F⁻ phenocopies: characterization of expression of the F transfer region in stationary phase

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The phenomenon of 'F⁻ phenocopies' in which F⁺ cells become transfer-deficient in stationary phase seems contradictory to the proposed role for F transfer in adaptive mutation during stationary phase induced by nutrient limitation. The expression of a range of transfer genes at the transcriptional and translational level in stationary phase has been characterized as well as the degree of nicking at the origin of transfer, *oriT*. Transfer efficiency rapidly decreased in mid-exponential phase, coincident with a decrease in *traM* transcripts. Approximately 2 h later, the transcript for *traA*, encoding F-pilin, also decreased to undetectable levels. The levels of TraA (pilin), TraD, TraJ and TraT remained fairly constant well into stationary phase while the levels of TraM and TraI decreased to undetectable levels in early stationary phase. A null mutation in the gene for the alternative σ factor, *rpoS*, did not affect mating efficiency or transcript levels but did increase the stability of TraM and TraI in stationary phase. Nicking at *oriT* was detected at maximal levels in early stationary phase and at low levels in late stationary phase. The results suggest that the F-pilus transfer apparatus is maintained in the cell envelope after transcription of the transfer region from the main promoter, *P_y*, has ceased with down-regulation of *traM* transcription being the first step detected in this process. The presence of a low level of nicking at *oriT* in stationary phase is consistent with a role for F in promoting adaptive mutation.

Keywords: F plasmid, conjugation, stationary phase, pili, F⁻ phenocopies

INTRODUCTION

The F plasmid was first identified by Lederberg & Tatum (1946) as a point on the chromosome of *Escherichia coli* that mediated gene exchange between differentially marked bacteria. This led to the full characterization of the F sex factor, including the physiological parameters that affect its role in conjugation and F-specific phage infection. With time, the F-pilus was identified as an essential feature for both these processes. Early research depended on mating efficiency assays, enumeration of the number of F-pili per cell by electron microscopic observation and phage binding assays. In general, it was concluded (reviewed by Tomoeda *et al.*, 1975) that F-pilus synthesis increased during exponential growth and was maximal in early stationary phase. F-piliation and mating efficiency decreased to undetectable levels in late stationary phase such that donor cells were able to act as recipients in

F⁺ × F⁺ crosses, a phenomenon termed 'F⁻ phenocopies' (Jacob & Wollman, 1961; Hayes, 1964). A procedure for generating F⁻ phenocopies is given in Miller (1972). This loss of piliation and transfer ability was found in well aerated cultures grown on rich medium. Anaerobic cultures grown on rich medium produced F-pili for extended time periods (several days). These pili were longer than usual and the mating efficiency was equal to or greater than aerobically grown cells (Curtiss *et al.*, 1969; Stallions & Curtiss, 1972). Biebricher & Düker (1984) also reported that synthetic media gave poorer levels of piliation in both aerobic and anaerobic cultures and that the addition of metabolic poisons affected aerobically grown cultures only.

Curtiss & Stallions (1967) reported that mating pair formation did not require energy and Novotny & Fives-Taylor (1974) reported that F-pili retract or withdraw into the host cell, a process that can be triggered by shifting the temperature (Novotny & Lavin, 1971) or by the addition of metabolic poisons such as arsenate or cyanide (Novotny & Fives-Taylor, 1974; O'Callaghan

Abbreviation: IHF, integration host factor.

et al., 1973). They suggested that F-pilus assembly required energy and that inhibitors that block energy production result in F-pilus retraction by default. Yamamoto *et al.* (1980, 1981) reported that filamentous phage absorption was not energy-dependent but that F-pilus formation required the proton motive force and ATP. Recently, Majdalani & Ippen-Ihler (1996) have shown that F-propilin insertion into the inner membrane requires TraQ, a putative chaperone, and the proton motive force.

The F plasmid is naturally derepressed for pilus synthesis and DNA transfer because of an IS3 insertion sequence within its *finO* gene which expresses the fertility inhibition protein, FinO (Cheah & Skurray, 1986). FinP antisense RNA, which requires FinO for activity, prevents translation of *traJ* mRNA (van Biesen & Frost, 1994). TraJ is the positive regulator of transcription from the Py promoter giving a long (33 kb) transcript encoding all of the transfer proteins except TraM. The first gene in this operon is *traY*, the product of which is important in formation of the nucleoprotein complex (relaxosome) at *oriT* (origin of transfer) and regulation of transcription. TraY and IHF (integration host factor) attract TraI to the *nic* site in *oriT* (Howard *et al.*, 1995) where TraI cleaves at *nic* in a strand- and sequence-specific manner. TraY also positively regulates the Py promoter (Maneewannakul *et al.*, 1996; Silverman & Sholl, 1996) as well as *traM* promoters, Pm1 and Pm2 (Penfold *et al.*, 1996). TraM is an essential transfer protein which is important in relaxosome formation and it appears to connect the relaxosome to the transfer apparatus via its interaction with TraD, an inner membrane protein involved in DNA transport (Disque-Kochem & Dreiseiklmann, 1997).

