Chapter 4

The Cyclic AMP-CRP Complex Regulates the Activity of the *traJ* Promoter of the *Escherichia coli* Conjugative Plasmid pRK100

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ABSTRACT

The TraJ protein is a central activator of F-like plasmid conjugal transfer. In search for regulators of *traJ* expression, we studied the possible regulatory role of the cAMP-CRP complex in *traJ* transcription using a *traJ-lacZ* reporter system. Comparison of the enzyme activities in the wild type *E. coli* strain MC4100 with that in a *cya* and *crp* mutant indicated that disruption of the formation of the cAMP-CRP complex negatively influenced the activity of the *traJ* promoter of the F-like plasmid pRK100. The defect in the *cya* mutant was partially restored by the addition of exogenous cAMP. Competitive RT-PCR using RNA isolated from the wild type and mutant strains showed that the cAMP-CRP complex influenced the level of TraJ transcript. Electrophoretic mobility shift assays with purified CRP demonstrated direct binding of CRP to the *traJ* promoter region. DNaseI footprint experiments mapped the CRP binding site around position –67.5 upstream of the assumed *traJ* promoter. Targeted mutagenesis of the *traJ* promoter region confirmed the location of the CRP binding site. Consistent with the demonstrated regulation of TraJ by the cAMP-CRP complex, mutants with defects in *cya* or *crp* exhibited reduced conjugal transfer from pRK100.

Key words: gene regulation, *traJ*, cAMP, CRP, conjugal transfer, F-like plasmid

1. INTRODUCTION

Conjugation leads to the transfer of genetic material from one bacterium to another and is directed by conjugative plasmids. One family of conjugative plasmids are the F-like plasmids present in *Escherichia coli* and related species. F-like plasmids carry an ~ 33 kb long transfer (*tra*) region that harbours approximately 40 genes responsible for conjugal transfer (Fig. 1A). The expression of the *tra* genes is tightly regulated by both plasmid and chromosomally encoded proteins (8), although subtle differences exist among the various F-like plasmids (5, 25). The main plasmid-encoded positive regulator of conjugation is the 27 kD protein TraJ, which is required for the initiation of high levels of transcription from the major *tra* promoter, P_{traY}. Full activation of P_{traY} of plasmids F and R1 also requires the chromosomally encoded ArcA protein, which is part of the ArcA/B two component system that responds to oxygen (30). In plasmid F and R100, the TraY protein further stimulates its own promoter and this autoactivation enhances the synthesis of proteins that form the scaffolding of the conjugation machinery. TraY also induces DNA bending and stimulates nicking at the origin of transfer in co-operation with IHF (22). This leads to the expression of the gene *traM*, which is essential for DNA transfer of F and R1. Eventually, TraM autorepression limits the activity of the *tra* operon. In R1 and R100, but not in F, *traJ* expression has been shown to be linked to *traM* (5, 25).

