Synthesis of FinP RNA by Plasmids F and pSLT Is Regulated by DNA Adenine Methylation

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ABSTRACT

DNA adenine methylase mutants of *Salmonella typhimurium* contain reduced amounts of FinP, an antisense RNA encoded by the virulence plasmid pSLT. Lowered FinP levels are detected in both Dam$^-$ FinO$^+$ and Dam$^-$ FinO$^-$ backgrounds, suggesting that Dam methylation regulates FinP production rather than FinP half-life. Reduced amounts of F-encoded FinP RNA are likewise found in Dam$^-$ mutants of *Escherichia coli*. A consequence of FinP RNA scarcity in the absence of DNA adenine methylation is that Dam$^-$ mutants of both *S. typhimurium* and *E. coli* show elevated levels of F plasmid transfer. Inhibition of F fertility by the *S. typhimurium* virulence plasmid is also impaired in a Dam$^-$ background.

METHYLATED bases are present in many genomes and participate in a wide range of biological processes, including gene regulation (Noyer-Weidner and Trautner 1993; Marinus 1996; Holliday 1996). One of the methylated bases found in the DNA of enteric bacteria and their phages is 6-methyl-adenine, which is formed by postreplicative modification of adenine at 5′GATC3′ sites (Marinus and Morris 1973; Hattman *et al.* 1978). Genes whose transcription is regulated by DNA adenine methylation have been known since the early 1980s; the current list includes *dnaA* (Braun and Wright 1986; Kücherer *et al.* 1986), *mioC* (Schauzu *et al.* 1987), *trpR* (Marinus 1985), *glnS* (Plumbridge 1987), *sulA* (Peterson *et al.* 1985), the *pap* operon of enteropathogenic *E. coli* (Blyn *et al.* 1990), the transposase genes of IS*10* (Roberts *et al.* 1985) and IS*50* (McCommas and Syvanen 1988), the *mom* gene of Mu (Huttman 1982), and the *cre* gene of P1 (Sternberg *et al.* 1986). Recently, a computer survey of the distribution of GATC sites in the *E. coli* chromosome has suggested that additional Dam-regulated genes can be expected to exist (Henaut *et al.* 1996). However, unless validated by reverse genetics, the predictive power to GATC site distribution analysis *in silico* faces potential limitations. First, it is not always obvious where the search for relevant GATC sites should be performed because Dam methylation can regulate a promoter from distant regulatory sites. This caveat is illustrated by the *mom* gene of bacteriophage Mu, in which Dam methylation regulates binding of the OxyR repressor to an upstream operator (Bölker and Kahmann 1989), and by the *pap* operon of *E. coli*, which possesses a regulatory GATC site located more than 100 bp away from the transcriptional start site (reviewed by van der Woude *et al.* 1996). In the *E. coli* chromosome, the average distance between GATC neighbor sites is 214 bp (Henaut *et al.* 1996), with the obvious consequence that GATC sites at distances potentially relevant for transcription are found in many promoters. Moreover, the presence of a GATC site in a promoter does not imply that the gene is regulated by Dam methylation. For instance, the P1 *cre* gene possesses two promoters that contain GATC sites, but only one of the two promoters is regulated by Dam methylation (Sternberg *et al.* 1986).

As an alternative approach to the combination of genome analysis and reverse genetics, we devised a screen for loci regulated by DNA adenine methylation based on classical genetic methods. The screen was designed for *Salmonella typhimurium* and involved a search for Lac fusions that showed different activity in Dam$^+$ and Dam$^-$ backgrounds (Torreblanca and Casadesús 1996). Transcriptional *lac* fusions were generated by transposition of a Mu derivative (Mu*dJ*) in a Dam$^+$ strain and classified according to their Lac phenotype (Lac$^+$ or Lac$^-$) on indicator plates. To avoid the analysis of individual fusions, isolates the same type (Lac$^+$ or Lac$^-$) were pooled. The fusion pools were transferred to an isogenic Dam$^-$ recipient by P22 HT transduction, selecting the kanamycin resistance marker of the Mu*dJ* element. Colonies that had changed their Lac phenotype were then sought among the Kan^r transductants. With this strategy, a locus repressed by Dam methylation was found in the virulence plasmid (pSLT) of *S. typhimurium* (Torreblanca and Casadesús 1996).

We describe below the molecular characterization of the original *zzv-6306*::Mu*dJ* fusion, followed by experiments that show that Dam methylation regulates the expression of the *tra* operon of pSLT. We propose that

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derepression of the pSLT *tra* operon in a Dam$^-$ background is an indirect effect caused by lowered synthesis of FinP RNA, and present evidence that Dam methylation also regulates synthesis of FinP RNA in the F plasmid. A consequence of FinP shortage in the absence of DNA adenine methylation is that Dam$^-$ mutants of both *S. typhimurium* and *E. coli* undergo F plasmid transfer at elevated levels.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and strain construction: The *S. typhimurium* and *E. coli* strains used in this study are listed in Table 1. Transductional crosses using phage P22 HT 105/1 *int201* (Schmieger 1972; G. Roberts, unpublished results), henceforth referred as P22 HT, were used for strain construction operations involving chromosomal markers and for transfer of small plasmids among Salmonella strains. To obtain phage-free isolates, transductants were purified by streaking on greed plates (Chan *et al.* 1972). Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5. Strain constructions involving F-prime transfer were achieved in rapid matings in which drops of the donor, the recipient, and the mating mixture were placed on a nutrient broth (NB) plate and allowed to dry. The plate was incubated at 37° for 4–8 hr, replica-printed to selective agar, and incubated again (until growth was observed in the area corresponding to the mating mixture). Transconjugants were purified twice on selective plates.

Plasmids and transposons: The episomes F′128 *pro*$^+$ lac$^+$, F′ *proAB lacI*^{q} *lacZ*Δ*M15*::Tn10, F′128 *pro*$^+$ *lac*$^+$ *zzf-1831*::Tn*10d*Tet, F′128 *pro*$^+$ *lac*$^+$ *zzf1836*::Tn10*d*Cam, and F′T80 *his*$^+$ were all obtained from J. R. Roth (University of Utah, Salt Lake City). Plasmid pACYC184 is a p15A derivative carrying Cam^r and Tet^r markers (Chang and Cohen 1978). Plasmid pMM40 (Amp^r) is an expression vector derived from pKK223-3 (Kleiner *et al.* 1988). The phagemid pBluescript II SK(+) is a product of Strategene (La Jolla, CA). pTP166, obtained from M. G. Marinus (University of Massachusetts, Worcester), is a pBR322 derivative carrying the *E. coli dam* gene under the control of a *tac* promoter (Marinus *et al.* 1984). pIC552 is a promoter-probe vector that permits the construction of transcriptional *lac* fusions (Macian *et al.* 1994). pMD1405 (Amp^r), obtained from M. Drummond (John Innes Institute, Norwich, England), is a ColE1 derivative engineered for the construction of translational fusions with a *lacZ* gene that lacks the first eight codons. Mu*d*I1734[KanLac] (Castilho *et al.* 1984) is a transposition-deficient Mu derivative that generates operon fusions upon insertion; the element was renamed Mu*dJ* by Hughes and Roth (1988). pIZ53 is a pUC19 derivative carrying the internal *Hin*dIII fragment of Tn5; this fragment includes the kanamycin resistance gene, which is the same Kan^r determinant carried by Mu*dJ* (Maldonado *et al.* 1992).

Construction of plasmid pIZ833: The *E. coli dam* gene was isolated from plasmid pTP166 (Marinus *et al.* 1984) by recovery of a *Xba*I-*Pvu*II fragment of 1.1 kb. Blunt ends were generated by treatment with Klenow DNA polymerase. The fragment was then cloned in the *Sma*I site of pMM40 (Kleiner *et al.* 1988). The orientation of the cloned fragment was analyzed by electrophoretic separation of DNA fragments digested with *Bam*HI. The correct location of the *dam* gene with respect of the *tac* promoter yields three distinct *Bam*HI fragments of 0.6, 0.5, and 0.3 kb, aside from the pMM40 backbone of ~5 kb (data not shown).

Construction of plasmids pIZ877, pIZ880, and pIZ903: For construction of a transcriptional fusion *traY::lac*, a 610-bp *Ssp*I-*Eco*RV fragment from pSLT was cloned on the *Sma*I site of pIC552 (Macian *et al.* 1994). The cloned fragment carries the 3′ end of *traJ* and the 5′ end of *traY*, properly oriented to permit *lacZ* expression from the putative *traY* promoter of pSLT. The resulting plasmid was designed pIZ903.

The initial step for the construction of a transcriptional fusion *traJ::lac* was cloning of a 240-bp *Dra*I-*Eco*RV fragment of pSLT on pBluescript II SK(+) digested with *Eco*RV and *Sma*I. The construct was then digested with *Bam*HI and *Xho*I, and cloned on pIC552 digested with *Bgl*II and *Xho*I. The resulting plasmid, pIZ877, carries the putative *traJ* promoter of pSLT and some 70 bp of the putative *traJ* ORF.

Construction of a transcriptional fusion *finP::lac* was as follows: a 300-bp *Bam*HI-*Hin*fI fragment containing the putative *finP* promoter was cloned on pBluescript II SK(+). In the fragment cloned, one *Hin*fI site is located in the putative *finP* gene, while the *Bam*HI site is part of vector DNA. After digestion with *Hin*fI and end filling with Klenow polymerase, the fragment was digested with *Bam*HI and cloned on pBluescript II SK(+) digested with *Eco*RV and *Bam*HI. The construct was then digested with *Bam*HI and *Xho*I and cloned on pIC552 digested with *Bgl*II and *Xho*I, to generate pIZ880. This plasmid contains a transcriptional *finP::lac* fusion and lacks the *traJ* promoter.