Several researchers in the field of adaptive mutation have suggested that the increase in mutations seen in stationary phase can be attributed to the F plasmid present in the test system for adaptive mutation. Typically, an F plasmid carrying the *lacI::lacZ33* fusion is used to detect the recombination-dependent mutations associated with this phenomenon (reviewed by Shapiro, 1995). Some researchers have suggested that F transfer itself was responsible for these mutations (Peters & Benson, 1995; Galitski & Roth, 1995; Radicella *et al.*, 1995) while others have suggested that the presence of a nicked species at *oriT*, which is nicked by the TraI relaxase, accelerated the adaptive mutation process by supplying a site for limited replication (Foster & Trimarchi, 1995). It has since been shown that F might indeed accelerate this process but is not solely responsible for it since similar types of mutations can be found on non-transmissible plasmids as well as throughout the chromosome (Torkelson *et al.*, 1997).

The debate on the role of F transfer in adaptive mutation in starved or stationary-phase cells seemed to be in direct opposition to the notion of F⁻ phenocopies. Using modern methodology, we wanted to determine whether the F transfer apparatus is completely dismantled during stationary phase, whether transcription of two primary

promoters, Py and Pm, in the transfer region are active and whether nicking at *oriT* could be detected.

METHODS

Bacterial strains, plasmids and general methods. pOX38-Km, which contains the *tra* region and RepFIA, represents the largest HindIII fragment of the F plasmid ligated together with a kanamycin cassette (Chandler & Galas, 1983). *Escherichia coli* MC4100 [F⁻*araD139* Δ (*argF-lac*)U169 *deoC1 flbB3501 relA1 rpsL150 ptsF25 rbsR*] was described by Casadaban (1976). RH90 is MC4100 *rpoS359::Tn10* (Lange & Hengge-Aronis, 1991); PD32 is MC4100 *hns206::Amp^R* (Dersch *et al.*, 1993) and SG22094 is MC4100 Δ *lonA clpP::CAT rcs::Kan^R* (Gottesman, 1990). *E. coli* ED24 is F⁻ *Lac⁻ Spc^R* and MC253 is F⁻ *ara* Δ (*lac-pro*) *gyrA metB argE rif thi supF* Δ 82(*himA*):Tn10 Δ 3(*hip*):Cam (Penfold *et al.*, 1996). All strains were grown in 50 ml Luria-Bertani broth from a 1:100 overnight culture in 500 ml baffled growth flasks at 37 °C in a shaking water bath to provide aeration. OD₆₀₀ was measured at the times indicated and cell samples equivalent to 0.4 OD₆₀₀ units (1.6 × 10⁸ cells ml⁻¹) were harvested at each time point for each sample for immunoblot analysis of proteins. Samples (3 ml) were removed for both RNA and DNA isolation. The presence of pOX38-Km in the various strains was occasionally confirmed by checking for phage sensitivity using a spot test (Anthony *et al.*, 1996).

Mating assay. At the time points indicated, 0.1 ml samples of cells were added to 0.1 ml ED24 recipient cells that had been pelleted and thoroughly drained of medium. ED24 cells were diluted at each time point and regrown until the next time point such that there were approximately 1 × 10⁹ cells ml⁻¹. Mating was performed in a 37 °C constant temperature block for 30 min. The cells were vortexed and immediately diluted via serial 10-fold dilutions and plated out as 10 µl drops on plates containing selective media. MC4100 and its derivatives were detected using streptomycin (200 µg ml⁻¹), ED24 was detected using spectinomycin (100 µg ml⁻¹) and pOX38-Km was detected using kanamycin (25 µg ml⁻¹). The results are expressed as mating efficiency (%) with a maximum mating efficiency of 73 transconjugants per 100 donor cells being set at 100%.

Immunoblot analysis. Immunoblot analysis was performed as described by Penfold *et al.* (1996). Cells from the same set of time samples were used for each immunoblot. TraD and TraI were resolved by 10% SDS-PAGE while TraA, TraJ, TraM and TraT were detected by immunoblots of proteins separated by 15% SDS-PAGE. Proteins were sized using BRL pre-stained protein standards (low molecular mass range) or Bio-Rad Kaleidoscope protein standards (high molecular mass range). All primary antibodies were diluted 1:5000 while secondary antibodies were diluted 1:1000 (horseradish-peroxidase-conjugated whole antibody from donkey; Amersham). Polyclonal anti-TraD, TraI, TraT and TraJ antisera were obtained from Karin Ippen-Ihler (Texas A & M University). All antibodies were checked for background reactivity with a negative control (MC4100 or RH90).

