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Cloning, mutation, and location of the F origin of conjugal transfer

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pED806, a pBR322 derivative carrying the origin of transfer (*oriT*) of F, was rapidly lost from cells carrying an F *tra*$^+$ plasmid. Instability was increased in a RecA$^-$ host, and depended in particular upon the F*traYZ* genes that produce the nick at *oriT* at which transfer is initiated. Instability was also correlated with the orientation of the *oriT* fragment in the vector plasmid. Mutants of pED806 selected as being stable in the presence of F*lac* proved to carry *cis*-dominant *oriT* mutations. The *oriT* site was subcloned from pED806 on a *Hae*II fragment including a *Hae*II-*Bgl*II segment of F DNA ~385 base pair (bp) long into the 2.25 kilobase (kb) vector plasmid pED825, giving pED822. pED822 was fully proficient for *oriT* function, and recircularised in recipient cells by a *recA*- and *tra*-independent *oriT*-specific ligation/recombination event. 'Phasmids' constructed by cloning pED806 or an *oriT*$^-$ mutant into a λ vector were used to confirm that the nick site in λ*oriT* phages grown in the presence of F*lac tra*$^+$ is indeed at *oriT*. The nick site in a further λ*oriT* phage (EDλ102) was then located 140 ± 20 bp from the *Bgl*II site forming one terminus of the F fragment cloned in pED806 and pED822.

Key words: conjugation/*oriT*/vector/mutagenesis

Introduction

Conjugal DNA metabolism by the *Escherichia coli* K12 plasmid F is initiated at a nick in a specific strand of the plasmid DNA within a specific site that is designated *oriT* (see review by Willetts and Skurray, 1980). This metabolism includes both transfer of the nicked strand to the recipient cell, and its replacement by conjugation-specific DNA synthesis using the non-transferred strand as template. Using a λ*oriT* transducing phage, Everett and Willetts (1980) showed that formation of the nick required the products of the conjugation genes *traY* and *traZ*, and suggested that this precedes the triggering (as a consequence of mating pair formation) of DNA transfer and synthesis by the products of further genes, possibly *traM* and/or *traI* (Kingsman and Willetts, 1978). The *oriT* site must therefore include DNA sequences recognised by at least some of these four *tra* gene products, as well as the nicked DNA sequence.

We have cloned a 540-bp *Bgl*II-*Sal*I fragment that includes *oriT* in pBR322, to give the plasmid pED806 (Johnson *et al.*, 1981). In this paper, the properties of pED806 are described in detail, including its instability in the presence of an F*lac tra*$^+$ plasmid, and the isolation of *cis*-dominant *oriT*$^-$ mutants by a method based upon this instability. Furthermore, *oriT* is shown to lie within a ~385-bp *Bgl*II-*Hae*II F fragment subcloned from pED806, and the nick site itself has been located within an ~40-bp region of this fragment using nicked λ*oriT* DNA and a refinement of the technique described by Everett and Willetts (1980).

Results

Instability of pED806 in the presence of Flac

pED806 is a derivative of pBR322 into which the 540-bp *Bgl*II-*Sal*I fragment of F that carries *oriT* has been cloned (Johnson *et al.*, 1981; Figure 1). At the time that it was shown that pED806 is transferred at about three times the frequency of F*lac* from a cell carrying both plasmids, it was noticed that pED806 was rapidly lost from such cells, especially if they were RecA$^-$. We have investigated this phenomenon in more detail.

Firstly, semi-quantitative data were obtained to confirm the initial qualitative observations (Table I). These showed that the instability of pED806 was indeed increased by a *recA* mutation in the host cell, while the parental pBR322 plasmid was (as expected) completely stable under these conditions. Furthermore, instability was reduced to the *rec*$^+$ level in a *recA recB* double mutant, indicating that the increased instability in a *recA* host is due to the rapid degradation of DNA by the *recBC* product, exonuclease V, as previously observed for bacterial DNA in such strains (Clark *et al.*, 1966; Willetts and Clark, 1969).

Secondly, the effects of various F*lac tra* mutations on the instability were tested (Table I). pED806 was completely stable in the presence of JCFL90 (F*lac traJ90*), and since the *traJ90* mutation prevents expression of essentially all the *tra* genes (Finnegan and Willetts, 1973; Willetts, 1977), this confirmed that instability was indeed due to an interaction between the F conjugation system and the *oriT* site of pED806. The products of four transfer genes are thought to act at *oriT*: those of *traY* and *traZ* to nick the "transferred" DNA strand at this point, and those of *traM* and/or *traI* to trigger the DNA transfer and replication process (Everett and Willetts, 1980). We therefore tested EDFL171 (F*lac traΔIZ337*), and pED806 was indeed completely stable in the presence of this mutant. By comparison, pED806 showed intermediate levels of instability in the presence of JCFL41 (F*lac traI41*) or JCFL102 (F*lac traM102*). Mutations in genes required for other conjugation functions such as pilus formation *(traA1)* and stabilisation of mating pairs *(traG42)* had little or no effect upon the instability of pED806. The role of *traD* in conjugal DNA metabolism is unclear, but the *traD38* missense mutation did not prevent pED806 instability, while the *traD83* amber mutation did so in part, presumably as a consequence of its partial polarity on expression of *traI* and *traZ* (Everett and Willetts, 1980). We conclude that only the *traYZ* nicking function is essential for pED806 instability, and that the *traM* and *traI* functions increase its extent.

