The F Plasmid Carries an IS3 Insertion Within *finO*

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DNA–DNA hybridization studies support the conclusion that coding sequences of the fertility inhibition gene, *finO*, are present on the F plasmid and map downstream of the transfer region, entirely within 99.19–2.0 F. In addition, the results indicate that the IS3a element at 100.0–1.26 F is inserted within the F *finO* coding region. This insertion may have inactivated the *finO* gene on the F plasmid.

INTRODUCTION

Electron microscopic heteroduplex studies have demonstrated that the transfer (*tra*) regions on F and on the F-like plasmids R1 and R6-5 are extensively, but not completely, homologous (Sharp *et al.*, 1973). The expression of the majority of the *tra* genes on these plasmids is positively controlled by the product of *traJ* (Willetts & Skurray, 1980). Expression of *traJ* is, in turn, negatively regulated by the FinOP fertility inhibition system, which depends upon the products of the *finO* and *finP* genes (Finnegan & Willetts, 1972; Willetts, 1977).

The *finP* gene of F maps between *traM* and *traJ* (Fig. 1) and it has been proposed that the product of *finP* is an RNA molecule, encoded within the *traJ* leader sequence, which interacts with the *finO* product to inhibit transcription and/or translation of *traJ* (Mullineaux & Willetts, 1985); *finP* on R6-5 is located in an analogous position to that on F (Timmis *et al.*, 1978). We have previously reported the cloning of the R6-5 *finO* region into pBR322, yielding the plasmid pRS3509; DNA deletion analysis and transposon Tn5 mutagenesis of pRS3509 have allowed us to precisely map the *finO* gene within the coordinates 94.0–94.85 R6-5 (Cheah *et al.*, 1984; Fig. 2). A 22 kDa protein has been tentatively identified as the R6-5 *finO* product (Timmis *et al.*, 1978), although it should be noted that further experimentation by these authors, and others (Dempsey & McIntire, 1983; Cheah *et al.*, 1984), has failed to resolve this polypeptide.

Since F-like plasmids such as R1, R6-5 and R100 constitutively specify the *finO* and *finP* products, the transfer systems of these plasmids are naturally repressed by the FinOP system (Finnegan & Willetts, 1972); the conjugal transfer frequency exhibited by these F-like plasmids is approximately 0.1–1% that of F. However, conjugally derepressed *FinO*[−] mutants, such as R100-1 (Egawa & Hirota, 1962) and R1*drd*-19 (Meynell & Datta, 1967), that transfer at a similar level to F have been isolated. The transfer proficient *finP*⁺ F plasmid is believed to be a naturally occurring *finO* mutant; in the presence of a co-resident *FinO*⁺ F-like plasmid, the F plasmid is also repressed for DNA transfer (Willetts, 1977).

To date there have been no reported attempts to clarify the exact status of *finO* sequences on the F plasmid, nor to establish if they are in fact present at all. Interpretation of the R6-5/F heteroduplex data of Sharp *et al.* (1973), in the light of our localization of *finO* sequences on R6-5 (Cheah *et al.*, 1984), did, however, lead us to speculate that *finO* sequences may well be present on F; a similar proposal has been put forward independently by Clark (1985). In this paper, we examine this hypothesis and describe the use of *finO*-specific DNA probes derived from R6-5 to locate *finO* sequences on F.

Fig. 1. Physical map of the F plasmid. The locations of *Eco*RI (E) and *Bam*HI (B) sites, as well as the positions of IS3 elements, taken largely from Skurray *et al.* (1978), have been adjusted to fit a 100 kb map of F. The *Bam*HI fragments of F, with the exception of fl1, have been cloned, and their map positions precisely determined; the location of a twelfth *Bam*HI fragment (0.075 kb) has not been determined but it must lie at either of the positions indicated by an asterisk (D. Cram & R. Skurray, unpublished data). The positions of *finP* and the transfer (*tra*) operons *traM*, *traJ* and *traY* → *Z* are shown (Willetts & Skurray, 1980); the size of IS3 (1.26 kb) is taken from the sequence data of Timmerman & Tu (1985). Recircularization of the largest *Hind*III (H) fragment (0.14–45.5 F) of F generated pED100, which is self replicative and carries the entire transfer region (Willetts & Johnson, 1981).

METHODS

Plasmids. Plasmids used, in addition to F, were pED100 (Fig. 1; consists of recircularized 0.14–45.5 F *Hind*III fragment of the F plasmid) (Willetts & Johnson, 1981); pSH2 (*Eco*RI fragment f2 of F cloned into RSF2124) (Achtman *et al.*, 1978); pRS3509 (92.75–95.55 R6-5 *finO*⁺ *Bam*HI fragment cloned into pBR322), pRS3509::Tn5 derivatives (pRS3565 and pRS3583) (Cheah *et al.*, 1984).