Northern blot analysis. RNA was isolated from 3 ml of cells using the modified hot-phenol method described by Frost *et al.* (1989). Total RNA was treated with DNase I at room temperature for 1 h, phenol-extracted and ethanol-precipitated. RNA was quantified spectrophotometrically and 30 µg was run in loading buffer on a denatured 8% polyacrylamide gel as described previously (Penfold *et al.*, 1996). The RNA

was stained with ethidium bromide and transferred to a Zetaprobe nylon membrane (Bio-Rad) and probed with ³²P-labelled oligonucleotide probes as described by Penfold *et al.* (1996). *traM* mRNA was detected using SPE8 (5' CATA-GGCATCATTGCTGATATACAG) and *traA* transcripts were detected using A2430 (5' GGAGTCCTTACCGAAG-GTCGC). The blots were stripped by placing them in boiling 0.1% SDS which was allowed to cool to room temperature. They were reprobed with primer JSA12 (5' CCGGTAG-AGTTGCCCTACTCCGGTTTTAG; He *et al.*, 1993), specific for tRNA^{ser}, to ensure even loading. RNA standard III was purchased from Boehringer Mannheim.

Nicking assay. pOX38-Km was isolated from 3–4 ml of cells, depending on the OD₆₀₀ taken at various time points, by the rapid alkali procedure of Birnboim & Doly (1979) without phenol extraction. The DNA was dissolved in 300 µl 0.5% SDS in 10 mM Tris/HCl, 2.5 mM EDTA, pH 8.0, and heated to 65 °C for 10 min. It was then treated with 50 µg Proteinase K ml⁻¹ (Boehringer Mannheim) for 45 min at 37 °C, phenol-extracted and ethanol-precipitated. The DNA was redissolved in 30 µl water and 15 µl were digested with *DraI* in the appropriate buffer (Boehringer Mannheim) for 1 h at 37 °C. For alkali denaturation, 2 µl 2 M NaOH in 2 mM EDTA was added for 5 min at room temperature and neutralized by the addition of 8 µl 1 M Tris/HCl, pH 7.4, 3 µl 3 M sodium acetate and 75 µl 95% ethanol to precipitate the DNA. The level of nicking was assayed by performing a unidirectional PCR with 10 pmol ³²P-end-labelled oligonucleotide SPE19 (5' CATCAGGCAGATGGCTAACATCC; nt 17–39; Frost *et al.*, 1994) using Vent polymerase and buffers (New England Biolabs) as described by the manufacturer. The reaction was performed with 35 cycles of 40 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C. To completely extend all products, 5 µl 2.5 mM dNTPs was added followed by four additional cycles of melting at 94 °C and elongation at 72 °C. The products of the PCR along with a sequencing reaction of *oriT*-containing DNA (pNY300; Frost *et al.*, 1989) using SPE19 were run on a 6% denaturing gel which was dried and exposed to both a phosphorimager for band quantification and film for autoradiography.

RESULTS

Illustration of the phenomenon of F⁻ phenocopies

The phenomenon of F⁻ phenocopies can be demonstrated by the loss of F transfer ability in stationary phase as illustrated in Fig. 1. *E. coli* MC4100 or RH90, containing the F derivative pOX38-Km, were grown with shaking at 225 r.p.m. at 37 °C in LB broth for 24 h. Samples were taken for OD₆₀₀ measurement and mating efficiency assays were performed at the time points indicated. The number of bacteria were also determined using a plate count assay. The mating efficiency assay was altered to avoid the introduction of fresh medium into the assay. Medium was removed from recipient cells (ED24) by centrifugation and the cells were resuspended in equivalent volumes of donor culture. The results for MC4100 and RH90 containing pOX38-Km were superimposable and reveal that the mating efficiency dropped dramatically during mid-exponential growth (4–5 h, OD₆₀₀ ~ 2.0) and was undetectable by early stationary phase. A 1:50 dilution of the culture into fresh medium at 24 h completely restored mating ability within 1 h. Addition of 1% glucose to the spent

Fig. 1. Mating efficiency and bacterial growth. MC4100 or RH90 cells containing pOX38-Km were grown as described in Methods. At the times indicated, samples for OD₆₀₀ measurement and mating efficiency assays were taken. ○, OD₆₀₀; ●, mating efficiency. The graphs for the two strains were superimposable.

medium also restored mating ability within 1 h. However, the addition of 1% glycerol had an intermediate effect (0.5% of the value for glucose). Thus, there appeared to be no inhibitors of conjugation in the spent medium but rather transfer ability was tied to the nutritional and energy status of the cell as previously determined.

Levels of transfer proteins in cells during the growth cycle

TraM and the other transfer proteins for which anti-serum was available were easily detected by immunoblot in cell samples equivalent to 0.4 OD₆₀₀ units with little to no background signal. The single copy F derivative, pOX38-Km, was used in all assays since the use of multicopy clones has been found to distort the results. The amount of TraM in MC4100(pOX38-Km) cells was assayed over the growth cycle and was shown to decrease dramatically in stationary phase. Repeating the experiment using RH90 cells as the host strain demonstrated that the presence of TraM was prolonged into late stationary phase (Fig. 2). This dramatic result initiated a more thorough analysis of expression of the transfer region in stationary phase.