Thirdly, the stabilities of other multicopy *oriT*$^+$ recombinant plasmids in the presence of F*lac* were tested. pED810 (pBR322 with the F *oriT Sal*I fragment), pED821 (pBR325 with the F *oriT traMJYALEKB Eco*RI fragment), pED819

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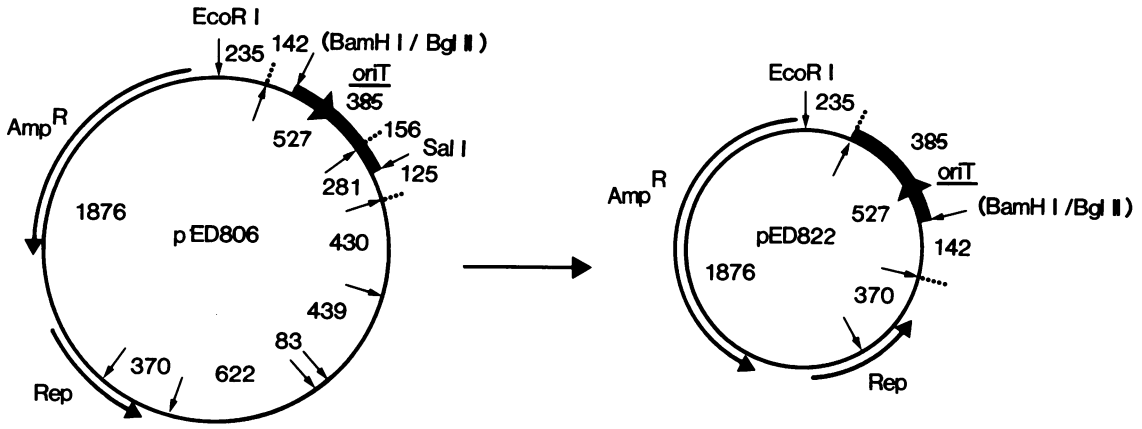

Fig. 1. Maps of the *oriT* plasmids pED806 and pED822. The heavy lines represent F DNA and *oriT* is shown as an arrow-head. *Hae*II sites are shown inside the circles.

Table I. The stability of pED806

Host strain *rec* genotype	Plasmids	% Ap^R cells after[a]	
		5 h	18 h
recA56	pED806	100	100
rec$^+$	pED806 + F*lac*	65	16
recA56	pED806 + F*lac*	0.4	0.04
recA56 recB21	pED806 + F*lac*	82	12
recA56	pBR322 + F*lac*	100	100
recA56	pED806 + JCFL90	100	100
recA56	pED806 + EDFL171	100	100
recA56	pED806 + JCFL41	26	4
recA56	pED806 + JCFL102	10	1
recA56	pED806 + JCFL1	3	0.19
recA56	pED806 + JCFL42	0.6	0.17
recA56	pED806 + JCFL38	1.7	0.15[b]
recA56	pED806 + JCFL83	86	22
recA56	pED810 + F*lac*	100	98
recA56	pED821 + F*lac*	100	98
recA56	pED819 + F*lac*	100	100
recA56	pED822 + F*lac*	100	99

[a]1 – 5 x 10^6 cells from a fresh single colony taken from a lactose-tetrazolium-ampicillin plate were incubated in 1 ml L broth for 5 h (allowing 5 – 7 generations) and, after a further 1:10 dilution, for 18 h (allowing 4 – 5 further generations). The proportion of Ap^R cells was determined by plating dilutions on nutrient plates with and without ampicillin, and by patching 100 colonies from the latter and replicating to nutrient-ampicillin plates.
[b]About 25% of the Ap^R cells were Lac^-; in all other cases the Ap^R cells were essentially all Lac^+.

(pBR325 with the F *oriT traM Bgl*II fragment) and pED822 (see below) were all lost at much lower frequencies than pED806 (Table I). We realised that the *oriT* segment in pED806 is inserted in the opposite orientation (II) with respect to the vector plasmid's *oriV* site, compared to the other four plasmids (I). Indeed, cloning of the F *oriT*$^+$ fragments in these four plasmids, or of the entire F transfer region in several others (Johnson and Willetts, 1980a, 1980b; Johnson *et al.*, 1981) had always given plasmids with insertions in orientation I, even though the equivalent single-strand extensions at the termini of the fragments should have allowed insertion in either orientation. Only in the case of the *Bgl*II-*Sal*I fragment cloned in pED806, was insertion 'forced' in orientation II. Possible reasons for the resultant instability

Table II. Properties of pED806 mutants

Plasmid[a]	% Ap^R cells after 18 h[b]	Transfer frequency (%)[c]
pED806	0.05	300
pED833	100	2
pED834	100	42
pED835	100	29
pED836	100	1

[a]In all cases the plasmids were present in JC6310 together with F*lac*.
[b]Measured as described in the legend to Table I.
[c]Expressed as a % of F*lac* transfer from the same strain.

of pED806 in the presence of F*lac* are considered below.