Construction of plasmid pIZ900: A 326-bp *Eco*RV fragment of pSLT, containing the *traJ* promoter and a 5′ portion of the *traJ* coding sequence, was cloned on the *Sma*I site of pBluescript II SK(+) to generate pIZ899. A translational fusion *traJ::lac* was obtained by cloning the *Eco*RI-*Xba*I fragment of pIZ899 on pMD1405 digested with the same enzymes, to generate pIZ900. In addition to the translational fusion *traJ::lac*, the cloned fragment contains the *finP* promoter and the complete *finP* gene.

Media and chemicals: The E medium of Vogel and Bonner (1956) was used as the standard minimal medium. NCE is E medium without citrate. Carbon sources were either 0.2% glucose or 1% lactose. The rich medium was nutrient broth (8 g/liter, Difco) with added NaCl (5 g/liter). MacConkey agar base was from Difco. Solid media contained agar at 1.5% final concentration. Auxotrophic requirements and antibiotics were used at the final concentrations described by Maloy (1990). Green plates were prepared according to Chan *et al.* (1972), except that methyl blue (Sigma, St. Louis) substituted for aniline blue. Isopropyl-β-d-thiogalactopyranoside (IPTG; Sigma) was used at a final concentration of 1 mm. For gel electrophoresis, agarose (SeaKem, FMC, Rockland, ME) was prepared in either TAE or TBE buffers (Sambrook *et al.* 1989). The final agarose concentration (0.7–1.0%) depended on the range of DNA fragments to be separated. E buffer contained 40 mm Tris-base and 2 mm EDTA; pH was adjusted to 7.9 with glacial acetic acid. The lysis solution contained 3% sodium dodecyl sulfate, 50 mm Trizma base, and 72 mm NaOH.

Virulence plasmid curing: Curing of the virulence plasmid of *S. typhimurium* was achieved by destabilization of the *par* locus with a kanamycin-resistant cartridge (Tinge and Curtiss 1990). The Km^r cartridge was introduced into the strain to be cured by P22 HT transduction, using strain X3918 as the donor. Km^r transductants were streaked on green plates, and individual phage-free colonies were scored for loss of kanamycin resistance.

β-Galactosidase assays: Levels of β-galactosidase activity were assayed as described by Miller (1972), using the $CHCl_3$-sodium dodecyl sulfate permeabilization procedure.

Matings: Saturated cultures of the donor and the recipient were harvested by centrifugation and washed with NCE medium without carbon source. Aliquots of both strains were then mixed and incubated at 37° for 2 hr, without shaking. After mating, the mixtures were diluted in NCE and spread

TABLE 1

Bacterial strains

Strain	Genotype or phenotype	Reference or source[a]
	Salmonella typhimurium	
LT2	Wild type	Laboratory stock
SV2049	*zij-6302*::Tn*10d*Cam	Laboratory stock
SV3000	*dam-201*::Tn*10d*Tet	Torreblanca and Casadesús (1996)
SV3003	*zzv-6306*::Mu*dJ*	Torreblanca and Casadesús (1996)
SV3006	Δ*dam-204*	Torreblanca and Casadesús (1996)
SV3044	*proAB47*	Laboratory stock
SV3052	*proAB47 zfi-6030* ::TN*10d* Tet	Laboratory stock
SV3069	*zzv-6306* ::Mu*dJ dam-201* ::Tn10*d*Tet	
SV3081	pSLT$^-$ (isogenic with LT2)	
SV3082	Δ*dam-204* pSLT$^-$ (isogenic with SV3006)	
SV3083	*dam-201*::Tn*10d*Tet pSLT$^-$ (isogenic with SV3000)	
SV3093	pSLT$^-$/pIZ832	
SV3094	Δ*dam-204* pSLT$^-$/pIZ832	
SV3095	*dam-201*::Tn*10d*Tet pSLT$^-$/pIZ832	
SV3096	pSLT$^-$/pIZ832, pIZ833	
SV3098	*dam-201*::Tn*10d*Tet pSLT$^-$/pIZ832, pIZ833	
SV4060	*proAB47 dam-201*::Tn*10d* Tet	
SV4061	*proAB47 dam-225*::Mu*dJ*	
SV4064	*his-646 proAB47 dam-201*::Tn*10d* Tet/F′128 *pro*$^+$ *lac*$^+$	
SV4065	*his-646 proAB47 dam-225*::Mu*dJ*/F′128 *pro*$^+$ *lac*$^+$	
SV4066	*proAB47 zfi-6303*::Tn*10d* Tet *dam-225*::Mu*dJ*	
SV4067	*proAB47 dam-225*::Mu*dJ*/F′128 *pro*$^+$ *lac*$^+$ *zzf-1836*::Tn*10d*Cam	
SV4068	*proAB47 zfi-6303*::Tn*10d* Tet pSLT$^-$	
SV4070	*proAB47*/F′128 *pro*$^+$ *lac*$^+$*zzf-1836*::Tn*10d*Cam pSLT$^-$	
SV4072	Δ*dam-204 zij-6302*::Tn*10d*Cam	
SV4073	*proAB47 dam-225*::Mu*dJ*/F′128 *pro*$^+$ *lac*$^+$ *zzf-1831*::Tn*10d*Tet	
SV4081	Δ*his-9533*/FT80 *his*$^+$	
SV4082	Δ*his-9533 dam-201*::Tn*10d* Tet/F′T80 *his*$^+$	
SV4098	pSLT$^-$/pIZ877	
SV4099	Δ*dam204* pSLT$^-$/pIZ877	
SV4104	pSLT$^-$/pIZ880	
SV4105	Δ*dam204* pSLT$^-$/pIZ880	
SV4107	LT2/pIZ900	
SV4108	Δ*dam204*/pIZ900	
SV4110	pSLT$^-$/pIZ900	
SV4111	Δ*dam204* pSLT$^-$/pIZ900	
SV4116	pSLT$^-$/pIZ903	
SV4117	Δ*dam204* pSLT$^-$/pIZ903	
TR35	Δ*his-712 ser-821 arg-501*/F′T80 *his*$^+$	J. R. Roth
TR1831	*hisD6419 proAB47*/F′128 *pro*$^+$ *lac*$^+$	J. R. Roth
TR2714	*serA790* Δ*his-644 recA1* Str^r	J. R. Roth
TR5878	r(LT2)$^-$ m(LT2)$^-$ r(S)$^+$ *ilv-542 met A22 trpB2* Fels2-*fliA66 thrA120 xyl-404 metE551 hspL56 hspS29*	SGSC[b]
TT10423	*proAB47*/F′128 *pro*$^+$ *lac*$^+$ *zzf-1831*::Tn*10d*Tet	J. R. Roth
TT10604	*proAB47*/F′128 *pro*$^+$ *lac*$^+$ *zzf-1836*::Tn*10d*Cam	J. R. Roth
X3918	Δ(*gal-uvrB*)*-1005 fla66 rpsL120 xyl-404 lamB*$^+$(*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29/parA* (89,1 kb *Sma*I::Kan) F$^-$	Tinge and Curtiss (1990)
	Escherichia coli	
GM28	F$^-$ *sup-85* (Am)	M. G. Marinus
GM33	F$^-$ *dam-3 sup-85* (Am)	M. G. Marinus
AB1157	F$^-$ *thr-1 leuB6 thi-1 argE3 hisG4 proA2 lacY1 galK2 mtl-1 xyl-5 ara-14 rpsL31 tsx-33 glnV44 rfbD1 kdgK51*	M. G. Marinus
GM3819	F$^-$ *dam-16*::Kan *thr-1 leuB6 thi-1 argE3 hisG4 proA2 lacY1 galK2 mtl-1 xyl-5 ara-14 rpsL31 tsx-33 glnV44 rfbD1 kdgK51*	M. G. Marinus

[a] Omitted for strains first described in this study.
[b] SGSC, Salmonella Genetic Stock Center, University of Calgary, Alberta, Canada.

(both diluted and undiluted) on selective plates. As controls, 0.1 ml of both the donor and the recipient cultures were also spread on selective plates.

Transformation of *S. typhimurium:* The transformable strain TR5878 was used as the recipient of plasmids; preparation of competent cells and transformation followed the procedures of Lederberg and Cohen (1974). For preparation of electrocompetent cells and electroporation of *S. typhimurium* we followed the procedures of Maloy (1990), using an Electro Cell Manipulator 600 from BTX (San Diego). Plasmids transformed into TR5878 were transferred to other strains by P22 HT transduction.

Extraction of the *S. typhimurium* virulence plasmid: One milliliter of an overnight culture in Luria-Bertani broth was centrifuged at 12,000 rpm for 2 min at 4°. The pellet was resuspended in 150 μl of E buffer; 300 μl of lysis solution were then added. After incubating at 65° for 1 hr, the lysate was chilled on ice and shaken for 10 min (until a white precipitate was formed). The preparation was then buffered by adding 150 μl of ice-cold 2 m Tris, shaken gently until it became transparent and centrifuged at 12,000 rpm for 20 min in the cold. The supernatant was transferred to a clean tube and mixed with one volume of nonsaturated phenol:chloroform:isoamyl alcohol (25:24:1). After two to three extraction cycles, DNA was precipitated with 2 m sodium acetate and absolute ethanol. The pellet was rinsed with 70% ethanol and resuspended in 10 μl of Tris-EDTA. All preparations were treated with ribonuclease (0.1 mg/ml, final concentration) before storage at −20°.

Standard isolation of plasmid DNA: Plasmid DNA for both clone analysis and DNA sequencing was obtained by alkaline lysis, without phenol extraction (Stephen *et al.* 1990).