The amounts of other transfer proteins were also assayed throughout the growth cycle (Figs 2 and 3). Cell samples equivalent to 0.4 OD₆₀₀ units were taken at the times indicated and immunoblots were performed using antisera against TraJ (27 kDa; positive regulator of the Py promoter), TraA (7.2 kDa; pilin), TraT (25 kDa; surface exclusion), TraD (81.7 kDa; DNA transport) and TraI (192 kDa; relaxase/helicase). Unfortunately, anti-TraY antiserum was unable to detect TraY expressed from the single copy pOX38-Km plasmid and cross-reacted with a protein of nearly identical size from the hosts MC4100 and RH90. Immunoblots for TraY using antisera pre-adsorbed against host proteins gave poor results.

Fig. 2. Immunoblots of TraI and TraM over the growth cycle. Samples equivalent to 0.4 OD₆₀₀ units were taken at the times indicated over each lane (h) for cultures of pOX38-Km in MC4100 (a) or RH90 (b). The position of the closest protein standard is shown on the right; the position of TraI and TraM is shown on the left.

Fig. 3. Immunoblots of TraD, TraT, TraJ and TraA (pilin) over the growth cycle. Samples were taken as described in the legend to Fig. 2 but only samples from MC4100(pOX38-Km) cells are shown with the exception of TraD which was from RH90(pOX38-Km) cells. The position of the closest protein standard is shown on the right; the position of the transfer proteins is shown on the left.

In nearly all cases the amount of each transfer protein was fairly constant throughout the growth cycle (Fig. 3) with the notable exceptions of TraM and TraI (Fig. 2). While TraM decreased to undetectable levels in early stationary phase (6 h), TraI was detectable until late

Fig. 4. Northern analysis of RNA isolated from MC4100(pOX38-Km) cells over the growth cycle and probed for (a) *traM* and (b) *traA* transcripts as described in Methods. The time of sampling (h) is given above each lane with the positions of the wells and the transcripts shown on the left and the positions of RNA markers (III, Boehringer Mannheim) on the right. (c) The amount of control tRNA^{Ser} is shown for the *traA* blot.

stationary phase (12 h). If pOX38-Km was introduced into RH90, the levels of TraJ, TraA, TraT and TraD remained the same as in MC4100 (data not shown). TraM remained detectable until 12 h while TraI was detectable throughout the growth cycle (Fig. 2b). The disappearance of TraM and TraI in MC4100(pOX38-Km) did not coincide with the loss in mating efficiency but occurred 1–2 and 8 h later, respectively.

Fig. 5. Nicking assay for pOX38-Km in MC4100 or RH90 cells over the growth cycle. The time of sampling is given (h) above each lane. The position of products I (*DraI* site), II (putative bend region) and III (*nic* site) are indicated on the left. The nature of the prominent band near the *DraI* site after 6 h for MC4100(pOX38-Km) is unknown.

Transcriptional analyses of the F transfer region

Northern analyses of *traM* and *traA* transcripts were performed to determine whether transcription from the P_m and P_y promoters was affected by stationary phase (Fig. 4a, b). The expected size for *traM* transcripts is 500 and 550 bases (Penfold *et al.*, 1996) while the size of the stable transcript for pilin (*traA*) that results from processing of the very long *tra* transcript (33 kb) is a cluster of species ranging from 0.5 to 1.0 kb. These stable transcripts result from endonucleolytic cleavage events in the region upstream of *traA* as well as the region of secondary structure downstream of *traA* in the mRNA which increases its overall stability (Koraimann & Högenauer, 1989). Transcripts for *traA* were detected at positions corresponding to 550 and 600 bases using an oligonucleotide probe derived from the sequence of *traA* (Fig. 4b). These transcripts were present in MC4100-(pOX38-Km) for 5 h (2.3 OD₆₀₀ units) at which time they decreased to undetectable levels. *traM* transcripts were present as two bands of 500 and 550 bases which decreased sharply in mid-exponential phase (data not shown). A repeat of the experiment using samples taken every 20 min showed that *traM* transcripts decreased to undetectable levels between 3.3 and 3.6 h (Fig. 4a) with a concomitant increase in non-specific binding of the probe to material in the wells. This was equivalent to the point in the growth cycle where transfer efficiency began to decrease sharply (Fig. 1). Results using RH90(pOX38-Km) were identical, suggesting that RpoS did not affect transcription of the transfer region (data not shown).

The level of nicking in stationary-phase F⁺ cells

A nicking assay for wild-type or *rpoS* cells containing pOX38-Km was devised based on the procedure of Perwez & Meyer (1996). Since TraI persists in the *rpoS*

host, nicking should also be detectable at later time points in RH90(pOX38-Km) compared to the wild-type MC4100(pOX38-Km). Cell samples were taken at various time points over a 24 h period and the DNA was isolated and treated with 0.5 % SDS and Proteinase K to remove TraI, which can bind covalently to *oriT* (Matson & Morton, 1991). The DNA was digested with *DraI* which cuts the plasmid 91 bp downstream from the *nic* site in *oriT*. A unidirectional PCR was performed to amplify the signal from either the *nic* site or the *DraI* site in the absence of cleavage at *oriT*. A 5' ³²P-labelled primer corresponding to a sequence upstream and complementary to the cleaved strand of DNA was used in a standard PCR (35 cycles). The products were ethanol-precipitated and run on a denaturing sequencing gel with a sequence of the *oriT* region generated by the SPE19 primer run next to the reactions (Fig. 5).

