Chapter 5

H-NS and Lrp Are Positive Modulators of Conjugal Transfer of the *Escherichia coli* Plasmid pRK100

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ABSTRACT

Conjugative transfer of F-like plasmids is a tightly regulated process. The TraJ protein is the main positive activator of the *tra* operon, which encodes products required for conjugal transfer of F-like plasmids. Nucleotide sequence analysis revealed potential Lrp and H-NS binding sites in the *traJ* regulatory region. Expression of a *traJ-lacZ* fusion in *hns* and *lrp* mutant strains showed that both are positive modulators of *traJ*. Competitive RT-PCR demonstrated that H-NS and Lrp exert their effect at the transcriptional level. Electrophoretic mobility-shift assays showed that H-NS and Lrp protein bind to the *traJ* promoter. Conjugal transfer of pRK100 was decreased 540- and 4-fold in *hns* and *lrp* mutant strains, respectively. Together, the results indicate H-NS and Lrp act as activators of *traJ* transcription.

Key words: TraJ, Lrp, H-NS, conjugation, F-like plasmids

1. INTRODUCTION

Conjugation is a process, which promotes DNA transfer from a donor to a recipient by forming direct cell-cell contact. In F-like plasmids the genes responsible for conjugative transfer are located in an approximately 33 kb long *tra* region. The *tra* region contains approximately 40 genes that are organised into three tightly regulated operons (Fig. 1). Two monocistronic operons encoding the *traM* and *traJ* genes are located immediately downstream of the origin of transfer (*oriT*) (14). TraM seems to link the control of transfer gene expression and the initiation of DNA transfer (27), while TraJ activates the transcription of the third multicistronic *tra* operon (31). This third operon is transcribed from the P_{traY} promoter and encodes genes involved in F pilus synthesis, pilus assembly as well as DNA unwinding and nicking (14).

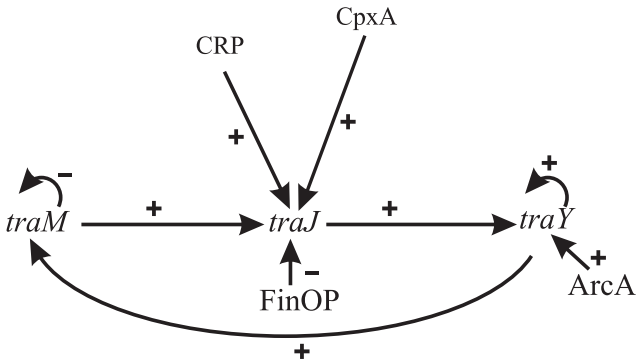

Fig. 1. **A general model for the regulation of expression of the *traM*, *traJ* and *traY* genes of F-like plasmids**
+ indicates a positive effect and – indicates a negative effect.

The expression of the *traJ* gene is subject to positive and negative regulation. We recently demonstrated that the cAMP-CRP complex activates transcription from the *traJ* promoter (Starčič M, Žgur-Bertok D, Jordi BJAM, Wösten MMSM, Gaastra W, van Putten JPM, in press) of pRK100, a conjugative ~145 kb F-like plasmid (1). Previously, CpxA of the two component signal transduction system CpxA/CpxR, which is activated by stress to the bacterial envelope (28), was reported to be required for efficient *traJ* expression (32). Negative regulation of TraJ expression occurs through the fertility inhibition FinOP system. FinP is an antisense RNA molecule complementary to part of the 5' untranslated region of *traJ* mRNA. FinO blocks FinP antisense RNA decay by promoting the formation of the *traJ*-FinP duplex. This duplex is subsequently degraded by RNase III preventing the synthesis of the TraJ protein (18). Repression of F plasmid transfer is also imposed by Dam methylation, which is required to sustain high levels of FinP RNA synthesis (37).

Increased expression of *traJ* stimulates $P_{traY,}$ the *traY* promoter. Full activation of the P_{traY} promoter requires, besides the TraJ protein, ArcA, which is part of the ArcA/B two component system sensing oxygen pressure (35). The TraY protein subsequently stimulates $P_{traY,}$ increasing the concentration of the *tra* operon proteins and enhancing expression of *traM*. TraY also induces DNA bending and stimulates nicking at the origin of transfer in co-operation with the integration host factor, IHF (24). Eventually, positive control of expression is interrupted by TraM autorepression. However, even though the *tra* regions of F-like plasmids have a common organisation, subtle differences in the regulation of transfer are evident (9, 27). Plasmid pRK100, used in our studies as a model system, was

isolated from a uropathogenic *Escherichia coli* strain. Partial nucleotide sequencing of the pRK100 *tra* region (*traM, finP, traJ, traY, traD,* and *finO*) showed highest similarity to F plasmid genes (14).