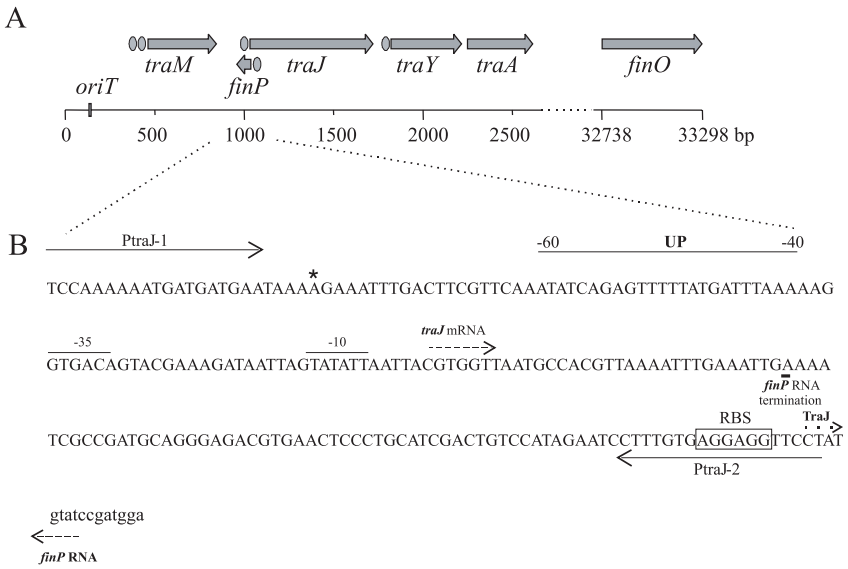

Fig. 1. **Schematic representation of the F-like plasmid transfer region**
(A) F-plasmid *tra* operon. Schematic presentation of the first 2700 bp and the last 600 bp of the 33.3 kb-sized *tra* operon (11). The origin of transfer (*oriT*), the *traM*, *traJ*, *traY*, *traA* and *finO* genes and the *finP* antisense RNA are indicated. Promoters are indicated as filled circles.
(B) Genetic organisation of the *traJ* promoter region of the F-like plasmid pRK100. The –35 and –10 promoter regions, the positions of transcription initiation (*traJ* mRNA and *finP* RNA) and termination (*finP* RNA termination), the ribosomal binding site (RBS) as well as the initiation of the TraJ translation (TraJ) are indicated. The upstream element (UP) from –40 till –60 is also depicted. Binding sites of the oligonucleotide primers PtraJ-1 and PtraJ-2 are marked with solid arrows. The indicated sequence of the pRK100 plasmid differs at one base (indicated by an asterisk) from the published sequence of the same region of the F plasmid (Genbank number U01159).

Even though TraJ is a central positive regulator of the transfer region, the knowledge of the mechanisms that regulate the expression of the *traJ* gene itself, is limited. TraJ expression is regulated at the translational level through the fertility inhibition FinOP system (7, 10). Fertility inhibition is imposed by the action of two *tra* gene products: FinP, the antisense RNA molecule complementary to the 5' untranslated region of *traJ* mRNA, and FinO, which increases the concentration of FinP and thereby promotes the formation of the *traJ*-FinP duplex. This *traJ*-FinP duplex is degraded by RNase III, thereby decreasing the amount of the *traJ* mRNA, and hence TraJ protein (15). The combined actions of FinO and FinP repress F transfer by 100 – 1000-fold while FinP, by itself, represses F transfer by only 6-fold (18). Further, it has been demonstrated for plasmid F that in *cpx* mutant strains the TraJ protein level is reduced (29).

Considering the importance of TraJ in bacterial conjugation promoted by F and other F-like plasmids, we focussed our work on the discovery of mechanisms of transcriptional regulation of *traJ* expression. In these studies, we used pRK100, a ~145-kb natural conjugative F-like plasmid isolated from a uropathogenic *Escherichia coli* strain as a model system. This plasmid has been partially characterised (1) and its *tra* region has been partially sequenced. At the nucleotide level, the sequenced *tra* genes (*traM, finP*, *traJ*, *traY*, *traD*, *finO*) were most similar to genes of the plasmid F (4). The regulation of the pRK100 *traJ* promoter was studied using a reporter system consisting of a transcriptional fusion of the *traJ* promoter and the *lacZ* gene. The expression of this construct was studied in the absence of the FinOP fertility inhibition system to facilitate the identification of factors acting at the level of *traJ* transcription initiation. Our data indicate that the expression of the *traJ* gene varies with the growth cycle and that the cyclic AMP (cAMP)-CRP complex is a positive regulator of *traJ* transcription. The regulatory role of this complex was supported by results from gel retardation assays, DNaseI footprinting experiments, targeted mutagenesis of the CRP binding site, and mating assays.

2. MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) medium with aeration at 37 °C, unless stated otherwise. When appropriate, bacteria were grown in minimal medium M63 (24) supplemented with 0.2% glucose, 0.1% casamino acids and 1 μg/ml thiamine. Conditioned medium was prepared by growing strain MC4100 in LB medium (12 h, 37 °C with aeration), removal of the bacteria by centrifugation, and filter sterilisation of the medium. Conditioned medium was used within 24 h. Starvation for glucose, casamino acids or phosphate was achieved by adding only 1/5 of the usual concentration of these compounds to M63 medium. When appropriate, cAMP (10 mM) was added to LB. Ampicillin (100 μg/ml) was added to the growth media, as needed.

General DNA manipulation techniques

Plasmid DNA isolation, ligation, and transformation experiments were performed using standard methods (27). 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 Analyser.

