this map are taken from Timmis *et al.* (12) and unpublished data; the locations of the *tra* and *RepA* functions are taken from Achtman *et al.* (14) and unpublished data. Single letters, such as M, J, etc., represent *tra* cistrons. The position of the *Hin*dIII fragment H10 relative to H3 is not currently known (12). Vertical solid lines represent restriction enzyme cleavage sites. *tra*E and *tra*C contain an *Eco*RI and a *Hin*dIII restriction site, respectively (14).

gels. The proteins synthesized by minicells containing the *finO*$^+$ plasmids pSC102 (E-2 + E-6 + E-8*), pKTO31 (pBR322 + P-2), and pKTO58 (pBR322 + P-2 in reverse orientation) and the *finO*$^-$ derivatives of pKTO31, pKTO59, and pKTO60 were therefore compared. As can be seen in Fig. 2*B*, the two protein profiles obtained from minicells containing the *finO*$^-$ mutants appear to differ from the profile of their *finO*$^+$ parent plasmid in only one respect—namely, that the mutant derivatives fail to direct the synthesis of a 22,000-dalton pKTO31-specified protein (arrow and dots). Instead of producing this protein, minicells containing the *finO*$^-$ mutant plasmids produced a new protein that migrated more slowly during electrophoresis. The 22,000-dalton protein encoded by pKTO31 is also determined by the other *finO*$^+$ plasmids pSC102 and pKTO58. pKT058 is similar to pKT031 except that the orientation of the pSC102 P-2 fragment within the pBR322 vector molecule is opposite to that in pKT031 (Fig. 2*A*). We suggest that the *finO* gene product corresponds to this 22,000-dalton protein. Note that a gene large enough to encode this protein would fill the 0.5–0.6 kb sequence identified as carrying *finO* (Fig. 2*C*). It should be mentioned that, because at least one P-2 determined protein has an electrophoretic mobility similar to that of the 22,000-dalton protein (Fig. 2*B*), it has not always been possible to resolve a *finO*$^+$ plasmid-specific protein in repeat experiments. For this reason our identification of the *finO* gene product must at present be tentative.

DISCUSSION

Biochemical analysis of the mechanism of fertility control by the FinOP repression system would be greatly facilitated by the identification of the genetic loci *finO* and *finP* and by the identification, isolation, and functional characterization of their gene products. In this study we have located *finO* and *finP* on specific *Eco*RI, *Hin*dIII, and *Sal* I fragments of R6-5 and have cloned a small (4.5 kb) *finO*-containing *Pst* I fragment of R6-5 on the high-copy-number plasmid vector pBR322. Detection of expression of *finO* activity by the mini R100 plasmid, pSM1, strongly suggests that the *finO* cistron is located on the 0.5-kb DNA segment that is common to pSM1 and to the cloned R6-5 *finO*$^+$ *Pst* I fragment present in pKTO31. This segment is delineated by R6-5 coordinates 96.3 and 96.8 kb. On the other hand, *finP* was located on an R6-5 DNA sequence with terminal coordinates of 54.0 and 60.6 kb. Surprisingly, therefore, the two cistrons responsible for the FinOP system of fertility control are separated by a segment of DNA greater than 35 kb in length. It is also surprising that *finO* is located very close to (within 1 kb of) the functions associated with autonomous replication of the R6-5 plasmid. One obvious question is, Does *finO* have a dual function, playing a role in both fertility inhibition and vegetative replication of R6-5? Such a role is not obligatory for the autonomous replication of R6-5 because mini R6-5 plasmids that do not contain the finO$^+$-containing *Pst* I fragment have been constructed (unpublished data).

The cloning into pBR322 of a small DNA segment that can code for only a few proteins can greatly simplify the identification of specific gene products. Comparison of the plasmid-specific proteins made by minicells containing the *finO*$^+$ hybrid plasmid pKTO31 with *finO*$^-$ mutant derivatives of this plasmid has enabled us tentatively to identify a specific protein (monomer molecular mass, 22,000 daltons) as the *finO* gene product. Because bacteria concurrently carrying F and the high-copy-number *finO*$^+$ plasmid pKTO31 are 10-fold more repressed for conjugal transfer than are bacteria concurrently carrying F and the low-copy-number R6-5 parent plasmid, it seems likely that the former cells contain higher levels of *finO* protein than do the latter, possibly due to a gene dosage effect. This finding should facilitate the isolation and purification of this protein and subsequent *in vitro* studies of its interaction with the *traJ* promoter sequence.

We are grateful to J. Timmis, B. Kusecek, and S. Hall for expert technical assistance and to H. Mayer for a gift of *Pst* I endonuclease. I.A. was the recipient of a postdoctoral fellowship from the Spanish Ministry of Education.

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