The PCR was expected to give two products, one of 124 bases resulting from termination at *nic* (III; Fig. 5) and another of 215 bases resulting from extension to the *DraI* site in the absence of cleavage at *nic* (I; Fig. 5). A third band, which corresponded to a product of 155 bases (II; Fig. 5), was routinely seen within a region defined as an intrinsic bend (Tsai *et al.*, 1990). This could result from breakage of the DNA during preparation of the sample. Interestingly, the amount of product II reflected the amount of product III (*nic*) at all time points, suggesting that breakage was due to the presence of the relaxosome complex bound to *oriT*. The percentage values of products I–III were determined by dividing the signal for each product by the sum of the signals for products I–III in each sample using a phosphorimager to quantify the intensity of the bands (Table 1). A nicked species could be detected at all time points during the growth cycle although it represented less than 2 % of the total signal at 24 h for both wild-

Table 1. Quantification of each product present over the growth cycle in the nicking assay for pOX38-Km in MC4100 or RH90

The intensity of each of the products at positions I, II and III (as defined in the legend to Fig. 5) after the times indicated (h) was calculated as a percentage of the total signal for all the products. A background value taken from a blank region of image was subtracted from the values given.

Position	Intensity of product (%)							
	3	4	6	8	10	12	18	24
MC4100(pOX38-Km)								
I	86.5	79.9	65.2	69.6	53.7	82.4	87.7	96.7
II	8.3	13.0	32.4	22.7	29.2	10.7	6.6	1.4
III	5.3	7.1	2.4	7.8	17.2	6.9	5.8	1.8
RH90(pOX38-Km)								
I	56.0	21.1	34	24.7	26.1	91.6	96.8	95.4
II	23.0	32.3	34.5	38.4	39.6	4.4	2.3	2.8
III	21.0	46.6	31.5	36.9	34.3	4.0	0.9	1.8

type and *rpoS* mutant cells. The amount of products II and III reached a maximum at 10 h and then quickly decreased at subsequent time points. In general the amounts of products II and III were greater for RH90(pOX38-Km) than for MC4100(pOX38-Km) which agreed with the larger amounts of TraI seen at every time point and for a longer time period in RH90(pOX38-Km). However, this effect could not be related to an increased mating efficiency.

Other factors affecting F transfer region expression

To determine whether the cellular proteases LonA or ClpP were involved in degradation, a strain containing mutations in *lonA* and *clpP* (SG22094) was tested for its effect on TraM and TraI levels *in vivo*. pOX38-Km was introduced into SG22094 as well as MC4100 and RH90, and an immunoblot of cell samples taken over a 24 h time period was performed. Since there was no change in the level of TraM compared to MC4100(pOX38-Km), neither LonA nor ClpP appeared to be involved in TraM degradation. Other host proteins which might affect TraM expression are IHF, which binds to two sites between *oriT* and the Pm promoters (Tsai *et al.*, 1990), or H-NS, which has a role in gene regulation in stationary phase (Dersch *et al.*, 1993; Hengge-Aronis, 1996). However, neither IHF-deficient (MC253) nor H-NS-deficient (PD32) strains were found to change the pattern of TraM expression as determined by immunoblotting (data not shown).

DISCUSSION

Our results suggest that F⁺ cells repress pilus assembly and transfer proficiency at the transcriptional level during early to mid-exponential phase. A decrease in mating efficiency coincided with a decrease in *traM* transcription which was followed by a steady decrease

in TraM to undetectable levels in late exponential phase. Using the level of stable *traA* transcripts as a measure of transcription of the entire *tra* operon (33 kb), transcription from the Py promoter decreased to undetectable levels in late exponential phase. Immunoblot analysis with antisera raised against a selection of transfer proteins showed that the pilin protein (TraA), TraD, TraJ and TraT remained present indefinitely in the cell during stationary phase while TraI, along with TraM, decreased as the cells entered stationary phase. This degradation was affected by a mutation in *rpoS*, encoding the alternative σ factor, but the mechanism of degradation is not known. RpoS is probably not important in F transfer since a null mutation in *rpoS* had no effect on mating efficiency or transcription throughout the growth cycle. However, the *rpoS* mutation did affect the amount of *nic*-cleaved DNA, perhaps by increasing the amount of TraI in these cells.