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		3
RH74	MC4100 Δ*cya851 ilv::*Tn*10*		20
SBS688	MC4100 Δ*crp*39		13
DH5α	*thi*-1 *hsdR*17 *gyrA*96 *recA*1 *endA*1 *glnV*44 *relA*1 Φ80d*lacZ*ΔM15 *phoA*8		NCCB
Plasmids	**Properties / Vector**	**Insert**	**Source or reference**
pCB267	*lacZ* promoter probe vector		28
pTJ1	pCB267	210 bp *traJ* promoter	This paper
pGEM-T Easy	T-vector for cloning of PCR products		Promega
pUC19	Multi-purpose cloning vector		33
pINP2	pGEM-T Easy	158 bp Δ*lacZ*	This paper
pYZCRP	CRP encoding		R. H. Ebright[1]

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Construction of the *traJ-lacZ* fusions

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 was amplified by PCR using primers PtraJ-1 (5'-CGGGATCC-TCCAAAAAATGATGATGAAT-3') and PtraJ-2 (5'-GCTCTAGA-ATAG-GAACCTCCTCACAAAG-3') (Fig. 1B). PtraJ-1 and PtraJ-2 contained *Bam*HI and *Xba*I restriction sites to facilitate cloning into the plasmid pCB267 (28), generating pTJ1. To generate mutations in the CRP binding site, primers with defined nucleotide substitutions were used in the PCR reactions. For mutagenesis of the left part of the CRP binding site (TTTGA → GTCGA) the primer PtraJ-1-ML (5'-CGGGATCC-TCCAAAAAATGATGATGAATAAAAGAAA**GTCGA**CTTCGTTCAAATATCA-GAG-3') was used in combination with PtraJ-2, while for mutagenesis of the right part of the CRP binding site (TCAAA → ATCGA) the primer PtraJ-1-MR (5'-CGGGATCC-TCCAAAAAATGATGA-TGAATAAAAGAAATTTGACTTCGT**ATCGA**TATCAGAG-3') was used in combination with PtraJ-2. The nucleotides that were altered are given in bold. To enable cloning into pCB267 both primers, PtraJ-1-ML and PtraJ-1-MR contained a *Bam*HI restriction site. DNA sequencing confirmed that the correct fragments had been cloned.

β-Galactosidase assays

For measurements of β-galactosidase activity, relevant strains were grown (37 °C) overnight in LB medium, diluted (1/500) into fresh LB or M63 medium, re-grown to an OD_{600} of 1, and again diluted (1/500) into fresh LB medium, M63 or conditioned medium. Samples were periodically removed and assayed for β-galactosidase activity. β-Galactosidase assays were performed essentially as described (21) with bacteria treated with sodium dodecyl sulfate (SDS)-chloroform and washed with Z buffer and *o*-Nitrophenyl-β-D-galacto-pyranoside (ONPG) as a substrate. Enzyme activity was defined in Miller units (MU) (21).

Generation of Δ*lacZ* RNA for use as RT-PCR competitive template

A 215 bp fragment of the *lacZ* gene was generated by PCR using plasmid pTJ1 as a template and the primers lacZ-1 (5'- ACGATGCGCCCATCTACACC-3') and lacZ-2 (5'- ACGACTGTCCT-GGCCGTAAC-3'). The generated DNA fragment was digested with *Mse*I and the restriction fragments were ligated with T4-ligase (Amersham Pharmacia Biotech). PCR amplification of the ligation

mixture with the primers lacZ-1 and lacZ-2 yielded several products, including the desired 158 bp Δ*lacZ* product. This fragment was isolated from a 4% NuSieve 3:1 agarose gel (BMA, BioWhittaker Molecular Applications) and cloned into pGEM-T Easy vector (Promega), generating the plasmid pINP2. Sequence analysis confirmed that, apart from the deletion, no other base changes had been introduced. pINP2 was cut with the restriction endonuclease *Pst*I and used in the Riboprobe In Vitro Transcription System (Promega) as the template for the Δ*lacZ* RNA. The amount of *in vitro* Δ*lacZ* RNA transcript was determined spectrophotometerically.

RNA isolation

RNA was isolated from log-phase grown bacteria passaged twice as indicated above, and then grown in LB medium for 2 hours. For each sample, RNA from the same amount of bacteria was purified with RNAzol™B (Campro Scientific). After isopropanol precipitation, RNA was dissolved in 40 µl of H_2O, and stored at −70 °C. Prior to analysis of the *traJ-lacZ* mRNA in the competitive RT-PCR (see below), 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) until no contamination of DNA was detected.

Competitive RT-PCR

In the competitive RT-PCR (12), 1 µl of DNA-free RNA sample of the relevant strain and 1 µl of competitive template (either 50, 5 or 0.5 pg of Δ*lacZ* RNA) were used together with the lacZ-1 and lacZ-2 oligonucleotide primers in the Access RT-PCR System (Promega). The RT-PCR involved 45 min of reverse transcription at 48 °C, 2 min of AMV reverse transcriptase inactivation and RNA/cDNA/oligonucleotide primer denaturation at 94 °C, and 40 cycles of denaturation (30 s at 94 °C), annealing (30 s at 58 °C) and extension (30 s at 72 °C), followed by one final extension step (10 min at 72 °C). The RT-PCR products were separated on 2% agarose gels. The competitive RT-PCR was performed twice on two different samples and gave similar results in all tests. The absence of DNA was verified by running the competitive RT-PCR with RNA as template but with the addition of water instead of reverse transcriptase.