Construction of oriT$^-$ point mutants

Whatever the reason for the instability of pED806 when present in the same cell as an F*lac* plasmid, it is clear that this is related to its possession of a functional *oriT* site. It should therefore be possible to select *oriT*$^-$ mutants of pED806 on the basis of their increased stability.

To this end, pED806 DNA was mutagenised *in vitro* with hydroxylamine. Separate cultures of JC6310 (F*lac*)$^+$ were transformed with the plasmid DNA, and Ap^R transformants selected in broth containing 40 μg/ml ampicillin. These cultures were serially sub-cultured four times in L broth, and Ap^R cells then selected on nutrient-ampicillin plates. These colonies contain putative *oriT*$^-$, stable pED806 mutants. After further testing to identify Lac^+ colonies in which the pED806 plasmid derivative was completely stable, the transfer frequencies were measured. The transfer frequency of F*lac* from the four independently isolated strains tested was normal, but transfer of the pED806 derivatives was substantially reduced. To ensure that the mutations causing the stability and reduced transfer were indeed carried by pED806 plasmids, these were transformed into fresh JC6310 (F*lac*)$^+$ cells and the measurements repeated. The results confirmed that the four plasmids (pED833 – 6) were stable in the presence of F*lac*, and were mobilized at frequencies reduced 7- to 300-fold compared to pED806 itself (Table II).

All four plasmids were similar in size to pED806 and gave similar restriction enzyme cleavage patterns; analysis of the sequences of their *oriT* regions is in progress.

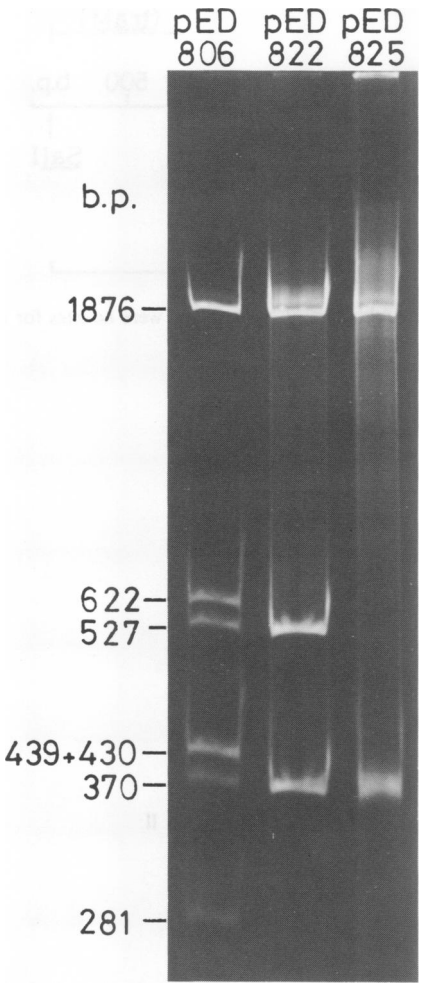

Fig. 2. *Hae*II digestion of pED806, pED822, and pED825. The fragments of the sizes (bp) marked, were separated by electrophoresis through a 5% polyacrylamide gel. It is notable that the 527-bp *oriT*$^+$ fragments run abnormally slowly in polyacrylamide, as opposed to agarose gels: this may be related to the several inverted repeat sequences in this region (unpublished data).

Subcloning of oriT from pED806

Previously, our relatively inaccurate experiments had located the site of the *oriT* nick at a point approximately midway between (and therefore 270 bp from) the *Bgl*II site to the left of *oriT* and the *Sal*I site in *traM* (see Figures 1 and 3). Thompson and Achtman (1979) have proposed an alternative location for *oriT*, between the same *Sal*I site and a *Hae*II site located 155 bp to its left. Their proposal is based upon similarities between F DNA sequences from this region and ColE1 DNA sequences near to its *nic* site (the relaxation complex *nic* and *oriT* nick sites are probably identical). We have therefore carried out further experiments to obtain a more precise location for *oriT*.

Firstly, we used the enzyme *Hae*II in *in vitro* recombination experiments designed to subclone the *oriT* region from pED806. pED806 was digested with *Hae*II, the mixture religated, and the DNA used to transform ED8654 (F*lac*)$^+$. Ap^R transformants were selected in broth containing 40 μg/ml ampicillin, and after growing to exponential phase the culture was mated with ED2196 and Ap^R[Nal^R] transconjugants were selected. This is the technique of Johnson *et al.* (1981) for selecting *oriT*$^+$ plasmids, since essentially only these will be transferred in the mating. Lac$^-$ Ap^R transconjugants were chosen, and screened using small plasmid preparations to identify those containing the smallest plasmids. Further analysis of one of these (pED822) showed that it contained only three *Hae*II fragments (Figure 2). These were the 1876-bp fragment of pBR322 that includes the β-lactamase gene and a part of the replication region, the 370-bp fragment carrying the rest of the replication region (the boundary between these fragments must be intact for replication to proceed), and a hybrid fragment of ~527 bp including the ~ 385-bp *Bgl*II-*Hae*II region of F, plus a 142-bp *Hae*II-*Bam*I segment of pBR322 (Figure 1). The hybrid fragment was shown to be in orientation I (see above) by digesting pED822 with *Hae*III (data not shown).