Preparation and analysis of DNA. Purified low copy number plasmid DNA (F and pED100) was obtained using a Triton X-100 cleared lysate polyethylene glycol precipitation procedure, followed by banding on caesium chloride/ethidium bromide density gradients, essentially as described by Humphreys *et al.* (1975) and Kahn *et al.* (1979). Isolation of amplifiable plasmid DNA, digestion with restriction endonucleases and agarose gel electrophoresis were done according to Ray & Skurray (1983). Fragment sizes of pBR322 DNA digested with *Taq*I and λ phage DNA cleaved with *Hind*III, for use as standards, were taken from Sutcliffe (1978) and Daniels *et al.* (1983), respectively.

DNA–DNA hybridization. Bidirectional transfer of DNA from an agarose gel to nitrocellulose filter papers (Schleicher and Schüll) was done by the method of Smith & Summers (1980). DNA fragments from low melting point agarose gel (Biorad) were extracted essentially as described by Langridge *et al.* (1980). Purified DNA was radiolabelled with [α -³²P]dATP (1800 Ci mmol⁻¹, 66600 GBq mmol⁻¹; Biotechnology Research Enterprises SA) by nick translation and hybridized with nitrocellulose filters containing target DNA (Maniatis *et al.*, 1982). Washed and dried filters were then exposed to Fuji RX film at –70 °C for sufficient time to produce an acceptable autoradiograph.

RESULTS AND DISCUSSION

Homology between F and the R6-5 *finO* region

To establish whether the F plasmid (Fig. 1) does indeed possess DNA sequences homologous to the R6-5 *finO* region, the pRS3509 *Bam*HI fragment that carries the entire R6-5 *finO* region (92.75–95.55 R6-5, Fig. 2) was labelled with [α -³²P]dATP by nick translation, and hybridized to a

Fig. 2. Physical map of R6-5 and F *finO* regions. The relevant restriction sites (B, *Bam*HI; Bg, *Bgl*II; Sm, *Sma*I), R6-5 coordinates and positions of Tn5 insertions (phenotype: ▼, *FinO*⁺; ▽, *FinO*⁻) on the *finO*⁺ 92.75–95.55 R6-5 *Bam*HI fragment of pRS3509 are taken from Cheah *et al.* (1984). The *Bgl*II (BgII) and *Sma*I sites of Tn5 (Jorgensen *et al.*, 1979) used in the construction of probes, as described in the text, are shown on the open triangles. The minimum span of R6-5 *finO* coding sequences is indicated by the open box (94.1–94.55 R6-5); broken arrowed lines on either side of *finO* (94.0–94.85 R6-5) depict the maximum extent of the gene. Only the relevant portion of *Eco*RI fragment f2 of F (89.4–2.6 F), which includes the distal part of the transfer (*tra*) region and IS3a (100/0–1.26 F), is shown; the restriction sites for *Eco*RI (E), *Hind*III (H), *Pst*I (P) and *Sma*I (Sm) are given (Willetts & Johnson, 1979; Willetts & Skurray, 1980; Manning *et al.*, 1982). Dots leading to the *Sma*I site to the right of the *Eco*RI site at 2.6 F denote 1.3 kb of RSF2124 vector sequences on pSH2, the source of F DNA used in the hybridization experiments described in Fig. 4. DNA fragment that hybridized to the *finO*-specific probe I (94.1–94.25 R6-5); DNA fragments that hybridized to the *finO*-specific probe II (94.25–94.55 R6-5); *Pst*I fragment that hybridized to both probes. Other probes used were III (92.75–94.3 R6-5) and IV (94.3–94.9 R6-5). The *finO* regions of R6-5 and F are aligned relative to the *Sma*I sites at 94.25 R6-5 and 99.75 F (see text). The open boxes at 99.6–100/0 F and 1.26–1.31 F indicate the presumed minimum span of *finO* sequences on F on either side of IS3a; the deduced maximum extent (99.5–1.61 F) of F *finO* coding sequences is indicated by broken arrowed lines.