EMSA

The electrophoretic mobility shift assay (EMSA) was performed as described (2). 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). CRP binding reactions were performed essentially as described (31). In brief, 20 ng of labelled DNA fragment were mixed with variable amounts of purified CRP (generously provided by G. S. Lloyd and S. Busby) in the presence of cAMP (18 mM) in a final volume of 20 µl, and incubated for 30 minutes at 37 °C. The 214 bp *Bam*HI–*Pvu*II fragment of pUC19 carrying the *lacZ* promoter was used as a positive control. After the binding reaction, 1 µl of loading buffer (0.1% brom-phenol blue and 50% glycerol in water) was added and samples were loaded onto a 5% PAGE gel and run at a constant voltage (240 V). The gels were transferred to Whatman 3MM paper, dried, and autoradiographed.

Mobility shift assays using agarose gels were performed as described (16) except that the reaction volume was 20 µl and incubations were performed for 15 min at 37 °C. The 100 bp DNA ladder (MBI Fermentas) was used as competitor DNA.

DNase I footprinting

DNaseI footprint experiments were essentially performed as described (32). For these experiments, the 226 bp *PtraJ* fragment was amplified using the non-labelled oligonucleotide primer PtraJ-1

and the T4 polynucleotide kinase (Gibco BRL) [γ-^{32}P]ATP end-labelled primer PtraJ-2. From the labelled PCR product, 3 µl were used in a 20 µl binding reaction with variable amounts of purified CRP protein as described for the EMSA. After incubation, 0.06 U of DNaseI (Amersham Pharmacia) was added and the mixture was incubated for 3 min at room temperature. The reaction was stopped by the addition of 1 µl of 60 mM EDTA followed by 10 min of incubation at 65 °C. DNA fragments were purified with QIAquick Nucleotide Removal Kit (Qiagen) and eluted in 30 µl of H_2O. Six µl of the final sample were analysed by denaturing polyacrylamide 6% gel (National Diagnostics) electrophoresis run at a constant power 60 W with a DNA sequence ladder in parallel. The DNA sequence ladder was generated with the appropriate primer using the Sequenase Version 2.0 DNA Sequencing Kit (USB).

Mating assay

Conjugation experiments were essentially carried out as described (9) except that the strains were grown in LB and overnight cultures of donor and recipient strains were diluted 100-fold and incubated for 2 hours with aeration at 37 °C, and that the mating mixture consisted of 0.05 ml of the donor and 0.45 ml of the recipient culture and 0.5 ml of fresh LB. When appropriate the mating mixture was supplemented with cAMP (10 mM). Mating was performed for 2 hours at 37 °C. Transconjugants were selected on LB media supplemented with the appropriate antibiotics. Conjugal transfer frequencies were expressed as the portion of transconjugants to recipient or donor cells.

3. RESULTS

Glucose starvation enhances *traJ* expression in a CRP dependent fashion

Measurement of β-galactosidase activity in *E. coli* strain MC4100 carrying the *traJ-lacZ* transcriptional fusion at various stages of growth in LB medium, demonstrated a gradual increase in enzyme activity with the duration of growth (Fig. 2A). To determine whether the increase in activity was caused by a depletion of nutrients, we assayed β-galactosidase activity after transfer of log-phase grown bacteria to conditioned LB medium. In this medium, in which the *E. coli* were unable to grow (Fig. 2A), a much stronger increase in enzyme activity was found, which was already apparent at 1 h of incubation and which reached its peak at 4 h of incubation (Fig. 2A).

The factors in the medium responsible for the induction of β-galactosidase activity were sought by monitoring bacterial enzyme activities during growth in M63 minimal medium with variable amounts of glucose, casamino acids or phosphate. These experiments indicated that *traJ-lacZ* transcription varied with the availability of glucose in the medium (Fig. 2B), while starvation for casamino acids or phosphate had no effect (data not shown).