Digestion, end modification, and ligation of DNA fragments: Restriction enzymes were purchased from Promega Biotech (Madison, WI), New England Biolabs (Beverly, MA), and Boehringer Mannheim (Mannheim, Germany). The buffers used were those provided by the supplier. For multiple digestions, we used the "One-phor-all" buffer (Pharmacia Biotech, San Francisco). Deoxyribonucleotides and and Klenow DNA polymerase were purchased from Pharmacia Biotech. T4 polynucleotide ligase was from Boehringer Mannheim; ligation was achieved by incubating >12 hr at 16°. For blunt-end ligation, a low-ATP buffer was used (New England Biolabs).

Electrophoretic separation of DNA fragments: Electrophoresis of DNA was carried out on agarose gels, and the usual buffer was TBE. However, TAE was used for recovery of DNA fragments from gels. The molecular weight markers used were *Hin*dIII-digested lambda DNA and/or the 1-kb ladder from GIBCO-BRL (New York). Gels were stained with ethidium bromide (final concentration, 0.5 μg/ml). For gel photographs, a Polaroid 3000/36° film (Polaroid Co., Cambridge, MA) was used.

DNA hybridization: Digestion of DNA with restriction enzymes, electrophoretic separation of restriction fragments, DNA denaturation, transfer of DNA from agarose gels to nylon filters, DNA labeling, and DNA hybridization followed the procedures of Southern (1975) and Sambrook *et al.* (1989). For probe preparation, recovery of DNA from agarose gels was achieved with the GeneClean system (Bio 101, La Jolla, CA). DNA probes were labeled with the DIG DNA labeling kit from Boehringer Mannheim. As a Mu*dJ* probe, we used the ~10-kb *Hin*dIII fragment of Mu*dJ* itself (Castilho *et al.* 1984), previously identified by hybridization against the *Hin*dIII fragment of plasmid pIZ53 (Maldonado *et al.* 1992). The composition of the 52-mer called "*traB* probe" was 5′AACGG CATCAAGGGTGAAGTGGTGATGCGTAATGGCAAAATCC TCGGCTGGG3′.

DNA sequencing and sequence analysis: Sequencing reactions were performed with the dideoxy chain termination procedure (Sanger *et al.* 1977), using the Sequenase kit 2.0 (United States Biochemical Corporation). For sequencing of inserts carried on pBluescript, T3 and T7 primers were used (Beuzón and Casadesús 1997). Sequencing of the boundaries of the *zzv-6306::*Mu*dJ* insertion was performed with primers MuL and MuR, whose composition was suggested by Michael J. Mahan, University of California, Santa Barbara: MuL, 5′CGAATAATCCAATGTCCTCC3′; MuR, 5′GAAGCGCGAA AGCTAAAG3′. Sequencing gels were prepared in TBE and contained 6% acrylamide and urea 500 g/liter. Gels were run in a Poker Face SE1500 sequencer (Hoeffer Scientific Instruments, San Francisco) dried in a Slab Gel Dryer, model SE1160 (Hoeffer), and developed by exposure to an X-ray film. Automated sequencing was performed by a DNA technology company (Medigene, Martinsried, Germany). For sequence analysis, we used the computer analysis package (Version 8, 1994) of the Genetics Computer Group, University of Wisconsin, Madison.

RNA extraction: RNA preparations were obtained by guanidine isothiocyanate lysis and phenol/chloroform extraction (Chomczynski and Sacchi 1987). Saturated cultures were immersed in liquid N_2, and 1.4 ml of lysis solution [5 m guanidinium isothiocyanate, 50 mm Tris (pH 7.5), 10 mm EDTA, 8% v/v β-mercaptoethanol] was added. Each mixture was incubated at 60° for 10 min, before 0.28 ml of chloroform was added. After gentle shaking and centrifugation at 9000 rpm for 10 min, 0.66 ml of isopropanol was added to the supernatant. The samples were incubated at −20° for 15 min, and centrifuged again at 9000 rpm for 15 min. The pellets were then rinsed with 70% ethanol and dried. After resuspension in 75 μl of water (with 0.1% v/v diethyl pyrocarbonate, henceforth DEP), the samples were subjected to standard treatments with deoxyribonuclease and proteinase K, followed by extraction with phenol:chloroform:isoamyl alcohol, and chloroform:isoamyl alcohol (Sambrook *et al.* 1989). The aqueous phase was precipitated with 1/10 volume of 3 m sodium acetate, pH 4.8, and 2.5 volumes of absolute ethanol. The samples were then kept at −78° for at least 30 min, centrifuged, and washed with 70% ethanol. Finally, the precipitates were dried and resuspended in 20–40 μl of DEP-water.

RNA electrophoresis in polyacrylamide gels: Samples contained 6 μl of the RNA preparation and 4 μl of loading buffer containing 50% formamide. After 2 min incubation at 94°, the samples were chilled on ice. Electrophoretic separation was carried out on gels prepared with TBE and containing 8% acrylamide and urea 7.5 m. The gels were 12 cm long and 0.75 mm thick. Vertical electrophoretic separation was performed at 250 V.

RNA hybridization against DNA probes: After electrophoretic separation of RNA, polyacrylamide gels were treated with cold TBE (0.5×) for 15 min. Transfer to nylon filters was achieved with a Transblot SD Semidry Transfer Cell system from BioRad Laboratories (Richmond, CA). Transfer was allowed to proceed for 1 hr at 400 mA, at intensities below 25 V. Prehybridization and hybridization were as described for DNA hybridization, except that the temperature was 38° and formamide was not used. After transfer, the filters were stained with a solution of 0.3% methylene blue in 0.3 m sodium acetate, pH 5.2, to confirm both the efficiency of transfer and the presence of equivalent amounts of RNA per lane. The probe was end-labeled with [γ^{32}P]ATP. After hybridization, the nylon filters were washed twice at room temperature with 6× SSC, 0.1% SDS for 5 min, and twice with 0.6× SSC, 0.1% SDS for 15 min. The latter washes were carried out either at room temperature or at 35°. The filters were then exposed to an X-ray film for 1–7 days. The FinP probe used was the

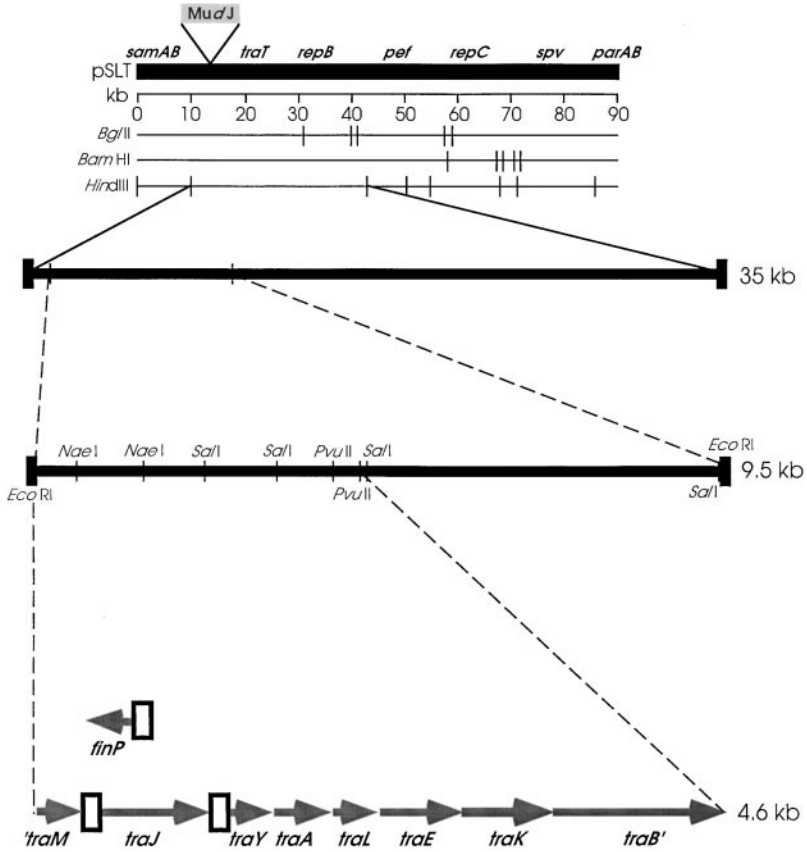

Figure 1.—Genetic and physical maps of the *S. typhimurium* virulence plasmid, shown at the top of the diagram, have been adapted from Sanderson et al. (1995). The position of the *traB1*::Mu*dJ* fusion on the pSLT map is also indicated. The *Eco*RI fragment of 9.5 kb described in the text is contained in the *Hin*dIII fragment of ~35 kb, which encompasses kb 11–45 on the pSLT map. The 4.6 kb of pSLT DNA sequenced correspond to the *Eco*RI-*Pvu*II fragment. Putative genes detected by computer analysis of DNA sequences are shown at the bottom. The boxes represent known promoters; their existence was first hypothesized from DNA sequence analysis and later confirmed by fusion construction.

20-mer 5′TAATCGCCGATACAGGGAG3′. This sequence is complementary to the 3′ end of F-encoded FinP RNA, and the region is 100% conserved in the pSLT plasmid.

RESULTS

Physical mapping of the fusion *zzv-6306*::Mu*dJ*: The location of the Dam-regulated fusion *zzv-6306*::Mu*dJ* on the virulence plasmid of *S. typhimurium* was established by a combination of restriction mapping and Southern hybridization. A map of *Hin*dIII, *Bam*HI, and *Blg*II sites in pSLT (Sanderson *et al.* 1995), as well as a map of *Hin*dIII, *Eco*RI, *Bam*HI, *Bgl*II, *Sal*I, and *Hpa*I restriction sites in Mu*dJ* (Castilho *et al.* 1984), provided coordinates for the initial mapping steps. Single and double digestions of the virulence plasmids pSLT and pSLT *zzv-6306*::Mu*dJ* (from strains LT2 and SV3003, respectively) with *Hin*dIII, *Eco*RI, *Bam*HI, and *Bgl*II showed that the insertion *zzv-6306*::Mu*dJ* was located in an ~10-kb *Eco*RI fragment. Restriction mapping also indicated that the insertion *zzv-6306*::*Mud*J mapped near kilobase 11 on the pSLT map (Sanderson *et al.* 1995). These conclusions were confirmed by Southern hybridization using the ~10-kb *Hin*dIII fragment of Mu*dJ* as a probe. The location of the insertion *zzv-6306*::Mu*dJ* on the pSLT map is depicted in Figure 1.