Genetic tests confirmed that pED822 carries the F *oriT* sequence, since it was transferred at about twice the level of F*lac* transfer from a JC6310 derivative carrying both plasmids; it was stable in such cells as expected for the orientation of its insertion (Table I). We therefore conclude that *oriT* is wholly contained within the *Bgl*II-*Hae*II fragment, and does not lie within the *Hae*II-*Sal*I fragment. When the Spc^R RecA$^-$ strain ED2030 was used as recipient in these matings, ~50% of the Ap^R transconjugants were Lac$^-$, and so had inherited pED822 but not F*lac*. This implied that pED822 could recircularise by a *recA*- and *tra*-independent mechanism. Similar results were obtained using JC6310 (pED100, pED822) as donor, where pED100 is a conjugative F plasmid derivative without insertion sequences (Willetts and Johnson, 1981), and where pED822 was present in the tetrameric form. The nature of the plasmids in Ap^R transconjugants that had inherited both plasmids were examined by electrophoresis of small-scale plasmid preparations through agarose gels: 43/45 contained the monomeric form of pED822, and the remaining two the dimeric form. Also, 12/13 Ap^R transconjugants that had received only pED822 contained the monomeric form. Monomerisation was therefore a frequent consequence of the transfer process, as might be expected if the 5′ terminus transferred first recognises the next *oriT* site (nicked or unnicked) to be transferred, as the site for recircularisation. Only the tetrameric form of pED822 could be found in the donor cells, showing that the process of transfer was essential for monomerization, and that this did not result from the reversible *oriT* nicking expected to take place in these Tra$^+$ cells even in the absence of mating pair formation (Everett and Willetts, 1980). No recombination between pED100 and pED822 was found during conjugation: this contrasts with the *oriT*-specific recombination between two plasmids both containing the ColE1 *oriT* (i.e., *bom*) site observed by Warren and Clark (1980).

The small size of pED822 (2.8 kb) compared to pED806 (4.9 kb), together with the known restriction enzyme cleavage sites of that part derived from pBR322 (Sutcliffe, 1978) has enabled us to construct a detailed restriction map of the *Bgl*II-*Hae*II region of F that includes *oriT*, without necessity for prior isolation of larger restriction enzyme fragments (Figure 3). Indeed, the 1876-bp and 370-bp *Hae*II fragments of pED822 that originate from pBR322 were subcloned together to give pED825. This is a useful vector plasmid, particularly for insertions into the *Hae*II site at nucleotide 235 and the *Cla*I site at nucleotide 26 (Figure 4). Its small size facilitates both mapping of restriction sites and sequencing,

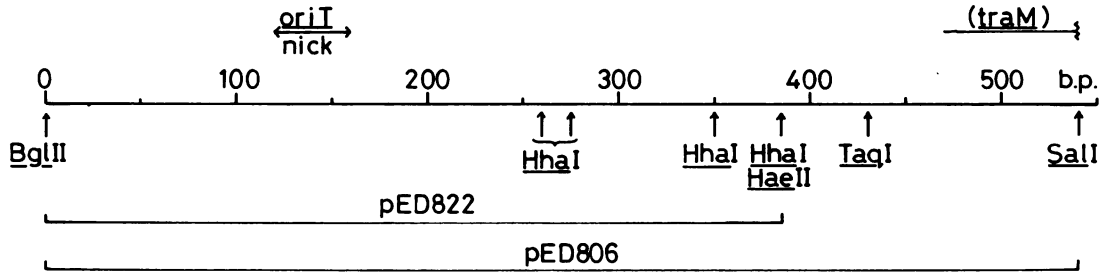

Fig. 3. Detailed restriction map of the ~540-bp *Bgl*II-*Sal*I fragment of pED806 that includes *oriT* and a part of *traM*. There were no sites for *Eco*RI, *Hind*III, *Bam*I, *Sma*I, *Hae*III, *Hpa*II, *Sau*3A, *Alu*I, or *Hinf*I.