Despite TraJ's role as a central positive regulator of the transfer region, the mechanisms that control the expression of the *traJ* gene itself are not well understood. Assembly of the transfer apparatus and transfer of DNA is energetically demanding therefore, global regulators co-ordinating cellular metabolism in response to environmental signals could be involved in regulation of TraJ synthesis. Analysis of the pRK100 *traJ* promoter region sequences revealed several potential binding sites for global regulators, namely Lrp and H-NS (Fig. 2).

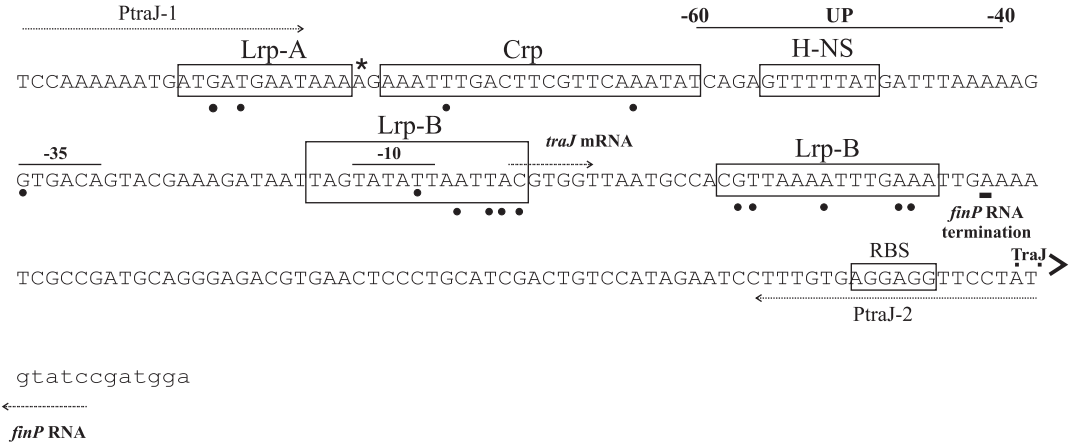

***Fig. 2.* Nucleotide sequence and locations of putative Lrp and H-NS regulatory sites in the *traJ* promoter region of plasmid pRK100**

The –35 and –10 promoter regions, the positions of transcription initiation and termination, the ribosomal binding site (RBS) as well as the initiation of the TraJ translation are indicated. Binding sites of the oligonucleotide primers PtraJ-1 and PtraJ-2 are marked. Putative binding sites for regulatory proteins Lrp and H-NS and for the CRP protein are indicated by boxes. The Lrp-A site is based on the consensus sequence described by Calvo and Matthews (5) and the Lrp-B sites are based on the consensus sequence described by Cui, *et al.* (8). The H-NS site is a putative DNA bending site as predicted by Lasergene PC, Dnastar, Inc. Madison, Wisconsin, USA. PC. The CRP site is based on results obtained with DNaseI footprinting and site-directed mutagenesis experiments (Starčič, *et al.,* in press). The nucleotides that differ from the known consensus binding sites are marked with dots. The Upstream element (UP) from –40 till –60 is also depicted. The indicated sequence of the pRK100 plasmid only differs at one base (indicated by an asterisk) compared with the published sequence of the same region of the F plasmid (accession number U01159).

The leucine-responsive regulatory protein (Lrp) is a host encoded regulator of *Escherichia coli* metabolism. Lrp influences expression of genes, whose protein products are involved in amino acid biosynthesis and degradation, nitrogen metabolism, carbohydrate degradation, synthesis of fimbriae and transport (25). In some cases leucine is required as an effector (21) while in others leucine has no effect *in vivo* (11). The histone-like nucleoid-structuring (H-NS) protein controls expression of many genes regulated by environmental signals (2). In the presented work we investigated the possible role of the global regulators Lrp and H-NS in *traJ* expression. The effect of the regulators was assessed using *in vitro* methods, such as a *lacZ* reporter system, RT-PCR, DNA binding assays, and also *in vivo* by conjugal transfer experiments. Our results demonstrate that H-NS and Lrp act as activators of pRK100 *traJ* transcription.

2. MATERIAL AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1.

Table 1. **Bacterial strains and plasmids**

Strains	Genotype	Source or reference*
MC4100	*ara*D139 Δ(*argF-lac*)U169 *rps*L150 *rel*A1 *flb*B5301 *pts*F25 *deo*C1	7
GE3653	MC 4100 *lrp*-201::Tn*10*	10
MC-HNS	MC 4100 *hns*::*kan*	40
TR8	MC4100 *cpxA*::*cam*	T. J. Silhavy[1]
TR51	MC4100 *cpxR*::*spc*	T. J. Silhavy
GE2897	MC4100 Δ*82*(*himA*)::*tet*	10
RH90	MC4100 *rpoS*::Tn*10*	R. Hengge-Aronis[2]
RH98	MC4100 *relA251*::*kan spoT207*::*cat*	20