TraM is an essential transfer protein which is thought to form a nucleosome-like structure that might facilitate strand opening in preparation for unwinding during DNA transfer (Penfold *et al.*, 1996; Kupelweiser *et al.*, 1998). It also controls *tra* operon expression at the transcriptional level by competing with FinP antisense RNA for *traJ* mRNA in the R1 (Polzleitner *et al.*, 1997) and R100 (Dempsey, 1994) transfer systems. In R1, *traM* mutations lead to low levels of piliation and phage resistance. In F, the fertility inhibition system of FinP antisense RNA in combination with FinO has been lost and thus the positive control exerted by *traM* transcription on *traJ* is superfluous. A null mutation in F *traM* reduces mating efficiency to undetectable levels; however, it does not affect the levels of F-pili or the sensitivity to F-specific phages (Penfold *et al.*, 1996). Thus, F might rely on *traM* transcription and TraM levels to control transfer efficiency by affecting relaxosome formation rather than regulation of the transfer region. It will be interesting to see whether the sudden

decrease in *traM* mRNA during the growth cycle is a feature of these related plasmids which have not lost fertility inhibition while in the wild.

Two promoters for F *traM* have been identified which result in transcripts of 500 and 550 bases, both terminating immediately downstream of *traM*. These promoters are negatively regulated by TraM itself and are positively regulated by TraY which is expressed by the first gene in the long (33 kb) transfer operon (Penfold *et al.*, 1996; Maneewannakul *et al.*, 1996). *traM* transcription decreases rapidly over a 20 min period in early exponential phase. The Pm1 promoter is repressed completely at this time while the Pm2 promoter is transcribed at low levels indefinitely, presumably to express sufficient TraM to effect repression of *traM* promoters. Expression from Pm2 was detectable only after prolonged exposure of the Northern blot (1 month; data not shown). The decrease in *traM* transcription was followed by a decrease in TraM and in mating efficiency which both reach undetectable levels in early stationary phase. Early stationary phase also marked the period when the stable transcript for *traA* disappeared, suggesting repression of the Py promoter. However, this repression was not due to a decrease in the level of the positive regulator, TraJ, which is fairly constant, albeit at diminished levels, throughout stationary phase.

Since *traM* transcription is stimulated by TraY, the most likely explanation for the decrease in *traM* mRNA is a decrease in TraY levels followed by repression of *traM* promoters by TraM itself. The Northern blot for *traA* mRNA, used as a reporter for *tra* operon expression, suggested that the level of Py transcripts decreased after *traM* mRNA levels had already fallen. One explanation could be that TraY is subject to post-translational controls such as selective degradation or modification which inactivates it or there is another level of control of *traM* transcription not yet identified. A number of host factors are known to affect F conjugation, including SfrA, SfrB (RfaH) and the Cpx proteins (for a review, see Firth *et al.*, 1996). Perhaps these systems act either directly or indirectly to affect TraY and TraM expression.

The amounts of TraD and TraT also remained fairly constant throughout the experiment. These proteins are expressed from genes distal to the Py promoter and have been shown to have their own promoters (Ham *et al.*, 1989a, b) which might be active during stationary phase. TraD is thought to be the protein that links pilus formation to DNA transport since it has been shown to interact with TraM which would target the relaxosome complex at *oriT* to the transport machinery (Disque-Kochem & Dreiseiklmann, 1997). Thus, a skeleton structure of TraD and other unidentified transfer proteins that form the secretion apparatus could be maintained in the cell envelope rather than being completely dismantled during stationary phase. When an appropriate carbon source such as glucose is introduced or the cells are diluted into fresh medium, transcription resumes with replenishment of pilin and

relaxosome proteins (TraM and TraI) and restoration of mating ability in a few minutes. We are currently investigating the mechanism of activation of the Pm promoters by TraY and perhaps other factors and the nature of the sudden repression of *traM* transcription in early exponential phase.

The cleavage of plasmid pOX38-Km at *nic* within *oriT* was very intense in RH90(pOX38-Km) cells which correlated fairly well with the amount of TraI in the cell. TraI is responsible for relaxase activity at *nic* in *oriT* as well as helicase activity during transfer. The decrease of TraI to very low levels during stationary phase is consistent with a small amount of TraI being available for cleavage at *nic* while the large amount of TraI needed for helicase activity would not be needed if pilus assembly and DNA transfer were not possible. This low level of *nic*-specific cleavage might contribute to the propensity of F⁺ cells to undergo adaptive mutation at an increased frequency compared to F⁻ cells.

Our results support the notion that it is the presence of F and not F plasmid transfer that contributes to adaptive mutation. Typically, the assay for adaptive mutation involves growing cells on plates containing selective minimal medium in the presence of a large number of scavenger cells (Lac⁻) which deplete all carbon sources except for lactose. Over the course of 3 or more days, -1 frameshift mutations occur within the *lacI::lacZ* fusion allowing the adaptive mutants to grow on lactose (Rosenberg *et al.*, 1994; Foster & Trimarchi, 1995). The recombination-dependent, error-prone replication that generates these mutations is thought to be accelerated by the presence of *nic* on F (Torkelson *et al.*, 1997). In this series of experiments, small amounts of nicked plasmid were present when transfer was undetectable, therefore some cleavage at *nic* is probably always present even in cells grown aerobically on rich medium that encourages the loss of mating efficiency and the appearance of F⁻ phenocopies.