nitrocellulose filter containing *Bam*HI and *Eco*RI restriction fragments of F DNA (Fig. 3). The resulting autoradiograph (Fig. 3b) showed that this *finO*⁺ fragment hybridized only to the 42 kb *Bam*HI fragment f1 of F (lane A), within which lies F *Eco*RI fragments f1, f2, f6, f15, f17, f19 and parts of f3 and f12 (Skurray *et al.*, 1978; Fig. 1). Of the latter *Eco*RI fragments, only f1 (14.2 kb) or f2 (13.2 kb), or both, showed homology with the *finO*⁺ fragment (Fig. 3b, lane B); the close migration of fragments f1 and f2 (Fig. 3a, lane B) made it difficult to distinguish between these possibilities. To resolve this, the *Eco*RI-digested products of pED100, a self-replicative Tra⁺ derivative of the F plasmid which consists of F *Eco*RI fragments f1, f3, f6, f15, f17, f19 and parts of f2 (f2̄) and f5 (f5̄) (Willetts & Johnson, 1981; Fig. 1), were hybridized with the *finO*⁺ fragment (Fig. 3, lane C). Whereas *Eco*RI fragment f1 did not hybridize to the *finO*⁺ fragment, the 21 kb *Eco*RI f2̄–f5̄ fusion fragment from pED100 (Willetts & Johnson, 1981) did. These data indicate that the *Eco*RI fragment which hybridized with the *finO*⁺ fragment in Fig. 3 (b) lane B is f2.

Fig. 3. Hybridization between the ^{32}P -labelled *finO*⁺ 92.75–95.55 R6-5 *Bam*HI fragment and F DNA. (a) Agarose gel (0.7%, w/v) electrophoresis of F DNA digested with either *Bam*HI (lane A) or *Eco*RI (lane B); lane C contains *Eco*RI-digested pED100. Six of the twelve *Bam*HI fragments of F are visible on this gel (lane A) as are eleven of the nineteen *Eco*RI fragments of F (lane B). λ phage DNA digested with *Hind*III was included in this gel; the positions of migration and sizes in kb of the DNA fragments generated are shown at the left. The positions of relevant *Bam*HI (B) and *Eco*RI (E) fragments of F are indicated at the right. (b) Autoradiograph of the nitrocellulose filter containing the DNA fragments shown in (a) after hybridization with the ^{32}P -labelled *finO*⁺ fragment.

Location of finO sequences on the F plasmid

To confirm that *Eco*RI fragment f2 of F possesses *finO* sequences, and to determine the organization of *finO* on F, two *finO*-specific DNA probes encompassing different segments of the R6-5 *finO* gene were chosen. DNA fragments containing *finO* sequences were obtained from two *FinO*[−] pRS3509 derivatives with transposon Tn5 insertions in R6-5 *finO* sequences at 94.1 R6-5 (pRS3565) and 94.55 R6-5 (pRS3583) (Cheah *et al.*, 1984; Fig. 2). Purification of the appropriate DNA fragments after *Bgl*II–*Sma*I digestion of pRS3565 and *Sma*I cleavage of pRS3583 provided the R6-5 *finO* : Tn5 fusion fragments containing 94.1–94.25 R6-5 (probe I) and 94.25–94.55 R6-5 (probe II) *finO* sequences, respectively (Fig. 2). Each probe also carried at least 1.55 kb of Tn5 sequences but, as expected, our preliminary hybridization experiments demonstrated that *Eco*RI fragment f2 of F lacked sequence homology with Tn5 (data not shown) and therefore any hybridization observed with the *finO* probes derived from either pRS3565 or pRS3583 should result from homology between F and sequences within the R6-5 *finO* gene.

^{32}P -Labelled probes I and II were incubated separately with nitrocellulose filters containing pSH2 DNA (*Eco*RI fragment f2 cloned into RSF2124; Achtman *et al.*, 1978) that had been digested with *Pst*I, *Sma*I or *Hind*III–*Sma*I (Fig. 4b and c); a summary of the hybridization results, aligned to the map of F, is presented in Fig. 2. Both of the *finO*-specific probes hybridized to the 1.7 kb (99.19–0.89 F) *Pst*I fragment (Fig. 4b and c, lane A) which encompasses

Fig. 4. Location of *finO* sequences on the F plasmid. (a) An agarose gel (1%, w/v) showing the products of pSH2 DNA digested with *Pst*I (lane A), *Sma*I (lane B) and *Hind*III-*Sma*I (lane C). λ pl age DNA and pBR322 DNA cleaved with *Hind*III and *Taq*I, respectively, were included in this gel and the positions of migration of relevant DNA fragments and sizes in kb are shown on the left. DNA from the agarose gel was transferred to nitrocellulose filters and hybridized with 32 P-labelled R6-5 *finO*-specific probe I (b) and probe II (c).

sequences to the left of IS3a and includes some of the IS3a sequence (Fig. 2). The 1.11 kb (0.89–2.0 F) *Pst*I fragment encompasses the right hand portion of IS3a and the adjacent F sequences (Fig. 2), and hybridized only to probe II (Fig. 4c, lane A). Neither probe I nor probe II hybridized to the 0.91 kb (0.14–1.05 F) *Hind*III fragment (Fig. 4, lane C) that is completely internal to IS3a (Fig. 2), but it should be noted that there are at least 0.14 kb of IS3a sequences on the 1.11 and 1.7 kb *Pst*I fragments that did hybridize with these *finO*-specific probes (Fig. 2). The fact that F *Eco*RI fragment f4, which contains the entire IS3b sequence (Hu *et al.*, 1975; Skurray *et al.*, 1978; Fig. 1), failed to hybridize to the *Bam*HI *finO*⁺ fragment (Fig. 3, lane B) precludes the involvement of IS3a sequences in the hybridization results shown in Figs 2 and 3.