Plasmids	Properties / Vector	Insert	Source or reference
pRK100	F-like plasmid		1
pCB267	*lacZ* promoter probe vector		30
pTJ1	pCB267	226 bp *traJ* promoter	This study
pUC19	Multi-purpose cloning vector		41
pINP2	pGEM-T Easy	158 bp Δ*lacZ*	This study
pBE18	*gltBDF* promoter		39
PSJ4	*proU* promoter		17

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Media

Strains were grown in Luria-Bertani (LB) medium or M63 medium, which consists of M63 salts (26) supplemented with 0.2% glucose, 0.1% casamino acids and 100 μg/ml thiamine with aeration at 37 °C, unless stated otherwise. When appropriate, ampicillin (100 μg/ml), kanamycin (30 μg/ml), chloramphenicol (20 μg/ml), spectinomycin (20 μg/ml), and tetracycline (10 μg/ml) were added to the medium.

DNA manipulation techniques

General DNA manipulation techniques, as DNA isolation, ligation, and transformation experiments were performed using standard methods (29). Restriction endonuclease digestions were carried out as specified by the manufacturer (Promega, Boehringer). DNA fragments were isolated from agarose gels using the QIAquick Gel Extraction kit (Qiagen). DNA sequencing was performed using a dye rhodamine terminator cycling reaction and an ABI PRISM™ 310 Genetic Analyzer automated sequencer and ABI PRISM™ software.

Construction of the *traJ-lacZ* fusion

PCR was performed to amplify a 210 bp fragment of pRK100 containing 208 nucleotides upstream of the *traJ* translation initiation site and the first two nucleotides of the *traJ* start codon ATG. For this purpose two primers, PtraJ-1 (5'-CGGGATCC-TCCAAAAAATGATGATGAAT-3') and PtraJ-2 (5'-GCTCTAGA-ATAGGAACCTCCTCACAAAG-3'), were used (Fig. 2). *Bam*HI and *Xba*I restriction sites in primers PtraJ-1 and PtraJ-2 facilitated cloning into the promoter probe plasmid pCB267 (30), generating pTJ1. Double-stranded nucleotide sequencing with PtraJ-1 and PtraJ-2 oligonucleotides as primers was carried out to confirm that no base changes had occurred while generating pTJ1.

β-Galactosidase assays

Overnight cell cultures of relevant strains were diluted 1:500 into fresh LB medium and grown with aeration at 37 °C to an OD_{600} of 1. Subsequently, cells were again diluted 1:500 into fresh LB medium and samples were periodically removed and assayed for β-galactosidase activity. β-Galactosidase assays were performed essentially as described (23). *o*-Nitrophenyl-β-D-galacto-pyranoside (ONPG) was used as a substrate in the β-galactosidase assays of cells treated with sodium dodecyl sulfate (SDS)-chloroform and washed with Z buffer (23). Enzyme activity is defined in units of optical density at 420 nm (OD_{420}) per minute per unit of OD_{600}, (23). At least two different colonies were tested in independent experiments.

Generating Δ*lacZ* RNA as competitive template for RT-PCR

PCR was performed to generate a 215 bp fragment of the *lacZ* gene by using plasmid pTJ1 as a template and primers lacZ-1 (5'- ACGATGCGCCCATCTACACC-3') and lacZ-2 (5'- ACGACTGTCCTGGCCGTAAC-3'). The obtained DNA fragment was digested with *Mse*I and the restriction fragments were ligated with T4-ligase (Amersham Pharmacia Biotech). This DNA was then used as a template in a PCR with primers lacZ-1 and lacZ-2. The different PCR products were separated onto a 4% NuSieve 3:1 agarose gel (BMA, BioWhittaker Molecular Applications) and a 158 bp Δ*lacZ* product with a deletion of the *Mse*I restriction fragment was purified from the gel. This fragment was cloned into the pGEM-T Easy vector (Promega), generating plasmid pINP2. The nucleotide sequence of the insert of pINP2 was determined to confirm, that apart from the deletion, no other base changes had been introduced. To obtain the RNA Δ*lacZ* competitive template for RT-PCR, pINP2, cut with *Pst*I, was used as the template in the Riboprobe In Vitro Transcription System (Promega).

RNA isolation

Overnight cell cultures were diluted 1:500 into fresh LB medium and grown with aeration at 37 °C to an OD_{600} of 1. The cell cultures were again diluted 1:500 into fresh LB medium and grown for 2 hours. For each sample, RNA was isolated from the same amount of cells with RNAzol™B (Campro Scientific) following the instructions of the manufacturer. After isopropanol precipitation, RNA was dissolved in 40 µl of H_2O, and stored at −70 °C. Prior to analysis of the *lacZ* mRNA in the competitive RT-PCR, samples were checked for DNA contamination by PCR using oligonucleotide primers lacZ-1 and lacZ-2. If DNA contamination was detected, samples were treated with RQ1 RNase-Free DNase (Promega).