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REFERENCES

- Anthony, K. G., Kathir, P., Moore, D., Ippen-Ihler, K. & Frost, L. S. (1996). Analysis of the *traLEKBP* sequence and the TraP protein from three F-like plasmids: F, R100-1, ColB2. *J Bacteriol* **178**, 3194-3200.
- Biebricher, C. K. & Düker, E.-M. (1984). F and type I piliation of *Escherichia coli*. *J Gen Microbiol* **130**, 951-957.
- van Biesen, T. & Frost, L. S. (1994). The FinO protein of IncF plasmids binds FinP antisense RNA and its target, *traJ* mRNA and promotes duplex formation. *Mol Microbiol* **14**, 427-436.
- Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7**, 1513-1523.
- Casadaban, M. (1976). Transposition and fusions of the *lac* genes

- to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *J Mol Biol* 104, 541–545.
- Chandler, M. & Galas, D. (1983).** Cointegrate formation mediated by Tn9. II. Activity of IS1 is modulated by external sequences. *J Mol Biol* 170, 61–91.
- Cheah, K. C. & Skurray, R. A. (1986).** The F plasmid carries an IS3 insertion within *finO*. *J Gen Microbiol* 132, 3269–3275.
- Curtiss, R., III (1969).** Bacterial conjugation. *Annu Rev Microbiol* 23, 69–136.
- Curtiss, R., III & Stallions, D. R. (1967).** Energy requirements for specific pair formation during conjugation in *Escherichia coli* K-12. *J Bacteriol* 94, 490–492.
- Dempsey, W. B. (1994).** Regulation of R100 conjugation requires *traM* in *cis* to *traJ*. *Mol Microbiol* 13, 987–1000.
- Dersch, P., Schmidt, K. & Bremer, E. (1993).** Synthesis of the *Escherichia coli* K-12 nucleoid-associated DNA-binding protein H-NS is subjected to growth-phase control and autoregulation. *Mol Microbiol* 8, 875–889.
- Disque-Kochem, C. & Dreiseikelmann, B. (1997).** The cytoplasmic DNA-binding protein TraM binds to the inner membrane protein TraD *in vitro*. *J Bacteriol* 179, 6133–6137.
- Firth, N., Ippen-Ihler, K. & Skurray, R. A. (1996).** Structure and function of the F factor and mechanism of conjugation. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 2377–2401. Edited by F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. Brooks Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter & H. E. Umbarger. Washington, DC: American Society for Microbiology.
- Foster, P. L. & Trimarchi, J. M. (1995).** Adaptive reversion of an episomal frameshift mutation in *Escherichia coli* requires conjugal functions but not actual conjugation. *Proc Natl Acad Sci USA* 92, 5487–5490.
- Frost, L. S., Lee, S., Yanchar, N. & Parachych, W. (1989).** *finP* and *fisO* mutations in FinP anti-sense RNA suggest a model for FinOP action in the repression of bacterial conjugation by the Flac plasmid JCFL0. *Mol Gen Genet* 218, 152–160.
- Frost, L. S., Ippen-Ihler, K. & Skurray, R. A. (1994).** Analysis of the sequence and gene products of the transfer region of the F sex factor. *Microbiol Rev* 58, 162–210.
- Galitski, T. & Roth, J. R. (1995).** Evidence that F plasmid transfer replication underlies apparent adaptive mutation. *Science* 268, 421–423.
- Gottesman, S. (1990).** Minimizing proteolysis in *Escherichia coli*: genetic solutions. *Methods Enzymol* 185, 119–129.
- Ham, L. M., Cram, D. & Skurray, R. A. (1989a).** Transcriptional analysis of the F plasmid surface exclusion region: mapping of *traS*, *traT* and *traD* transcripts. *Plasmid* 21, 1–8.
- Ham, L. M., Firth, N. & Skurray, R. A. (1989b).** Nucleotide sequence of the F plasmid transfer gene, *traH*: identification of a new gene and a promoter within the transfer operon. *Gene* 75, 157–165.
- Hayes, W. (1964).** *The Genetics of Bacteria and Their Viruses*. New York: Wiley.
- He, L., Soderbom, F., Wagner, E. G., Binnie, U., Binns, N. & Masters, M. (1993).** PcnB is required for the rapid degradation of RNAI, the antisense RNA that controls the copy number of ColE1-related plasmids. *Mol Microbiol* 9, 1131–1142.
- Hengge-Aronis, R. (1996).** Back to log phase: σ^s as a global regulator in the osmotic control of gene expression in *Escherichia coli*. *Mol Microbiol* 21, 887–893.
- Howard, M. T., Nelson, W. C. & Matson, S. W. (1995).** Stepwise assembly of a relaxosome at the F plasmid origin of transfer. *J Biol Chem* 270, 28381–28386.
- Jacob, F. & Wollman, E. L. (1961).** *Sexuality and Genetics of Bacteria*. New York: Academic Press.
- Koraimann, G. & Högenauer, G. (1989).** A stable core region of the *tra* operon mRNA of plasmid R1–19. *Nucleic Acids Res* 17, 1283–1298.
- Kupelweiser, G., Schwab, M., Högenauer, G., Koraimann, G. & Zechner, E. L. (1998).** Transfer protein TraM stimulates TraI-catalyzed cleavage of the transfer origin of plasmid R1 *in vivo*. *J Mol Biol* 275, 81–94.
- Lange, R. & Hengge-Aronis, R. (1991).** Growth phase-regulated expression of *bolA* and the morphology of stationary-phase *Escherichia coli* cells are controlled by the novel sigma factor σ^s . *J Bacteriol* 173, 4474–4481.
- Lederberg, J. & Tatum, E. L. (1946).** Gene recombination in *Escherichia coli*. *Nature* 158, 558.
- Majdalani, N. & Ippen-Ihler, K. (1996).** Membrane insertion of the F-pilin subunit is Sec independent but requires leader peptidase B and the proton motive force. *J Bacteriol* 178, 3742–3747.
- Maneewannakul, K., Kathir, P., Endley, S., Moore, D., Manchak, J., Frost, L. & Ippen-Ihler, K. (1996).** Construction of derivatives of the F plasmid pOX-*tra715*: characterization of *traY* and *traD* mutants that can be complemented *in trans*. *Mol Microbiol* 22, 197–205.
- Matson, S. W. & Morton, B. S. (1991).** *Escherichia coli* DNA helicase I catalyzes a site- and strand-specific nicking reaction at the F plasmid *oriT*. *J Biol Chem* 266, 16232–16237.
- Miller, J. H. (1972).** *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Novotny, C. P. & Fives-Taylor, P. (1974).** Retraction of F pili. *J Bacteriol* 117, 1306–1311.
- Novotny, C. P. & Lavin, K. (1971).** Some effects of temperature on the growth of F pili. *J Bacteriol* 107, 671–682.
- O'Callaghan, R. J., Bundy, L., Bradley, R. & Paranchych, W. (1973).** Unusual arsenate poisoning of the F pili of *Escherichia coli*. *J Bacteriol* 115, 76–81.
- Penfold, S. S., Simon, J. & Frost, L. S. (1996).** Regulation of the expression of the *traM* gene of the F sex factor of *Escherichia coli*. *Mol Microbiol* 20, 549–558.
- Perwez, T. & Meyer, R. (1996).** MobB protein stimulates nicking at the R1162 origin of transfer by increasing the proportion of complexed plasmid DNA. *J Bacteriol* 178, 5762–5767.
- Peters, J. E. & Benson, S. A. (1995).** Redundant transfer of F' plasmids occurs between *Escherichia coli* cells during nonlethal selections. *J Bacteriol* 177, 847–850.
- Polzleitner, E., Zechner, E., Renner, W., Fratte, R., Jauk, B., Högenauer, G. & Koraimann, G. (1997).** TraM of plasmid R1 controls transfer gene expression as an integrated control element in a complex regulatory network. *Mol Microbiol* 25, 495–507.
- Radicella, J. P., Park, P. U. & Fox, M. S. (1995).** Adaptive mutation in *Escherichia coli*: a role for conjugation. *Science* 268, 418–420.
- Rosenberg, S. M., Longerich, S., Gee, P. & Harris, R. S. (1994).** Adaptive mutation by deletions in small nucleotide repeats. *Science* 265, 405–407.
- Shapiro, J. A. (1995).** Adaptive mutation: who's really in the garden? *Science* 268, 373–374.
- Silverman, P. M. & Sholl, A. (1996).** Effect of *traY* amber mutations on F-plasmid *traY* promoter activity *in vivo*. *J Bacteriol* 178, 5787–5789.