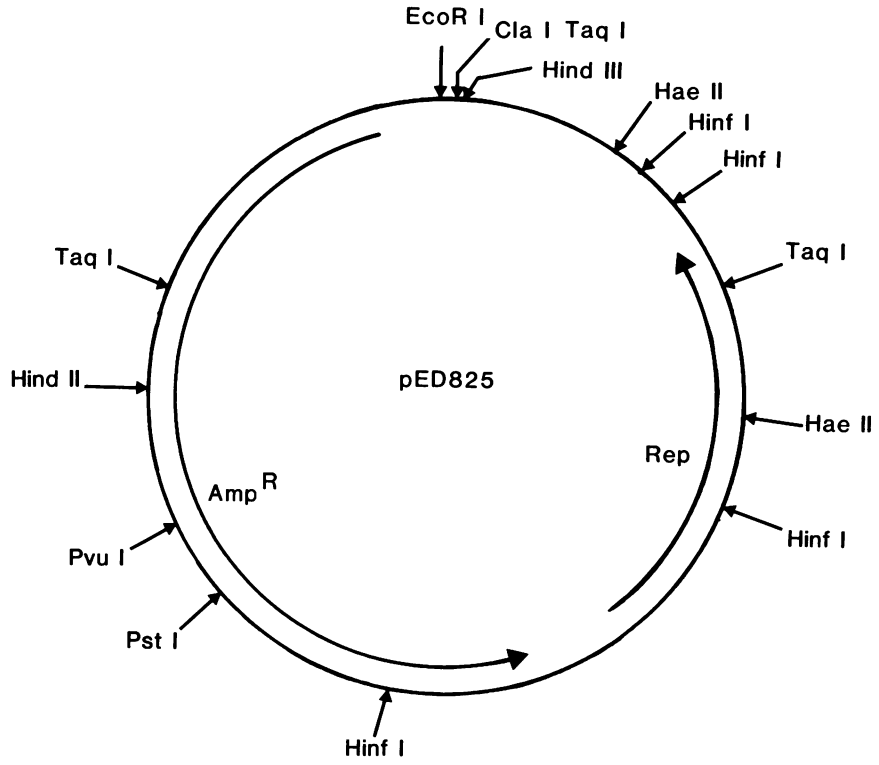

Fig. 4. Map of the 2246-bp cloning vector pED825. This is composed of the two *Hae*II fragments located from positions 2349–2719 and 2719–235 on the pBR322 map (4362 bp; Sutcliffe, 1978). Coordinates are those of the base to the right of the dyad axis of the restriction endonuclease cleavage site. In pED825 these coordinates are as follows, reading clockwise: *Eco*RI (1), *Cla*I and *Taq*I (26), *Hind*III (32), *Hae*II (235) *Hinf*I (259.5 and 334.5), *Taq*I (458), *Hae*II (603), *Hinf*I (730.5 and 1246.5), *Pst*I (1493), *Pvu*I (1620), *Hind*II (1791), *Taq*I (1901). The *Hae*II site at nucleotide 603 must be reconstituted in order for the plasmid to replicate, and *Taq*I, *Msp*I, or *Hpa*II fragments can be cloned into the unique *Cla*I site.

and since it lacks the *oriT* region of pBR322, it has the same increased copy number and improved biological containment properties as pAT153 (Twigg and Sherratt, 1980) and pBR327 (Soberon *et al.*, 1980).

Authenticity and location of the nick site in λoriT phages

We have described a λ*oriT* transducing phage, EDλ102, and shown that when (F*lac tra*$^+$) cells are infected with this phage, a proportion of the progeny phage genomes are nicked at a site which we believe to be *oriT* (Everett and Willetts, 1980). Further evidence that this is indeed the case has been obtained by cloning pED806 and its *oriT*$^-$ mutant pED833 *via* their *Eco*RI sites into the λ vector λNM781 to give EDλ806 and EDλ833, respectively (see Materials and methods). Using the assay of Everett and Willetts (1980), one strand of EDλ806 DNA but not of EDλ833 DNA was found to be nicked when grown on cells carrying an F*lac tra*$^+$ plasmid (Figure 5). This confirmed that nicking was dependent upon the λ*oriT* phage possessing a functional *oriT* site. EDλ806 is an interesting phage since although not containing an *att* site, it lysogenised a λ$^-$ *E. coli* K12 strain, presumably by virtue of its ability to replicate as a pBR-based replicon. Furthermore, this λ plasmid was unstable in the presence of F*lac*, and was transferred by conjugation from such cells.

The inaccuracy of our previous experiments to locate the nick at *oriT* was due to the difficulty of measuring the precise sizes of 4–5 kb *Eco*RI cleavage fragments (Everett and Willetts, 1980). We therefore modified our protocol so that the important fragments would be ~1 kb in size. For this, nicked EDλ102 DNA was briefly treated with exonuclease III, followed by digestion with *Bam*I + *Sal*I or *Bam*I + *Hind*III, and then with endonuclease S1. Other samples were not treated with exonuclease III plus endo-