Competitive RT-PCR

1 µl of DNA-free RNA sample of the relevant strain and 1 µl of the Δ*lacZ* competitive template, were used together with the lacZ-1 and lacZ-2 oligonucleotide primers in the competitive RT-PCR (16) performed with the Access RT-PCR System kit (Promega). The RT-PCR program was as follows: reverse transcription – synthesis of first cDNA strand at 48 °C for 45 min, AMV RT inactivation

and RNA/cDNA/oligonucleotide primer denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 30 seconds, and extension at 72 °C for 30 seconds, and one final extension for 10 min at 72 °C. The RT-PCR products were separated on 2% agarose gels. Competitive RT-PCR was performed twice on two different samples and similar results were obtained in all tests.

EMSA

The electrophoretic mobility shift assays (EMSA) were based on the method described in "Current Protocols in Molecular Biology" (4). The 226 bp PCR product, *ptraJ*, obtained with oligonucleotide primers PtraJ-1 and PtraJ-2 (see above), was labelled with [α-^{32}P] at the *Bam*HI site using the Klenow enzyme (USB). 20 ng of the labelled DNA fragment were used in a volume of 20 µl. Lrp binding reactions were performed essentially as described by Stauffer and Stauffer (34). However, there was no 5 min incubation before addition of purified Lrp and binding was allowed to proceed 30 min at 37 °C. The 480 bp *Eco*RI–*Pst*I fragment of pBE18 carrying the *gltBDF* promoter, ^{32}P-end labelled at the *Eco*RI site with the Klenow enzyme (USB), was used as a positive control.

H-NS binding reactions were performed essentially as described by Jordi, *et al*. (19) with protein binding proceeding for 15 min at 37 °C. The 317 bp *Bam*HI–*Pst*I fragment of pSJ4 (17) carrying the *proU* promoter was used as a positive control. After the binding reactions 1 µl of loading buffer (0.1% brom-phenol blue and 50% glycerol in water) was added to samples prior to loading onto a 5% PAGE gel, which was run at constant voltage (240V). The gels were subsequently transferred to Whatman 3MM paper, dried, and autoradiographed. The amounts of DNA and protein that were used are indicated in the Figures.

Competitive mobility shift assay using agarose gels

The mobility shift assay using agarose gels was based on the method described (19). However, the reaction volume was 20 µl and the incubation was performed for 15 min at 37 °C. The 100 bp DNA ladder (MBI Fermentas) was used as competitor DNA.

Mating assay

Conjugation experiments were performed essentially as described by Franklin and Möllby (13). Overnight cultures of donor and recipient strains were diluted 100-fold and incubated for 2 hours with aeration at 37 °C. A mating mixture consisting of 0.5 ml of the donor and 4.5 ml of the recipient culture was incubated for 2 hours at 37 °C. Transconjugants were selected on LB media supplemented with the appropriate antibiotics. Conjugal transfer frequencies were calculated per donor cells.

3. RESULTS

***traJ* expression is positively affected by Lrp and H-NS**

DNA sequence analysis of the *traJ* promoter region in plasmid pRK100 revealed putative H-NS and Lrp binding sites (Fig. 2). To test whether these two global regulators, as well as some other regulators (CpxA/R, ppGpp, IHF, σ^S), influence *traJ* expression, β-galactosidase activity of the *traJ-lacZ* transcriptional fusion was assayed in mutant strains defective for the above mentioned global regulators.

A significant decrease in β-galactosidase activity of the *traJ-lacZ* fusion was observed in the *hns* and *lrp* mutant strains throughout the growth cycle. The maximal difference between the wild type

strain and the *lrp* and *hns* mutants was observed in the early exponential phase, when activities were 6.5-fold and 5-fold lower, respectively, than in the wild type strain (Fig. 3A and B). Since Lrp in some cases requires leucine as an effector, β-galactosidase activity of the *traJ-lacZ* fusion was also tested in minimal medium with and without 50 mM leucine. Expression of the fusion was not affected by the addition of leucine (data not shown).

In contrast to the apparent positive effects of H-NS and Lrp on *traJ* expression, mutation in *cpxA* had only a small effect while mutations in *cpxR*, *relA spoT*, *himA* and *rpoS* had no significant effect on the β-galactosidase activity of the *traJ-lacZ* fusion (data not shown).