Sequencing of the boundaries of the insertion *zzv-6306*::Mu*dJ*: Sequencing of the regions flanking the insertion *zzv-6306*::Mu*dJ* was performed using pSLT DNA extracted from strain SV3003. Sequencing with the MuL primer permitted the identification of 203 nucleotides on one side of the insertion *zzv-6306*::Mu*dJ* (not shown), while priming with MuR failed. A search in the EMBL database using the program FASTA indicated that the stretch of 203 nucleotides sequenced was 84.5% homologous to nucleotides 5070–5272 of the F plasmid, which correspond to the coding sequence of the *traB* gene (EMBL accession no.: U01159). The finding that the insertion *zzv-6306*::Mu*dJ* was located in a gene homologous to the *traB* gene of F prompted a change of the designation *zzv-6306*::Mu*dJ* to *traB1*:Mu*dJ*.

Physical mapping of the *traB* region of pSLT: An oligonucleotide that could serve as a probe in Southern hybridization experiments was derived from the *traB* sequence (see materials and methods). The region selected is extremely similar in F and pSLT (only three differences in 52 nucleotides) and corresponds to nucleotides 5167–5218 of the F sequence (EMBL accession no.: U01159). The resulting *traB* 52-mer, henceforth called "*traB* probe," was used in Southern hybridization experiments against virulence plasmid DNA from strains LT2 (pSLT) and SV3003 (pSLT *traB1*::Mu*dJ*). Relevant results were as follows:

i. The *traB* probe hybridized against a single *Hin*dIII fragment, larger than the 21 kb of the DNA size marker, in both pSLT and pSLT *traB1*::Mu*dJ*. This result permitted us to map the Mu*dJ* insertion within

the *Hin*dIII fragment of ~35 kb located between kilobase 11 and kilobase 45 on the pSLT map, which is the only "large" *Hin*dIII fragment of pSLT (Sanderson *et al.* 1995).

ii. Single and double digestions of pSLT DNA with *Eco*RI, *Sal*I, and *Hin*dIII, followed by Southern hybridization, indicated that the *traB* probe hybridized against a *Eco*RI fragment of 9–10 kb, as well as with a *Sal*I fragment of 1.2 kb. The *Eco*RI fragment, whose exact size is 9.5 kb, does not contain any *Hin*dIII site, and the 1.2 *Sal*I fragment is devoid of both *Eco*RI and *Hin*dIII sites. These data are shown in the diagram of Figure 1.

Reconstruction of the insertion *traB1*::Mu*dJ* in a pACYC184 derivative: The 9.5-kb *Eco*RI fragment of pSLT (Figure 1) was cloned onto pACYC184, a vector compatible with ColE1 derivatives (Chang and Cohen 1978), to generate plasmid pIZ830. Because this plasmid was devised to reconstruct the *traB1*::Mu*dJ* fusion by homologous recombination (see below), candidates were subjected to restriction analysis to investigate the orientation of their inserts with respect to the promoter of the tetracycline resistance gene of pACYC184. The goal was to obtain a plasmid with the *lacZ* gene of the *traB1*::Mu*dJ* fusion opposite to the strong *cam* promoter of pACYC184 (see below).

Plasmid pIZ830 contains ~4.5 kb of DNA homologous to the 5′ boundary of the *traB1*::Mu*dJ* insertion of pSLT, and ~5 kb corresponding to the 3′ side of the insertion. These sizes largely exceed the 20 bp estimated as the minimal size for homologous recombination in enteric bacteria (Watt *et al.* 1985). Taking advantage of these homologies, we introduced the insertion *traB1*::Mu*dJ* in pIZ830 by homologous recombination. Rescue of the *traB1*::Mu*dJ* insertion was as follows:

i. pIZ830 was transformed into *S. typhimurium* TR5878, and a P22 HT lysate was obtained on the resulting strain.

ii. The lysate of TR5878 / pIZ830 was then used to transduce SV3003 (pSLT *TraB1*::Mu*dJ*), selecting Tet^r transductants.

iii. After purification and lysogen elimination, one transductant was lysed with P22, and the lysate was used to transduce a pSLT-cured strain (SV3081), selecting Kan^r transductants. These were replica-printed to tetracycline plates to score coinheritance of the plasmid-borne *tet* gene. An isolate carrying Kan^r and Tet^r markers (a putative recombinant that had incorporated the fusion *traB1*::Mu*dJ* into pIZ830) was the source of plasmid pIZ832. Restriction analysis confirmed that the recombination process had not generated unwanted rearrangements, and that pIZ832 carries the *traB1*::Mu*dJ* insertion in the chloramphenicol gene of pACYC184, with the *lacZ* gene of the fusion oriented opposite to the *cam* promoter (Figure 2).

Effect of DNA adenine methylation on the expression of the *traB1*::Mu*dJ* fusion carried by plasmid pIZ832: Plasmid pIZ832 was transduced to strains SV3081 (pSLT$^-$ *dam*$^+$) and SV3083 (pSLT$^-$ *dam*-201::Tn*10d*Tet), as well as to derivatives of these strains that carried the compatible, methylase-producing plasmid pIZ833. Batch cultures of the resulting six strains were used to measure β-galactosidase activities. Figure 3 shows that the β-galactosidase activity of the *traB1*::Mu*dJ* fusion carried by plasmid pIZ832 was >10-fold higher in the absence of DNA adenine methylation. Thus the impaired levels of expression of the fusion *traB1*::Mu*dJ* in Dam$^+$ and Dam$^-$ backgrounds (Torreblanca and Casadesús 1996) was reproduced with a pACYC184-derivative carrying the *traB1*::Mu*dJ* fusion in a 9.5-kb pSLT fragment. The main conclusion from these experiments was that the 9.5-kb pSLT fragment contained all the elements necessary for the regulation of the *traB1*::Mu*dJ* fusion by DNA adenine methylation. A side observation was that the fusion was repressed at similar levels in a Dam$^+$ host and in the presence of the multicopy plasmid pIZ833, indicating that the level of DNA adenine methylase present in the wild type was sufficient to repress expression of the *traB1*::Mu*dJ* fusion when carried on a medium-copy-number plasmid. Smaller plasmids lacking regions located near the 5′ end of the 9.5-kb *Eco*RI insert did not show Dam-dependent expression (data not shown), indicating that the target(s) of Dam regulation were located on the 5′ side of the *traB1*::Mu*dJ* fusion carried on pIZ832. These hypothetical elements must therefore be present in the ~4.5-kb left half of the insert of pIZ830.

Cloning, subcloning, sequencing, and sequence analysis of a 4.6-kb fragment of the *tra* region of pSLT: The 9.5-kb *Eco*RI fragment of pSLT was subjected to restriction analysis, to search for sites that might facilitate subcloning. The fragment could be divided into two *Eco*RI-*Sal*I fragments of 2.4 kb and <0.2 kb, and three *Sal*I fragments of 4.8 kb, 1.2 kb, and 1.1 kb (Figure 1). For the purpose of our study, the most interesting fragments were the 2.4-kb *Eco*RI-*Sal*I fragment and the *Sal*I fragments of 1.2 kb and 1.1 kb, because most of their DNA sequences lie on the 5′ side of the insertion *traB1*::Mu*dJ* (which is located in the 1.2-kb *Sal*I fragment). Thus, further restriction analysis was concentrated on this ~4.7 kb region; relevant sites are shown in Figure 1.

Plasmids carrying subclones of 0.3–0.9 kb were generated by subcloning on pBluescript II SK(+). Their inserts were sequenced using T7L and T3/pBS primers. In total, the *Eco*RI-*Pvu*II region of pSLT sequenced has a length of 4649 bp, of which only 450 correspond to the 3′ side of the fusion *zzv-6306*::Mu*dJ*. The sequence has been deposited in the EMBL database with the accession number AJ011572.