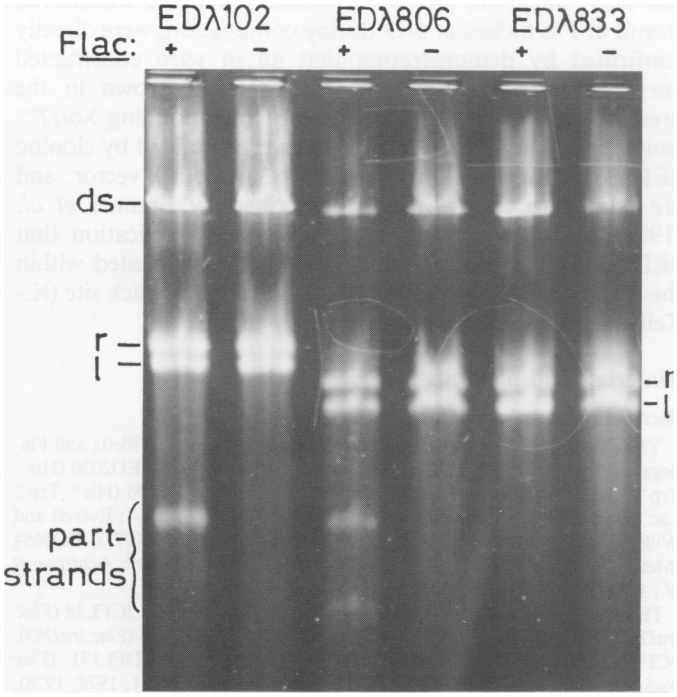

Fig. 5. Nicking of λ*oriT*$^+$ but not of λ*oriT*$^-$. EDλ806 (*oriT*$^+$) and EDλ833 (*oriT*$^-$) were grown on ED3823 or ED3823 (F*lac*), their DNA isolated, denatured, and electrophoresed in 0.7% agarose gels as described by Everett and Willetts (1980). The positions of double-strand (ds), 'right' (r) and 'left' (l) single-strands, and of part-strand bands are indicated. In the case of EDλ102, these had similar mobilities and were not separated.

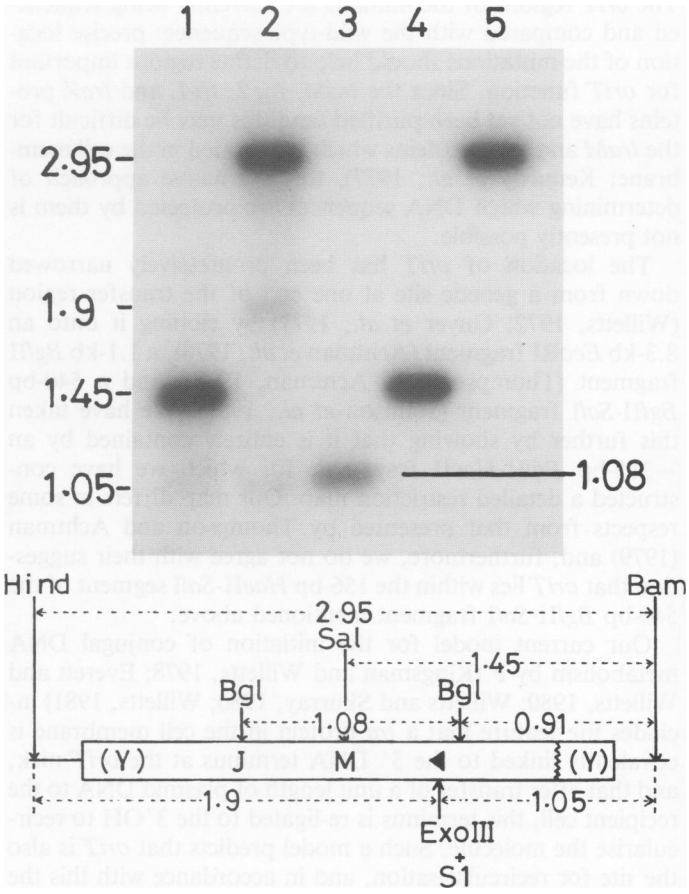

Fig. 6. Location of the λ*oriT* nick. Nicked EDλ102 DNA was digested with *Bam*HI and either *Sal I* (**tracks 1** and **4**) or *Hind*III (**tracks 2** and **5**), and in addition (**tracks 1** and **2**) with exonuclease III (briefly) and endonuclease S1. **Track 3** contains EDλ102 DNA digested with *Bgl*II. Bands were transferred from the agarose gel to a nitrocellulose filter and hybridised with ^{32}P-labelled pRS27 DNA. The relevant portion of the resultant autoradiogram is shown, and the sizes of the labelled bands are marked. The diagram below the photograph shows the provenance of the fragments giving rise to these labelled bands.

nuclease S1, which together cleave the DNA at the *oriT* nick. After separation on an agarose gel, the fragments were transferred to nitrocellulose paper (Southern, 1975) and hybridised with ^{32}P-labelled pRS27 DNA (which includes the F *oriT* region). The resultant radioautogram (Figure 6) showed that cleavage at *oriT* gave the expected extra DNA fragments, and that the *oriT* to *Bam*I distance in EDλ102 is 1.05 kb (by comparison with the 1.08-kb F *Bgl*II fragment). In other digestions (not shown) the *Bgl*II to *Bam*I fragment included in this 1.05-kb fragment was measured as 0.91 kb. The distance between the left-hand *Bgl*II site in F and *oriT* is therefore 140 bp, with a likely accuracy of ±20 bp. This location is shown in Figure 3.