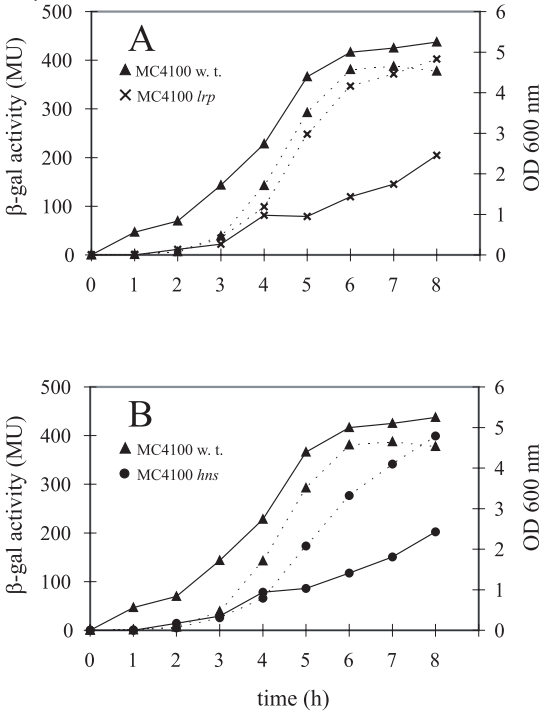

Fig. 3. **β-Galactosidase activity of the *traJ-lacZ* fusion**
At various time points (0-8 h), the optical density (OD600) of the culture was measured (dotted lines) and aliquots were assayed for β-galactosidase activity (expressed in Miller Units, MU) (solid lines).
(A) β-Galactosidase activity of the *traJ-lacZ* fusion in the MC4100 w. t. strain (▲) and in the MC4100 *lrp* mutant strain (×).
(B) β-Galactosidase activity of the *traJ-lacZ* fusion in the MC4100 w. t. strain (▲) and in the MC4100 *hns* mutant strain (●).
The experiments were performed in duplicate and a representative result is shown.

Competitive RT-PCR reveals lower mRNA levels in *hns* and *lrp* mutants

To ascertain whether, H-NS and Lrp act at the level of transcription of *traJ*, comparative analysis of mRNA in corresponding mutant strains was performed using competitive RT-PCR. Competitive RT-PCR is based on co-amplification of a competitive template, competing for the same oligonucleotide primers as the target RNA. The amplified products can be distinguished by a difference in size (16). The FinOP system reduced *traJ* mRNA levels below the level of "quantifiable" RT-PCR detection (data not shown). pTJ1 *lacZ* mRNA was therefore used as the target and the Δ*lacZ* RNA as a competi-

tive template. By comparing the intensity of the band due to *lacZ* mRNA with the band due to the defined amounts of the Δ*lacZ* RNA that were added to the sample, semi-quantitative results were obtained. Analysis of the RT-PCR results demonstrated that the Δ*lacZ* band was much more intense for both mutant strains than for the wild type strain (Fig. 4), indicating reduced levels of *lacZ* mRNA. These data, which are consistent with the observed reduction in β-galactosidase activity in the mutant strains, suggest that H-NS and Lrp likely exert their effect at the transcriptional level.

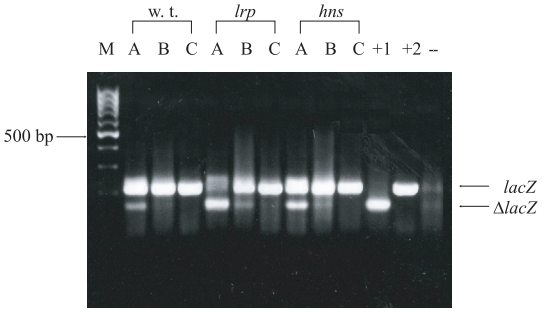

Fig. 4. **Semi-quantitative determination of *traJ-lacZ* mRNA in the wild type, *lrp* and *hns* mutant strains with competitive RT-PCR**

RT-PCR was performed on mRNA templates isolated from the wild type MC4100 (w. t.), *lrp* (*lrp*) and *hns* mutant (*hns*) in the presence of 50 pg (lanes A), 5 pg (lanes B) or 0.5 pg (lanes C) of competitive Δ*lacZ* RNA . +1 and +2 are positive controls, plasmid pINP2 with Δ*lacZ* and pTJ1 with the intact *lacZ* fragment, respectively. – is the negative control consisting of RT-PCR with water instead of mRNA. M – 100 bp DNA ladder (MBI Fermentas).

Note the change in *lacZ*-Δ*lacZ* ratio indicating a reduction in *traJ* mRNA in the *lrp* and *hns* mutant strains.

Lrp and H-NS proteins bind to the *traJ* promoter region

To further unravel the mechanism of regulation of *traJ-lacZ* expression by Lrp and H-NS, we assessed the abilities of the proteins to bind to the *traJ* promoter region. Mobility shift DNA-binding experiments were performed with pure Lrp and H-NS proteins and a 226 bp DNA fragment corresponding to the *traJ* promoter. As shown in Fig. 5, a 5 nM concentration of the Lrp protein was sufficient to promote significant retardation in the electrophoretic mobility of the 226 bp fragment. The fragment was also retarded by H-NS when the concentration of H-NS reached 500 nM. Shifts at similar concentrations of protein were observed for the respective positive controls, binding of Lrp to the *gltBDF* promoter and H-NS to the *proU* promoter (data not shown).