The sequence of the 4.6-kb *Eco*RI-*Pvu*II fragment of pSLT was aligned with the *tra* region of F using the

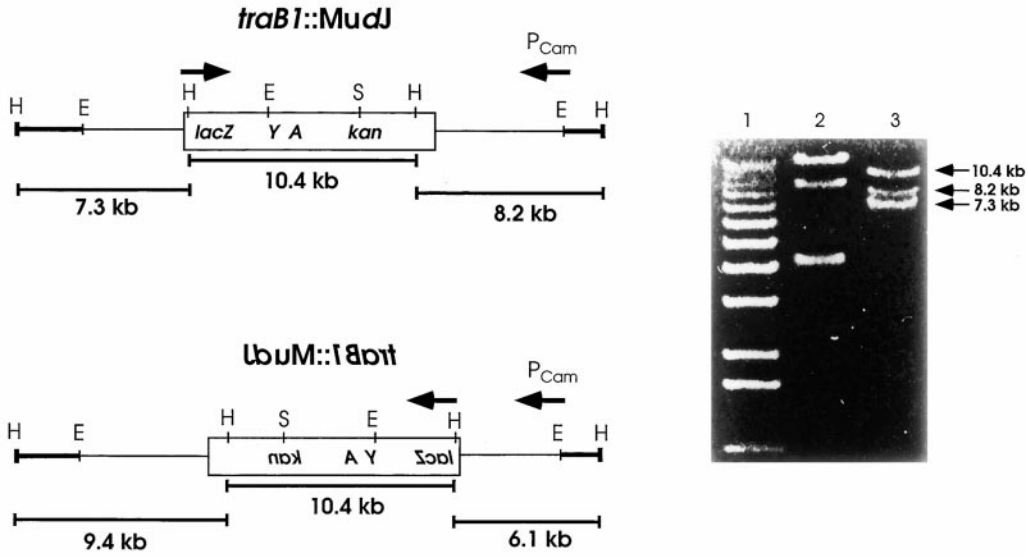

Figure 2.—(Left) A diagram of fragments generated by *Hin*dIII digestion of pIZ832, depending on the orientation of its insert relative to the promoter of the chloramphenicol-resistance gene of pACYC184. For simplicity, a linear plasmid map is shown. Symbols are as follows: H, *Hin*dIII; E, *Eco*RI; S, *Sal*I. pACYC184 contains a single *Hin*dIII site (Chang and Cohen 1978). (Right) Electrophoretic separation of fragments generated by digestion of pIZ832 with *Eco*RI (lane 2) and *Hin*dIII (lane 3). Lane 1 contains the 1-kb DNA ladder.

programs Clustal W 1.60 and Seq Vu 1.0.1. Sequences homologous to the *traM, traJ, traY, traA, traL, traE, traK,* and *traB* genes of F (nucleotides 656–5466, EMBL accession no. U01159) were found. The overall homology was of 72.30%, albeit with significant variations from one region to another. The highest homology degrees were found in the intervals *traM-traJ* and *traA-traB* (73.24 and 82.22%, respectively) and the lowest, in the *traY-traA* interval (45.79%, with gaps and insertions). A survey of potential open reading frames (ORFs) using the program Strider 1.1 indicated that the region contained putative ORFs identical to those found in F: *traJ, traY, traA, traL, traE,* and *traK,* as well as the 3′ end of *traM* and the 5′ end of *traB* (see Figure 1). A putative *finP* gene was also found overlapping with the *traJ* gene, an arrangement analogous to that found in F (Mullineaux and Willetts 1985; Frost *et al.* 1994).

Strategy for the identification of a Dam-regulated promoter in pSLT: In F, the main promoter of the *tra* operon is located upstream of *traY* (Willetts 1977; Gaffney *et al.* 1983). The *traJ* gene has its own promoter, and another promoter drives the overlapping *finP* gene (Mullineaux and Willetts 1985; Frost *et al.* 1989). If the structural conservation found between F and pSLT is indicative of functional analogy, the following scenarios are conceivable to explain the derepression of the *tra* operon of pSLT observed in the absence of Dam methylation:

i. The *traY* promoter might be directly repressed by Dam methylation. This promoter does not contain GATC sites (neither in F nor in the corresponding region of pSLT). In the latter, the closest GATC lies approximately at position −250 (data not shown, EMBL entry AJ011572). Although this location is farther than any of the known GATC sites that regulate promoters from a distance (Kahmann 1983; Plasterk *et al.* 1983; Blyn *et al.* 1990; van der Woude *et al.* 1996), the possibility that the *traY* promoter was directly regulated by Dam methylation was not excluded *a priori*.

ii. The *finP* promoter might be activated by Dam methylation; thus, reduced synthesis of FinP RNA would derepress *tra* expression in a Dam$^-$ background. Figure 4 shows the *traJ* and *finP* promoters of the F plasmid aligned with the corresponding regions of pSLT. Aside from their high homology, a relevant observation is the presence of a GATC site in the −10 module of the *finP* promoter of F. This Dam site is also found in the putative *finP* promoter of pSLT. The *finP* promoter of F contains a second GATC site near the −35 module; this Dam site is not present in pSLT.

iii. The *traJ* promoter might be repressed by Dam methylation; thus increased TraJ synthesis would derepress *tra* expression in a Dam$^-$ background. pSLT contains a GATC site upstream of the putative *traJ* promoter; this Dam site is not found in F (Figure 4).

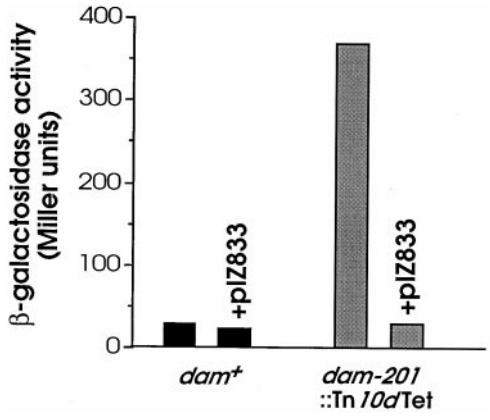

Figure 3.—β-Galactosidase activity of the fusion *traB1*::Mud*J* of plasmid pIZ832 in Dam$^+$ and Dam$^-$ backgrounds (strains SV3093, SV3095, SV3096, and SV3098).

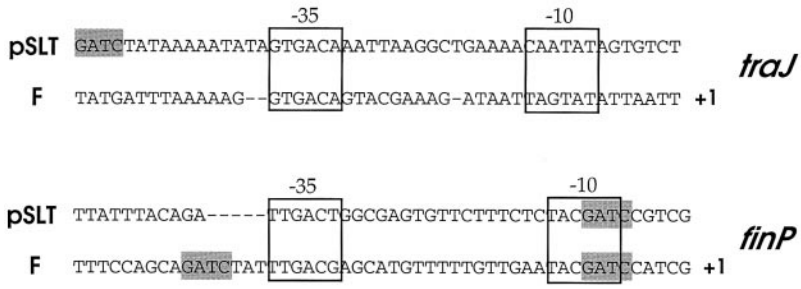

Figure 4.—Alignment of the *finP* and *traJ* promoters of F with the corresponding regions of pSLT. GATC sites located in or near the promoters are highlighted. The transcription start sites known in the F plasmid are also indicated.

The search for active promoters in pSLT DNA involved the construction of transcriptional *traY::lac*, *traJ::lac*, and *finP::lac* fusions using the promoter-probe vector pIC552. The background expression of this plasmid in *E. coli* and *S. typhimurium* is low (usually, <30 Miller units of β-galactosidase) and thus facilitates the detection of fair and weak promoter activities (Macian *et al.* 1994). Plasmids bearing fusions driven by pSLT promoters were then introduced in Dam$^+$ and Dam$^-$ strains cured of the pSLT plasmid, and the expression pattern of each fusion in response to DNA adenine methylation was analyzed (see below).

Construction of a transcriptional fusion *traY::lac*: Plasmid pIZ903 carries the 3′ end of *traJ* and the 5′ end of *traY*, properly oriented to permit *lacZ* expression from the putative *traY* promoter of pSLT. The fusion proved to be active, thereby indicating the existence of a promoter in the DNA fragment cloned. However, significant differences were not found between Dam$^+$ and Dam$^-$ backgrounds, indicating that the *traY* promoter of pSLT is not directly regulated by DNA adenine methylation (data not shown).

Construction of transcriptional fusions *traJ::lac* and *finP::lac*: Plasmid pIZ877 carries the putative *traJ* promoter of pSLT and some 70 bp of the putative *traJ* ORF. The construction generates a transcriptional *lac* fusion driven by the putative *traJ* promoter (Figure 5A). The activity of this fusion must reflect only the activity of the *traJ* promoter, because the *lacZ* gene of the vector possesses its own ribosome-binding site that cannot be occluded by FinP RNA. In turn, plasmid pIZ880 contains a transcriptional *finP::lac* fusion and lacks the *traJ* promoter (Figure 5A).

The effect of DNA adenine methylation on the activity of the *traJ::lac* and *finP::lac* fusions carried by plasmids pIZ877 and pIZ880 is shown in Figure 5B. The *traJ::lac* fusion did not show significant differences in Dam$^+$ and Dam$^-$ hosts. In contrast, the *finP::lac* fusion was more active (around fourfold) in a Dam$^+$ background. These results indicate that the Dam-regulated gene is *finP*. Not surprisingly, the putative *finP* promoter of pSLT contains a GATC site in its −10 module (Figure 4), like other Dam-regulated promoters (Noyer-Weidner and Trautner 1993; Marinus 1996). The experiments shown were carried out in the absence of the pSLT plasmid; similar results were obtained in a pSLT$^+$ background (not shown).

Construction of a translational fusion *traJ::lac*: Plasmid pIZ900 carries a translational fusion *traJ::lac*, as well as the *finP* promoter and the complete *finP* gene (Figure 6A). The effect of DNA adenine methylation on the activity of the *traJ::lac* fusion of pIZ900 is also shown in Figure 6B. Unlike the transcriptional *traJ::lac* fusion, which was insensitive to Dam methylation, the translational *traJ::lac* fusion becomes derepressed in a Dam$^-$ background. This expression pattern suggests that translation of *traJ* mRNA encoded by the pSLT plasmid is inhibited by FinP RNA, as in F (Mullineaux and Willetts 1985; Frost *et al.* 1989). This is a relevant result, because derepression of TraJ translation in a Dam$^-$ background provides further evidence that absence of DNA adenine methylation causes FinP scarcity. The slightly higher levels of TraJ expression in a pSLT$^-$ background (observed both in Dam$^+$ and Dam$^-$ hosts) may reflect the absence of FinO product (Finnegan and Willetts 1973; Frost *et al.* 1989), and the putative FinO effect may be small because pMD1405 is a high-copy-number plasmid.