Taken together, these results confirm that *Eco*RI fragment f2 of F carries *finO* sequences. In addition, the data indicate that the IS3a sequences between 100/0 F and 1.26 F divide the F *finO* sequences into two portions (Fig. 2).

Both probe I and II were derived by digestion at the 94.25 R6-5 *Sma*I site (Fig. 2). Whereas probe I hybridized to the 1.06 kb (98.69–99.75 F) *Sma*I fragment (Fig. 4 b, lanes B and C), probe II hybridized with the 0.39 kb (99.75–0.14 F) *Hind*III-*Sma*I fragment (Fig. 4 c, lane C) as well as

with the 4.15 kb *Sma*I and the 2.85 kb *Hind*III-*Sma*I fragments, which contained the 99.75-2.6 F and 1.05-2.6 F sequences of the F plasmid, respectively (Fig. 2; Fig. 4 c, lanes B and C). These hybridization data coupled with the fact that the probed F *finO* fragments map leftwards and rightwards of the *Sma*I site at 99.75 F, suggest that the *Sma*I sites at 94.25 R6-5 and 99.75 F are equivalent; they have been aligned accordingly in Fig. 2.

Defining the boundaries of finO sequences on F

To determine the physical limits of *finO* coding sequences on either side of IS3a, two further DNA probes (III and IV, Fig. 2), which together encompassed the entire R6-5 *finO* gene, were used. ³²P-Labelled probe III and probe IV were incubated separately with pSH2 DNA which had been digested with *Pst*I (data not shown). Probe III hybridized only to the 99.19-0.89 F *Pst*I fragment thus delimiting the maximum leftward boundary of *finO* sequences on F to 99.19 F (Fig. 2). Probe IV, however, hybridized to the 0.89-2.0 F *Pst*I fragment, as well as to the 99.19-0.89 F *Pst*I fragment; this indicates that the rightwards limit of *finO* on F must be between 0.89 F and 2.0 F (Fig. 2). Besides confirming that IS3a sequences at 100/0-1.26 F are bounded by *finO* coding sequences, these results conclusively demonstrate that *finO* sequences are entirely contained within 99.19 and 2.0 F (Fig. 2).

The maximum and minimum limits of the R6-5 *finO* gene have previously been determined to be 94.0-94.85 R6-5 and 94.1-94.55 R6-5, respectively (Cheah *et al.*, 1984; Fig. 2). By assuming that R6-5 and F *finO* genes are of approximately the same size, we have been able to align the F and R6-5 *finO* coding sequences as shown in Fig. 2. On this basis, we propose (i) that the site of IS3a insertion into the F *finO* coding region at 100/0 F is equivalent to 94.5 R6-5 in terms of R6-5 coordinates — indicating that IS3a is indeed inserted into the F *finO* structural gene — and (ii) that the maximum and minimum boundaries of *finO* coding sequences on F are between 99.5-1.61 F and 99.6-1.31 F, respectively (Fig. 2).

Evolution of the F finO gene

The conjugative plasmids F and R6-5 probably originated from a common ancestor but followed different evolutionary pathways, during which the *finO* gene of F was inactivated by an insertion of IS3. Since the excision frequency of IS3 is low (5×10^{-9} per cell per generation) (Sommer *et al.*, 1979), a *finO*::IS3a mutation would be expected to be relatively stable. In accordance with this, no *finO*⁺ revertant of the F plasmid was detected in studies reported by Beutin & Achtman (1979). However, the apparent stability of the *FinO*⁻ state of F could also result if part of the *finO* gene had been deleted, an event which might have occurred in addition to the IS3a insertion; the exact extent of *finO* sequences on F must await a comparative DNA sequence analysis of the *finO* regions on R6-5 and F.

A bacterium harbouring the derepressed *FinO*⁻ F plasmid suffers two disabilities. Firstly, such a cell synthesizes F pili and is therefore subject to infection by male-specific bacteriophages, and secondly, unnecessary constitutive synthesis of *tra* gene products would be expected to place the cell in an energetically unfavourable situation relative to F⁻ cells. The survival of the F plasmid suggests that properties expressed by this plasmid over-ride these disadvantages.

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