A competitive agarose gel shift assay was performed to determine the specificity of Lrp and H-NS protein binding to the *traJ* promoter. At concentrations needed to retard the *ptraJ* fragment both proteins also retarded some, but not other "random" DNA fragments of a 100 bp ladder used as competitive DNA (data not shown).

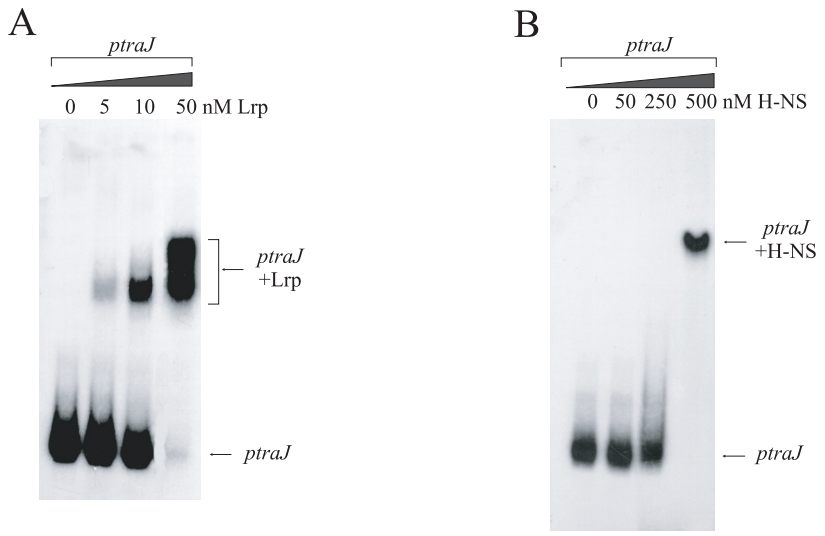

***Fig. 5*. Lrp and H-NS bind to the *traJ* promoter region**
Electrophoretic mobility shift assay with purified Lrp (A) and H-NS (B) and a 226 bp DNA fragment encompassing the P_{traJ} regulatory region (*ptraJ)*. The arrows indicate the ^{32}P-end labelled *ptraJ* fragment and the shifted protein-DNA complex. The amount of added protein is indicated.

Mutations in *hns* and *lrp* reduce conjugal transfer frequencies of pRK100

To confirm that Lrp and H-NS are activators of pRK100 conjugal transfer, mating experiments were performed and transfer frequencies from *hns, lrp* and the isogenic wild type strain MC4100 were compared (Table 2).

***Table 2.* Conjugal transfer frequencies of plasmid pRK100 from the MC4100 w. t. and mutant strains**

Strain	Conjugal frequency[a]	Strain	Conjugal frequency	Strain	Conjugal frequency
Serial A		**Serial B**		**Serial C**	
MC4100 w. t.	1.5×10^{-4}	MC4100 w. t.	5.1×10^{-4}	MC4100 w. t.	3.9×10^{-4}
MC4100 *lrp*	4.1×10^{-5}	MC4100 *hns*	9.4×10^{-7}	MC4100 *himA*	3.3×10^{-4}
MC4100 *rpoS*	2.2×10^{-4}			MC4100 *relA spoT*	5.0×10^{-6}

[a] Plasmid transfer frequencies were calculated per donor cells; experiments were performed in duplicate and a representative experiment is presented.

From both *hns* and *lrp* mutant strains conjugal transfer frequencies were significantly reduced, compared with transfer from the wild type MC4100 strain. Transfer frequencies, calculated per donor cell, were approximately 540-fold lower from the *hns* mutant and 4-fold lower from the *lrp* mutant, compared with the wild type MC4100 strain. Mutations in *cpxA*, *cpxR*, *himA*, *rpoS* and *sdiA* had no significant affect on conjugal transfer, but transfer was significantly reduced in a *relA spoT* mutant strain, producing no ppGpp. Together, these results are consistent with H-NS and Lrp being activators of conjugal transfer of plasmid pRK100.