Effect of Dam methylation on the production of pSLT-encoded FinP RNA: Northern hybridization experiments were carried out to compare the production of FinP RNA in Dam$^+$ and Dam$^-$ hosts of *S. typhimurium*. The probe used was an oligonucleotide complementary to FinP RNA (see materials and methods). Total RNA was extracted from strains SV3003 and SV3069. Twenty micrograms of RNA per lane was loaded and RNA molecules were separated by electrophoresis on an 8% polyacrylamide gel in the presence of 7.5 m urea. The results, exemplified by the autoradiogram of Figure 7, were unambiguous: higher amounts of FinP RNA were detected in a Dam$^+$ background. Differences in FinP RNA content were also detected in Dam$^+$ and Dam$^-$ hosts that carried pIZ832 but not pSLT (SV3093 and SV3095, respectively; data not shown). These pSLT-lacking strains do not produce the FinP-stabilizing protein FinO (Gasson and Willetts 1971; Finnegan and Willetts 1973). Thus reduced FinP content in a Dam$^-$ background likely reflects a difference in FinP synthesis rather than in FinP half-life. This conclusion agrees with the results obtained with *lac* fusions (see above). In F, FinP is a negative regulator of TraJ, and the latter is an activator of the *tra* operon (Frost *et al.* 1994; Firth *et al.* 1996). If this framework is applied to pSLT, derepression of the original *traB1*::Mu*dJ* fusion can be tentatively

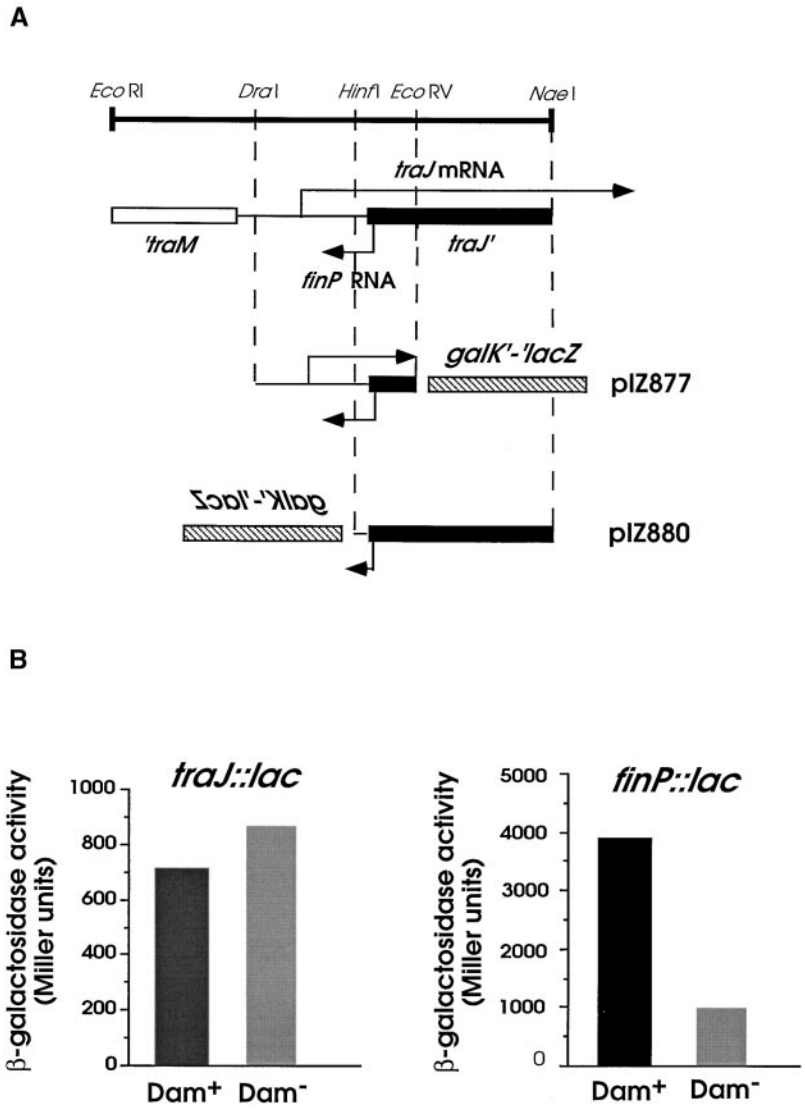

Figure 5.—(A) Construction of transcriptional fusions *traJ::lac* (carried on plasmid pIZ877) and *finP::lac* (carried on plasmid pIZ880). (B) β-Galactosidase activities of the transcriptional fusions *traJ::lac* and *finP::lac* of pIZ877 and pIZ880 in Dam$^+$ and Dam$^-$ backgrounds (strains SV4098, SV4099, SV4104, and SV4105).

attributed to reduced synthesis of FinP RNA in the absence of DNA adenine methylation.

Effect of Dam methylation on the production of F-encoded FinP RNA: The effect of Dam methylation on the synthesis of F-encoded FinP RNA was investigated in *E. coli*, using derivatives of strains AB1157 and GM3819 in which we had introduced the episome F′ *proAB lacI*^{q} *lacZ*Δ*M15*::Tn*10*. Total cellular RNA was extracted from both strains, and Northern hybridization was performed as described above, except that higher amounts of RNA were used (100 μg of RNA per well). Larger amounts of FinP RNA were detected in the Dam$^+$ strain (Figure 8). A side (but highly reproducible) observation was that the levels of F-encoded FinP RNA were consistently smaller than those of pSLT, both in Dam$^+$ and Dam$^-$ backgrounds (compare Figures 7 and 8). This observation may reflect the absence of a functional *finO* gene in the F plasmid (Cheah and Skurray 1986).

Effect of Dam methylation on F plasmid transfer: The detection of lower amounts of F-encoded FinP RNA in Dam$^-$ mutants of *E. coli* suggested the possibility that F plasmid transfer was derepressed in the absence of DNA adenine methylation. The hypothesis received indirect support from the observation that the *traB1*::MudJ fusion of pSLT, located in a region highly homologous to F, is expressed at elevated levels in a Dam$^-$ background (Torreblanca and Casadesús 1996). F has been traditionally considered a derepressed plasmid because it carries an IS3 element inserted in the *finO* gene (Cheah and Skurray 1986). Lack of FinO protein reduces the half-life of FinP RNA (Lee *et al.* 1992); as a consequence, FinP-mediated repression of *traJ* mRNA becomes inefficient. This scenario does not exclude the possibility that further derepression could occur in a Dam$^-$ background, if mutations that reduce FinP synthesis were epistatic over the *finO* mutation of the F plasmid.

To examine the effect of Dam methylation on F plasmid transfer, matings between *E. coli* strains were performed. Use of F primes instead of the wild-type F element facilitated the detection of transconjugants by either complementation or selection of dominant anti-

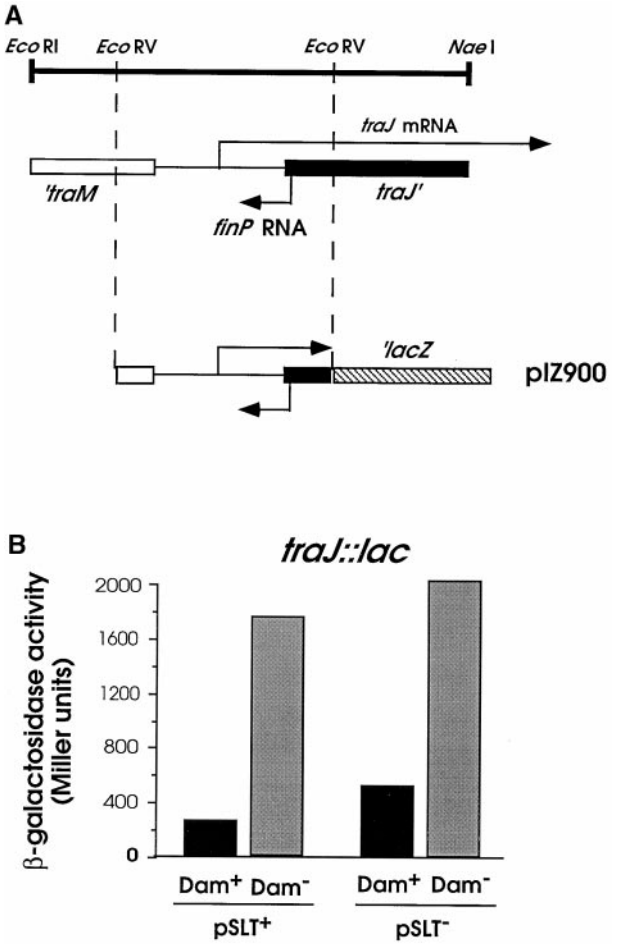

Figure 6.—(A) Diagram of the construction of plasmid pIZ900. (B) Activity of the translational *traJ::lac* fusion of pIZ900 in Dam$^+$ and Dam$^-$ backgrounds, measured both in the presence and in the absence of plasmid pSLT (strains SV4107, SV4108, SV4110, and SV4111).