Discussion

Our investigations of pED806, the pBR322 derivative carrying the F origin of transfer on an ~540-bp *Bgl*II-*Sal*I fragment (Johnson *et al.*, 1981) have confirmed that it is rapidly lost from cells that in addition carry an F*lac tra*$^+$ plasmid. Substitution of F*lac tra* mutants for F*lac tra*$^+$ showed that the *oriT* nicking function of *traZ* (and presumably of *traY*; Everett and Willetts, 1980) was essential for instability, and that the triggering or other functions of *traM* and *traI* contributed to its maximal expression. The second important factor was the orientation of the *oriT* fragment insertion relative to that of the *oriV* replication origin of the vector plasmid, since the opposite orientation was invariably obtained in cloning other *oriT* fragments, and these plasmids were relatively stable in the presence of F*lac tra*$^+$. Increased instability in a RecA$^-$ host compared to Rec$^+$ or RecA$^-$ RecB$^-$ suggested that nicking at *oriT* results in the conversion of the plasmid to a form that is susceptible to degradation by exonuclease V.

Attempts to visualize pED806 DNA by agarose gel electrophoresis of cleared lysates prepared from Rec$^+$ (F*lac tra*$^+$) cells grown in the presence of ampicillin simply showed a very small amount of superhelical closed-circular form. The orientations of the *oriV* and *oriT* sites on pED806 are such that lagging strand discontinuous DNA synthesis during vegetative replication uses the *oriT*-nicked strand as template. We have previously suggested that the *traYZ* endonuclease is able to nick and religate the *oriT* sequence to give an equilibrium between superhelical covalently closed and open circular plasmid forms, even in the absence of mating pair formation (Everett and Willetts, 1980). An *oriT* 'nicking complex' might interfere with lagging, but not leading, strand replication, or a nick at *oriT* might give a 3'OH terminus on the lagging strand and so result in its detachment from the replication fork and the subsequent degradation of the plasmid molecule.

Whatever the reason for the instability, we have been able to exploit this to isolate stable point mutants of pED806 that proved to have simultaneously become *oriT*$^-$. The correlation between instability and possession of a functional *oriT* site further confirms that these two properties are related.

The *oriT* regions of the mutants are currently being sequenced and compared with the wild-type sequence: precise location of the mutations should help to define regions important for *oriT* function. Since the *traM, traY, traI*, and *traZ* proteins have not yet been purified (and this may be difficult for the *traM* and *traY* proteins which are located in the cell membrane; Kennedy *et al.*, 1977), the alternative approach of determining which DNA sequences are protected by them is not presently possible.

The location of *oriT* has been progressively narrowed down from a genetic site at one end of the transfer region (Willetts, 1972; Guyer *et al.*, 1977) by cloning it onto an 8.3-kb *Eco*RI fragment (Achtman *et al.*, 1978), a 1.1-kb *Bgl*II fragment (Thompson and Achtman, 1978), and a 540-bp *Bgl*II-*Sal*I fragment (Johnson *et al.*, 1981). We have taken this further by showing that it is entirely contained by an ~385-bp *Bgl*II-*Hae*II fragment, for which we have constructed a detailed restriction map. Our map differs in some respects from that presented by Thompson and Achtman (1979) and, furthermore, we do not agree with their suggestion that *oriT* lies within the 156-bp *Hae*II-*Sal*I segment of the 540-bp *Bgl*II-*Sal*I fragment mentioned above.

Our current model for the initiation of conjugal DNA metabolism by F (Kingsman and Willetts, 1978; Everett and Willetts, 1980; Willetts and Skurray, 1980; Willetts, 1981) includes the feature that a *tra* protein in the cell membrane is covalently linked to the 5′ DNA terminus at the *oriT* nick, and that after transfer of a unit length of plasmid DNA to the recipient cell, this terminus is re-ligated to the 3′OH to recircularise the molecule. Such a model predicts that *oriT* is also the site for recircularisation, and in accordance with this the recircularisation site was indeed present on the 385-bp *Bgl*II-*Hae*II fragment of F, currently the smallest cloned region carrying *oriT*. In addition, the tetrameric form of pED822 in RecA$^-$ (F*lac*)$^+$ donor cells was recircularised in RecA$^-$ recipient cells mainly in the monomeric form. This suggests that either a unit length DNA strand was transferred after nicking in the donor cell at two contiguous *oriT* sites, or that the first incoming unnicked *oriT* site was recognised and nicked as it entered the recipient cell: in either case the leading 5′ terminus was then ligated to the 3′ terminus of the unit length linear molecule. These alternatives are similar to those proposed by Warren and Clark (1980) for recircularisation of ColE1 monomers after transfer. However, in contrast to the F *oriT* system, monomerisation of ColE1 dimers was not so efficient, and could be reversed to form dimers from monomers during transfer. Either model for recircularisation might explain the formation, at low frequency during conjugation by Hfr donor strains, of Δ*tra* F prime plasmids (Guyer *et al.*, 1977; Hadley and Deonier, 1980), by occasional recognition of a chromosomal DNA sequence resembling *oriT*. Finally, pED822 includes no *tra* genes, and its efficient recircularisation in recipient cells that had not simultaneously received F*lac tra*$^+$ is in accordance with the observation of Hiraga and Saitoh (1975) that F DNA was recircularised in rifampicin-sensitive recipient cells in the presence of rifampicin; these findings show that synthesis of *tra* protein in the recipient cell is not required for recircularisation.