4. DISCUSSION

Synthesis of the conjugative apparatus as well as DNA transfer itself represent a heavy metabolic burden to bacteria and the presence of receptors on the surface of conjugative pili bears the risk of bacteriophage infection. These factors demand conjugation to be a tightly regulated process. Regulation of F-like transfer has been extensively studied and has been demonstrated to be affected by environmental stimuli as well as plasmid and host factors (12). Yet, the molecular mechanisms underlying the regulation through environmental signals and host factors are still poorly understood. Recently, we demonstrated that the cAMP-CRP complex enhances *traJ* promoter activity in the F-like plasmid pRK100 (Starčič, *et al*., in press). The TraJ protein is the central positive activator of F-like *tra* genes activating the P_{traY} promoter (Fig. 1). In the present study, we identified two additional host encoded regulators of *traJ* promoter activity. Nucleotide sequence analysis revealed potential Lrp and H-NS binding sites in the *traJ* promoter region. Comparison of β-galactosidase activity of a transcriptional fusion of the pRK100 *traJ* promoter and the *lacZ* gene in the wild type with that in *lrp* and *hns* mutant strains indicated that both regulators positively affect *traJ* expression. Competitive RT-PCR suggested that the regulatory proteins enhanced *traJ* transcription.

Both H-NS and Lrp are global regulators of gene expression as well as nucleoid-structuring proteins. Lrp has been shown to be a sequence specific DNA binding protein while H-NS is a sequence-independent DNA-binding protein with preference for curved DNA (36, 38). Because of their function as global regulators, theoretically Lrp and H-NS may exert their effect on *traJ* transcription through direct binding to the *traJ* promoter region or indirectly by affecting genes that control *traJ* promoter activity. The results of our mobility shift assays indicate that both proteins directly bind to the *traJ* promoter region. It should be noted that the specificity of binding was not high, as particularly H-NS was found to bind to some fragments used as competitor DNA in the assay. This result is not surprising, considering the sequence-independent binding properties of H-NS. Both Lrp and H-NS proteins have been shown to have multiple binding sites around promoters and form nucleoprotein structures, which influence transcription (3, 33). Lrp and H-NS may form a complex that influences *traJ* transcription. The relevance of the *in vitro* data indicative of positive regulatory effects of Lrp and H-NS on *traJ* transcription was demonstrated by the mating experiments that plasmid pRK100 conjugal transfer was considerably reduced from the *lrp* and *hns* mutants (Table 2).

Recently, Lrp has been shown to be an activator of conjugation by promoting *traJ* transcription of the *Salmonella enterica* F-like virulence plasmid pSLT. The Lrp binding site described by Camacho and Casadesús (6), is immediately upstream of the region studied in our investigation, and is also conserved in plasmid pRK100 (Fig. 6). Nucleotide sequence analysis of the pRK100 *traJ* regulatory region revealed three additional potential Lrp binding sites (Fig. 2). Comparative nucleotide sequence analysis shows that the potential Lrp and H-NS binding sites described in this work are conserved in other F-like plasmids (Fig. 6).

From the other potential regulatory genes that we investigated, only *cpxA* showed a minor effect on *traJ-lacZ* transcription. This result seems at variance with work that showed that several *cpx* mutations affected the accumulation of TraJ (32). At this point however, it should be noted that this work was performed with plasmid F, while we adopted the plasmid pRK100 as a model system. Even though the *tra* regions of F-like plasmids have a common organisation, subtle differences in the regulation of transfer appear to exist. For example in R1 and R100 but not in F, *traJ* expression has been shown to be linked to *traM* (9, 27). Thus, caution is warranted in comparing the regulatory effects in different F-like plasmids.

A second noteworthy observation from the experiments with the other regulator mutants was the apparent absence of a regulatory effect of *relA spoT* on *traJ* transcription. Mutation of *relA spoT*,

which prevents the production of the stress alarmone ppGpp, has been demonstrated to reduce conjugal transfer in pRK100 (Table 2). Apparently this effect is not caused via regulation of *traJ* promoter activity. This finding once more illustrates the complexity of the regulation of the conjugation machinery in which TraJ is an important, but not the only factor, that is being regulated. How ppGpp affects conjugation awaits further investigation.