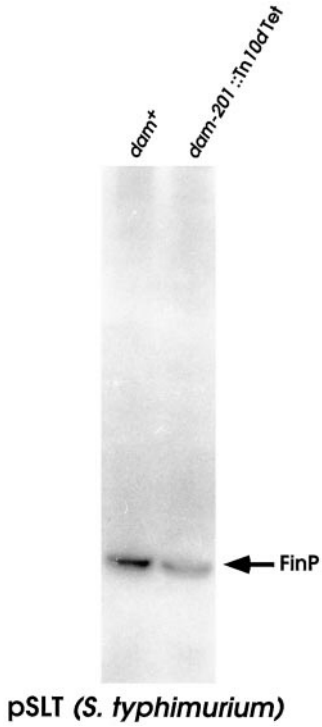

Figure 7.—Northern hybridization of total RNA isolated from Dam$^+$ and Dam$^-$ strains of *S. typhimurium* carrying plasmid pIZ832 (strains SV3093 and SV3094, respectively). RNA separation was performed on 8% polyacrylamide and hybridized against the *finP* probe.

biotic-resistance markers. Donors were derivatives of the isogenic strains AB1157 and GM3819 carrying either of the F primes F′128 *pro*$^+$ *lac*$^+$ *zzf-1831*::Tn*10d*Tet or F′128 *pro*$^+$ *lac*$^+$ *zzf-1836*::Tn*10d*Cam. The recipients were GM28 and GM33. Prototrophic, Tet^r or Cam^r transconjugants were selected. Figure 9 shows data for F′128 *pro*$^+$ *lac*$^+$ *zzf-1836*::Tn*10d*Tet. The frequencies of F-prime transfer increased around fourfold when one of the mating strains were Dam$^-$, and one order of magnitude in the Dam$^-$ × Dam$^-$ mating. Crosses involving F′128 *pro*$^+$ *lac*$^+$ *zzf-1831*:Tn*10d*Cam gave similar results, and the highest transfer frequency was detected in the Dam$^-$ × Dam$^-$ cross (data not shown). The latter observation can be explained as an amplification effect: in Dam$^-$ × Dam$^-$ crosses, derepression of F transfer, combined with the presence of excess recipients and the long mating times allowed, permits a swift increase of the donor population. Differential growth is unlikely to be involved, because the matings were performed in buffer. Moreover, Dam$^+$ and Dam$^-$ strains in *E. coli* do not show significant differences in viability (Marinus and Morris 1973). The possibility that the elevated conjugation frequencies found might be related to the hyperecombinogenic ability of Dam$^-$ mutants (Marinus and Konrad 1976) seems also unlikely, because F-prime transfer is RecA-independent (Low 1968). Lastly, we have also discarded the possibility that altered plasmid replication is involved, because F and pSLT do not exhibit differences in stability or copy number between Dam$^+$ and Dam$^-$ hosts (data not shown). On

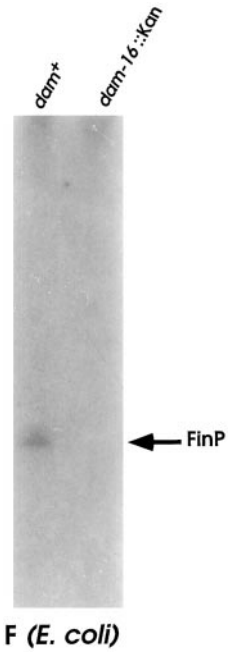

Figure 8.—Northern hybridization of total RNA isolated from Dam$^+$ and Dam$^-$ strains of *E. coli* carrying the episome F′ *pro*AB$^+$ *lacI*^{q} *ZΔM15*::Tn*10 (GM28 / F′ proAB*$^+$ *lacI*^{q} *ZΔM15*::Tn*10 and GM33 / F′ proAB*$^+$ *lacI*^{q} *ZΔM15*::Tn*10*, respectively). RNA separation was performed on 8% polyacrylamide and hybridized against the *finP* probe.

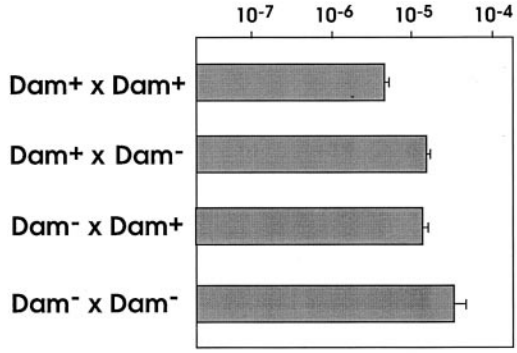

Figure 9.—Transfer frequencies of the episome F′ 128 *pro*$^+$ *lac*$^+$ *zzf-1831*::Tn*10d*Tet between Dam$^+$ and Dam$^+$ strains of *E. coli* (averages from four independent matings). The donors were AB1157 / F′ 128 *pro*$^+$ *lac*$^+$ *zzf-1831*::Tn*10d*Tet and GM3819 / F′ 128 *pro*$^+$ *lac*$^+$ *zzf-1831*::Tn*10d*Tet. The recipients were GM28 and GM33. Crosses are described in the form "donor × recipient." The selection applied was tetracycline resistance.

these grounds, we interpreted that the increase in the number of transconjugants directly reflected an increase in F-prime transfer, confirming the prediction that a *dam* mutation would be epistatic over *finO*.

Effect of Dam methylation on pSLT-mediated inhibition of F fertility: If derepression of F plasmid transfer in a Dam$^-$ background is indeed caused by reduced synthesis of FinP RNA, a prediction is that the virulence plasmid of *S. typhimurium* should fail to inhibit F fertility in a Dam$^-$ background: pSLT-encoded FinO will not be able to protect FinP RNA is the latter is absent or scarce. The prediction was tested by comparing the frequencies of F-prime transfer between Dam$^+$ and Dam$^-$ strains of *S. typhimurium*. The F primes used were F′128 *pro*$^+$ *lac*$^+$, F′128 *pro*$^+$ *lac*$^+$ *zzf-1836*::Tn*10dCam*, F′128 *pro*$^+$ *lac*$^+$ *zzf-1831*:Tn*10d*Tet, and F′T80 *his*$^+$. To construct the donor strains, the F primes were conjugally transferred to pairs of isogenic Dam$^+$ and Dam$^-$ strains that carried either of the deletions Δ*proAB47* or Δ*his-9533* (and thus permitted the selection of F-primer transfer by complementation). The recipients were pairs of isogenic Dam$^+$ and Dam$^-$ strains whose genotype permitted easy donor counterselection.

Experiments of transfer of F′128 *pro*$^+$ *lac*$^+$ *zzf-1836*::Tn*10d*Cam between Dam$^+$ and Dam$^-$ strains of *S. typhimurium* are summarized in Figure 10. Transconjugants were selected on LB supplemented with chloramphenicol (to select plasmid transfer) and tetracycline (to counterselect the donor, because the recipient carried the insertion *zfi-6303*::Tn*10d*Tet). In each experiment, the frequency of F-prime transfer was calculated as the quotient between the number of transconjugants (per milliliter of mating mixture) and the number of donors (per milliliter of culture). The highest conjugation frequencies, ~100 times over the Dam$^+$ × Dam$^+$ cross, were obtained in matings in which both the donor

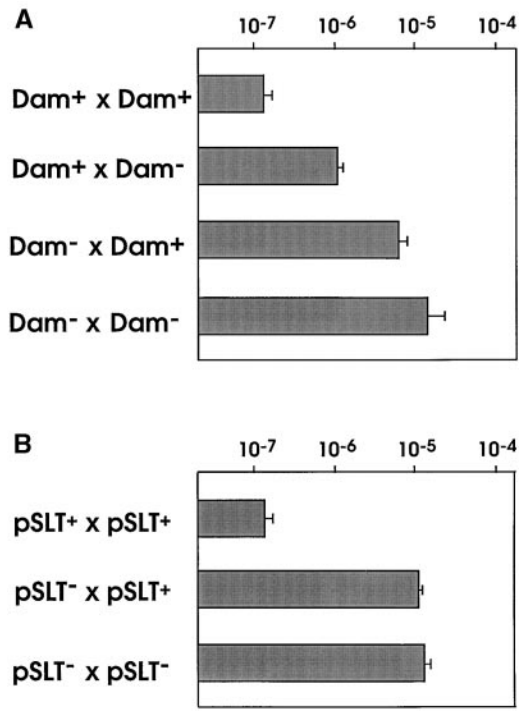

Figure 10.—(A) Transfer frequencies of the episome F′ 128 *pro*$^+$ *lac*$^+$ *zzf-1831*::Tn*10d*Cam between Dam$^+$ and Dam$^-$ strains of *S. typhimurium* (averages from six independent matings). The donors were TT10604 and SV4067. The recipients were SV3052 and SV4066. Crosses are described in the form "donors × recipient." The selection applied was chloramphenicol resistance. (B) Transfer frequencies of the episome F′ 128 *pro*$^+$ *lac*$^+$ *zzf-1831*::Tn*10d*Cam between pSLT$^+$ and pSLT$^-$ strains of *S. typhimurium*, selecting Cam^r transconjugants (average from six independent matings). The donors were TT10604 and SV4070. The recipients were SV3052 and SV4068. Cross description is as in A.

and the recipient were Dam$^-$ (Figure 10A). However, the conjugation frequency also increased in crosses in which only one of the mating strains was Dam$^-$, especially when the Dam$^-$ partner happened to be the donor (40-fold increase over the wild-type cross). Crosses involving F primes F′128 *pro*$^+$ lac$^+$, F′128 *pro*$^+$ *lac*$^+$ *zzf-1831*:Tn*10d*Tet, and F′T80 *his*$^+$ gave similar results: the absolute transfer frequencies ranged from $<10^{-7}$ to $<10^{-4}$, depending on both the F prime assayed and the genotype (Dam$^+$ or Dam$^-$) of the mating strains. The relative conjugal transfer frequencies were higher when one of the partners was Dam$^-$, and the highest rates were found, as in the *E. coli* matings, in the Dam$^-$ × Dam$^-$ cross (data not shown).

An interesting difference between the *E. coli* and *S. typhimurium* matings affects the wild-type (Dam$^+$ × Dam$^+$) cross, which yielded higher frequencies of F-prime transfer in *E. coli*. This observation, made for the first time four decades ago (Zinder 1960; Mäkelä *et al.* 1962), reflects the inhibition of F fertility in the presence of pSLT. Not surprisingly, an increase of F-prime transfer is observed in a pSLT$^-$ background

(Figure 10B). The latter results served as an internal control to confirm the prediction that pSLT would be unable to inhibit F fertility in a Dam$^-$ background. The simplest interpretation is that FinO action is prevented by scarcity of FinP RNA.