Stallions, D. R. & Curtiss, R., III (1972). Bacterial conjugation under anaerobic conditions. *J Bacteriol* **111**, 294–295.

Tomoeda, M., Inuzuka, M. & Date, T. (1975). Bacterial sex pili. *Prog Biophys Mol Biol* **30**, 23–56.

Torkelson, J., Harris, R. S., Lombardo, M. J., Nagendran, J., Thulin, C. & Rosenberg, S. M. (1997). Genome-wide hypermutation in a subpopulation of stationary-phase cells underlies recombination-dependent adaptive mutation. *EMBO J* **16**, 3303–3311.

Tsai, M. M., Fu, Y. H. & Deonier, R. C. (1990). Intrinsic bends and integration host factor binding at F plasmid *oriT*. *J Bacteriol* **172**, 4603–4609.

Yamamoto, M., Kanegasaki, S. & Yoshikawa, M. (1980). Effects of temperature and energy inhibitors on complex formation between *Escherichia coli* male cells and filamentous phage fd. *J Gen Microbiol* **119**, 87–93.

Yamamoto, M., Kanegasaki, S. & Yoshikawa, M. (1981). Role of membrane potential and ATP in complex formation between *Escherichia coli* male cells and filamentous phage fd. *J Gen Microbiol* **123**, 343–349.

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