More precise mapping of the nick in λ*oriT* phage genomes grown in (F*lac tra*$^+$) cells showed that this is located nearer (140 ± 20 bp) to the *Bgl*II site forming one terminus of the F fragment cloned in pED806 and pED822, than we had previously stated (Everett and Willets, 1980). The authenticity of this λ*oriT* nick site and the hypothesis that the transferred strand of F is nicked at *oriT* during conjugation, were directly confirmed by demonstrating that an *in vitro* constructed λ*oriT*$^+$ mutant genome was nicked when grown in the presence of F*lac tra*$^+$, whereas the corresponding λ*oriT*$^-$ genome was not. These two phages were obtained by cloning pED806 and its *oriT*$^-$ mutant pED833 into a λ vector, and are related to the 'phasmids' described by Brenner *et al.* (1982). It will be shown in a subsequent publication that pED833 does indeed carry a point mutation located within the 40-bp region that we find to include the *oriT* nick site (K.-Kelly, personal communication).

Materials and methods

Bacterial strains, plasmids, and phages

The *E. coli* K12 bacterial strains were ED24 (Lac$^-$ Spc^R; Willetts and Finnegan, 1970), ED92 (JC3272 *recA56 recB21*; Willetts, 1975), ED2030 (His$^-$ Trp$^-$ Lac$^-$ Spc^R RecA$^-$; Foster and Willetts, 1976), ED2196 (His$^-$ Trp$^-$ Lac$^-$ Spc^R Nal^R; Gasson and Willetts, 1977), ED3823 (EndoI$^-$; Everett and Willetts, 1980), ED8538 (Su$^-$ *lacZ* amber; Murray *et al.*, 1977), ED8654 (Met$^-$; Borck *et al.*, 1976), JC3272 (His$^-$ Trp$^-$ Lys$^-$ Lac$^-$ Str^R; Achtman *et al.*, 1971) and JC6310 (JC3272 *lys*$^+$ *recA56*; Willetts, 1975).

The F*lac* plasmids were JCFL0 (F*lac*) JCFL1 (F*lac traA1*), JCFL38 (F*lac traD38*), JCFL41 (F*lac traI41*) JCFL42 (F*lac traG42*), JCFL83 (F*lac traD83*), JCFL90 (F*lac traJ90*), JCFL102 (F*lac tra*M102) and EDFL171 (F*lac traΔIZ337*); these have been described by Achtman *et al.* (1971, 1972, 1978), McIntire and Willetts (1978) and Everett and Willetts (1980). pED100 (a conjugative mini-F) was described by Willetts and Johnson (1981). Chimeric plasmids were pED806 (pBR322 + *oriT Bgl*II-SalI fragment of F; Johnson *et al.*, 1981), pED810 (pBR322 + *oriT traMJYALEKBV Sal*I fragment of F; Johnson *et al.*, 1981), pED819 (pBR325 + *oriT traM Bgl*II fragment of F; our unpublished data), pED821 (pBR325 + *oriT traMJYALEKB Eco*RI fragment of F; Johnson *et al.*, 1981) and pRS27 (pSC101 + *oriT traMJYALEKBV Eco*RI fragment of F; Clark *et al.*, 1976); others constructed during the course of this work are described in the text.

The λ*tra* transducing phage was EDλ102 (λ*oriT traMJ*; Everett and Willetts, 1980; Johnson and Willetts, 1982). λNM781 is a c1857 *nin5 supE*$^-$ *Eco*RI replacement vector (Murray *et al.*, 1977).

Media, reagents, and enzymes

These were described by Everett and Willetts (1980), except for hydroxylamine (obtained from British Drug Houses Ltd) and other restriction endonucleases (obtained from New England Biolabs, Boehringer-Mannheim or Bethesda Research Laboratories, and used in the buffers recommended by the suppliers).

Plasmid and phage methodology

Plasmid and phage DNA preparation and manipulation and genetic techniques were as described by Everett and Willetts (1980) and Johnson *et al.* (1981). Small scale plasmid DNA preparations were made using the technique of Willetts *et al.* (1981), and then incubated for 10 min with 1% SDS at 65°C.

Hydroxylamine mutagenesis of plasmid DNA

The method used was that of Humphreys *et al.* (1976).

Construction of EDλ806 and EDλ833

0.5 μg of pED806 (or pED833) DNA and 2.5 μg λNM781 DNA were digested with *Eco*RI, mixed, and ligated overnight. The mixtures were used to transfect ED8654, and recombinant phages identified by their Lac$^-$ response on stabbing into a film of the amber *lacZ* mutant ED8538 on a lactose-McConkey indicator plate. One such phage from each mixture was shown to transduce a lambda-lysogenic *E. coli* K12 strain to Ap^R at a high frequency, and these phages were designated EDλ806 and EDλ833, respectively. Digestion of DNA prepared from these phages with *Eco*RI gave the three predicted fragments, and with *Sal*I showed that the orientations of the insertions were the same. Both phages were then grown on both ED3823 and its F*lac* derivative as described by Everett and Willetts (1980), and the phage DNAs prepared for single-strand analysis.

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