DISCUSSION

The fusion *zzv-6306*::Mu*dJ*, originally described as a novel locus repressed by DNA adenine methylation (Torreblanca and Casadesús 1996), disrupts an ORF homologous to the *traB* gene of the F plasmid; accordingly, the fusion has been renamed *traB1*::Mu*dJ*. The Dam-dependent expression pattern of the original fusion was still observed when the *traB1*::Mu*dJ* fusion was reconstructed in pIZ832, a plasmid smaller and easier to handle than the 90 kb of pSLT. pIZ832 carries 9.5 kb of pSLT DNA, and about half of this length corresponds to sequences located on the 5′ side of the *traB1*::Mu*dJ* fusion (Figure 1). Analysis of plasmids carrying smaller inserts indicated that all the elements necessary for Dam-dependent expression of the fusion were located on the left half of the pIZ832 insert. Sequence analysis of 4.6 kb of pSLT DNA, of which roughly 4 kb lie on the 5′ side of the fusion *traB1*::Mu*dJ* (EMBL accession number AJ011572), indicated that the region was highly homologous to the *tra* operon of the F plasmid, with the presence of putative ORFs homologous to the *traM*, *traJ*, *traY*, *traA*, *traL*, *traE*, *traK*, and *traB* genes of F (Figure 1). A putative *finP* gene was also found overlapping with *traJ*, an arrangement identical to that found in F (reviews: Frost *et al.* 1994; Firth *et al.* 1996). Another relevant finding was the high homology between the *finP* and *traJ* promoters of F and the corresponding regions of pSLT (Figure 4).

Search for *traY*, *traJ*, and *finP* promoters in pSLT DNA was carried out by constructing *lac* fusions *in vitro*, using DNA sequence data as a chart for cloning. Transcriptional *lac* fusions constructed in the promoter-probe vector pIC552 were then assayed in Dam$^+$ and Dam$^-$ hosts. All the fusions were active, indicating that *traY*, *traJ*, and *finP* promoters do exist in pSLT. However, neither of the *traJ* or *traY* promoters showed Dam-dependent activity (Figure 5 and data not shown). In contrast, the *finP*::*lac* fusion showed higher activity in Dam$^+$ background, a pattern opposite to that described for the original *traB1*::Mu*dJ* fusion (Figure 5). The finding that the Dam-regulated promoter is *finP*, rather than *traJ* or *traY*, receives direct support from sequence data: the putative *finP* promoter of pSLT contains a GATC site overlapping with its −10 module (Figure 4).

The opposite effects of Dam methylation on the expression of *finP*::*lac* and *traB1*::lac fusions can be tentatively explained by analogy with the F plasmid. The main promoter of the *tra* operon of F is located upstream of *traY* (Gaffney *et al.* 1983), and the regulatory genes *traJ* and *finP* exert opposite effects on *tra* expression. The TraJ product is a positive regulator of *tra* expression and acts at the *traY* promoter (Willetts 1977; Silverman *et al.* 1991). Translation of *traJ* mRNA is negatively regulated by an antisense RNA encoded by the overlapping *finP* gene (Mullineaux and Willetts 1985; Finlay *et al.* 1986; Frost *et al.* 1989). FinP RNA is short-lived unless stabilized by the F-encoded product FinO (Finnegan and Willetts 1973). If the same circuitry operates in pSLT, increased expression of the *traB1*::Mu*dJ* fusion in a Dam$^-$ background must result from elevated activity of the *traY* promoter. However, the latter does not appear to be directly regulated by Dam methylation (data not shown), and the same conclusion applies to the *traJ* promoter (Figure 5). The key observation is that the *finP*::*lac* fusion is less active in a Dam$^-$ background (Figure 5). Lowered synthesis of FinP RNA can be expected to increase translation of *traJ* mRNA; increased TraJ synthesis will then enhance *tra* operon expression. This model, summarized in Table 2, is supported by several lines of evidence:

i. Direct RNA quantitation by Northern hybridization confirms that FinP RNA is scarce in a Dam$^-$ background (Figure 7).
ii. Translational (but not transcriptional) *traJ*::*lac* fusions become derepressed in a Dam$^-$ background, indicating that reduced synthesis of FinP RNA can be correlated with increased translation of *traJ* mRNA (Figures 5 and 6).
iii. Variations of FinP levels in response to Dam methylation are observed in the absence of FinO (*e.g.*, in a pSLT$^-$ host strain), suggesting that the effect of Dam methylation is exerted upon FinP synthesis, and not upon FinP half-life.

The existence of a GATC site in the *finP* promoter of F (Figure 4, data from EMBL entry U01159), exactly at the same position found in pSLT, raised the question of whether synthesis of FinP RNA in the F plasmid might be likewise controlled by Dam methylation. Northern hybridization experiments showed that synthesis of FinP RNA by the F plasmid is impaired in Dam$^-$ mutants of *E. coli* (Figure 8). Thus a tentative conclusion is that the methylation state of the *finP* promoter regulates FinP RNA synthesis in both F and pSLT. The model that the *finP* promoter is only active in the methylated state has an intriguing side, because the GATC site found in the −10 module of the IS*10* transposase gene has been previously shown to exert an opposite effect on promoter activity: the IS*10* transposase promoter is inactive when the GATC site is methylated (Roberts *et al.* 1985). However, it must be noted that the GATC sites of the IS*10* and *finP* promoters overlap with opposite edges of the −10 module, thus leaving open the possibility that this difference may explain their opposite effects on promoter activity. An alternative possibility is that these GATC sites exert different functions: while the GATC

TABLE 2

Model for regulation of *tra* operon expression by DNA adenine methylation

State of the *finP* promoter	Synthesis of FinP RNA	Translation of *traJ* mRNA	Activity of the *traY* promoter	Expression of the *tra* operon
Methylated	High	Low	Low	Off
Unmethylated	Low	High	High	On

site of the IS*10* promoter appears to modify directly the affinity of the promoter for RNA polymerase (Roberts *et al.* 1985), below we consider the possibility that the GATC site of the *finP* promoter might prevent binding of an hypothetical repressor.

If different levels of F-encoded FinP RNA are synthesized by Dam$^+$ and Dam$^-$ hosts, a prediction is that F plasmid transfer should be affected by the methylation state of host DNA. This hypothesis was investigated by performing matings between Dam$^+$ and Dam$^-$ mutants of *E. coli*, and the transfer frequencies of F-prime plasmids were found to increase 4- to 10-fold in the absence of DNA adenine methylation (Figure 9). This observation can be easily accommodated in the regulatory circuit of F-plasmid transfer: lack of Dam methylation reduces the level of the main transfer inhibitor, FinP RNA, and a consequence is that the *tra* operon of F becomes derepressed. Although the F plasmid is naturally derepressed because of lack of FinO product (Finnegan and Willetts 1973; Willetts 1977; Cheah and Skurray 1986), additional derepression occurs in a Dam$^-$ host because mutations causing FinP scarcity are epistatic over *finO*.

The effect of *dam* mutations on F-plasmid transfer is even better observed in *S. typhimurium*, where repression of F fertility by the pSLT plasmid reduces by more than one order of magnitude the frequencies of F-prime transfer among Dam$^+$ hosts (Figure 10). Like other virulence plasmids from Salmonella, pSLT is nonconjugative (Sanderson and MacLachlan 1987) but can inhibit the conjugative ability of other plasmids, namely, of the f factor (Zinder 1960; Mäkelä *et al.* 1962; Smith *et al.* 1973; Spratt *et al.* 1973). The fertility inhibition phenotype of pSLT relies on the possession of an intact *finO* gene (Finnegan and Willetts 1973). In the Dam$^-$ background, failure of F-encoded FinP RNA synthesis increases F-prime transfer by one to two orders of magnitude (Figure 10). A simple interpretation is that pSLT-encoded FinO product is only efficient in a Dam$^+$ background because FinO action requires the presence of FinP.

At the present stage of knowledge, the physiological significance of controlling FinP synthesis by DNA adenine methylation is unknown. If molecular analysis confirms that Dam methylation acts directly at the *finP* promoter, the following two alternative models will emerge.

i. Unmethylation and hemi-methylation may be equivalent signals, and the *finP* promoter may be inactive when hemi-methylated. In this case, a tentative model might be that Dam methylation is used to couple *finP* expression to plasmid replication. Transient hemi-methylation after passage of the replication fork would cause a brief repression of FinP synthesis and thus an increase of *tra* expression during a short lapse of the replication cycle. A mechanism of this kind would be analogous (but opposite) to that described for IS*10* transposase synthesis (Roberts *et al.* 1985).
ii. The methylation state of the *finP* promoter might be controlled by cellular factors. Stably undermethylated GATC sites have been detected in the *E. coli* genome (Ringquist and Smith 1992; Wang and Church 1992; Hale *et al.* 1994), and current evidence suggests that undermethylation is a consequence of protein binding (Wang and Church 1992; Hale *et al* 1994; Casadesús and Torreblanca 1996). Hindrance of GATC remethylation during two consecutive rounds of DNA replication generates unmethylated Dam sites (Braaten *et al.* 1991, 1994). Regulation of the *finP* promoter might involve binding of a hypothetical repressor to the GATC site of the −10 module, thereby preventing RNA polymerase binding and/or transcription initiation. A mechanism of this kind would be analogous to that described for *mom*, with the difference that OxyR binds to GATC sites located upstream of the *mom* promoter (Bölker and Kahmann 1989). An attractive aspect of this model is that it envisages the possibility of regulating the methylation state of the *finP* promoter. If this view were correct, further investigation might lead to the discovery of mechanisms that regulate FinP synthesis (and hence conjugal transfer of DNA) in response to physiological or environmental signals.

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