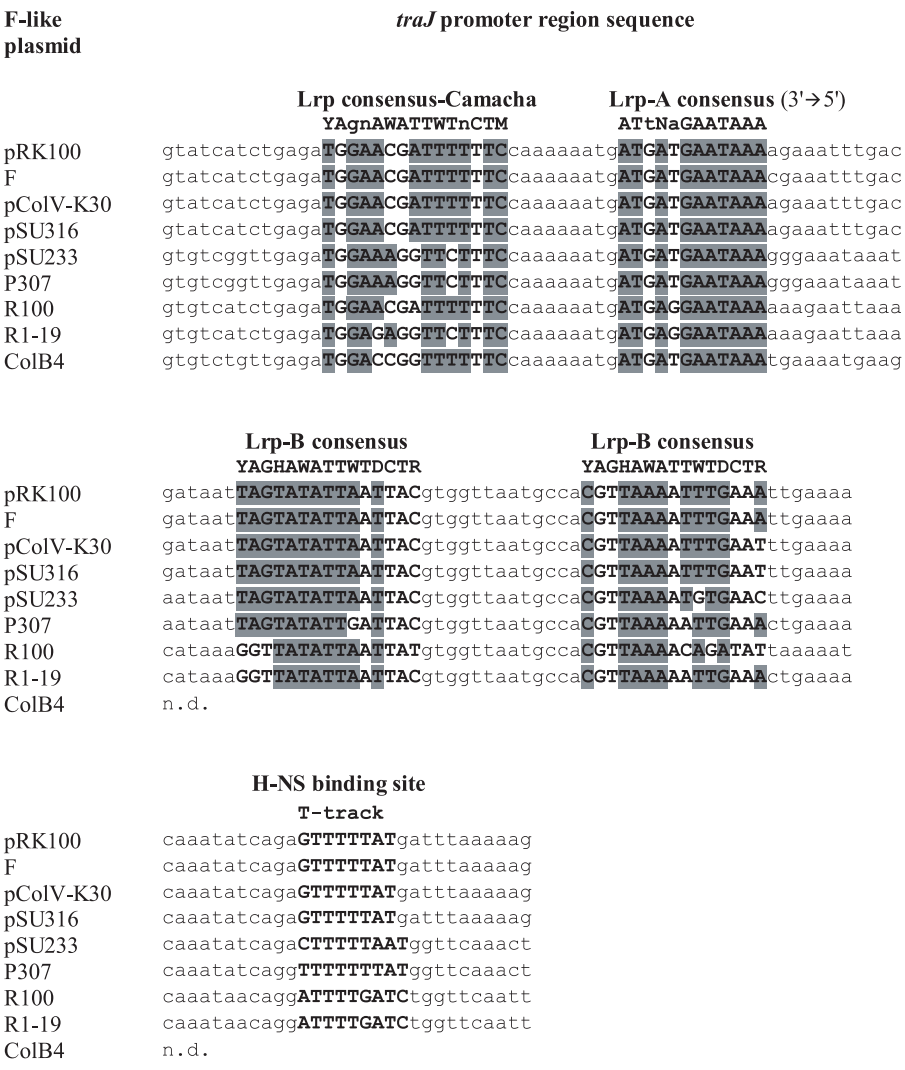

Fig. 6. **Sequences of the *traJ* regulatory region of several F-like plasmids, which are similar to the consensus Lrp and H-NS binding sites**

The Lrp-Camacho site was described by Camacho and Casadesús (6). The Lrp-A site is based on the consensus sequence described by Calvo and Matthews (5) and the Lrp-B sites are based on the consensus sequence described by Cui, *et al.*(8).

D = A or G or T; H = A or C or T; K = G or T; M = A or C; N = A or C or G or T; R = A or G; S = C or G; W = A or T and Y = C or T.

n.d. not determined

The mechanism by which Lrp and H-NS enhance *traJ* promoter activity remains to be defined. In a previous study we demonstrated that the cAMP-CRP complex enhances *traJ* promoter activity and that the CRP binding site is centred around position –67.5 upstream of the *traJ* transcription start site in pRK100 (Starčič, *et al*., in press). A cAMP-CRP binding site centred at –68.5, has also been demonstrated for *csiD*, a stationary phase inducible σ^S-dependent gene in *E. coli* (22). In a recent publication Germer, *et al*. (15) observed that, for the *csiD* promoter, the location of the activator site at –68.5 is a factor contributing to the pronounced $E\sigma^S$ selectivity at the *csiD* promoter. On the basis of β-galactosidase activity of the *traJ-lacZ* fusion and results of mating experiments we can conclude that, *traJ* expression in pRK100 is independent of σ^S and thus that positioning of the CRP binding site in the *traJ* regulatory region is not connected with σ factor selectivity. Despite this difference, it is interesting to note that cAMP-CRP, H-NS and Lrp are involved in the expression of *csiD* with H-NS and Lrp modulating, probably directly, activation of *csiD* by cAMP-CRP (15). Thus, it can be imagined that, in pRK100, binding of H-NS and Lrp to the DNA causes a conformational change in the *traJ* promoter, which facilitates binding of the cAMP-CRP complex to the CRP binding site centred at –67.5. As comparative sequence analysis indicates that the CRP binding site and the consensus Lrp and H-NS sequences are conserved among a number of F-like plasmids, this mechanism may also operate in other F-like plasmids.

Gene exchange in bacteria promotes adaptation to environmental challenges and conjugation is one of the main mechanisms responsible for horizontal gene transfer. Incorporation of Lrp, H-NS, cAMP-CRP, and positioning of the *traJ* CRP binding site at –67.5, into regulation of *tra* function in pRK100 ensures fine tuning of conjugation to specific environmental conditions. As F-like plasmids frequently encode antibiotic resistances and virulence factors, the identification of new regulatory factors as well as variations in regulation of conjugal transfer, is crucial to develop approaches to prevent dissemination of plasmid-encoded genes.

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