Since a well established bacterial response to the level of glucose is via the cAMP-CRP complex, we measured *traJ-lacZ* expression levels in a *cya* and *crp* mutant background. As illustrated in Fig. 2C, β-galactosidase activity in both mutants was considerably lower than in the parent strain. Complementation experiments demonstrated a strong increase in β-galactosidase activity in the *cya* mutant when grown in the presence of 10 mM cAMP (Fig. 2D), while no significant effects were observed for the parental strain (data not shown). Complementation of the *crp* mutant via introduction of a plasmid encoding CRP (pYZCRP) was not successful as it led to a loss of the pTJ1 plasmid from the cell. Overall, the results support the notion that the cAMP-CRP complex is required for the induction of *traJ* expression during conditions of glucose starvation.

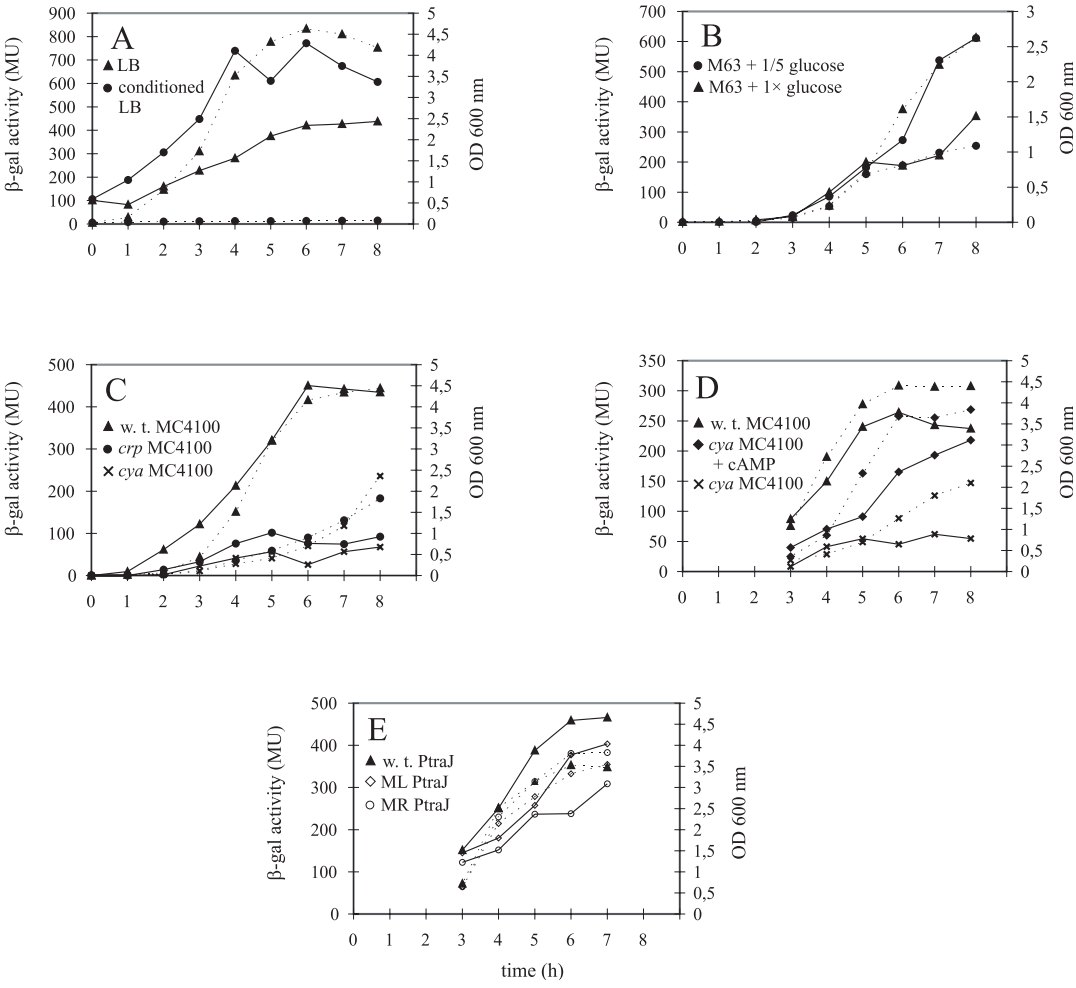

Fig. 2. **β-Galactosidase activity of the *traJ-lacZ* fusion**

At various time points (0-8 h), the optical density (OD_{600}) 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 w. t. strain MC4100 grown in LB (▲) and in conditioned LB medium (●).

(B) β-Galactosidase activity of the *traJ-lacZ* fusion in the w. t. strain MC4100 grown in M63 medium with 1× glucose (▲) and with 1/5 glucose (●).

(C) β-Galactosidase activity of the *traJ-lacZ* fusion in the w. t. strain MC4100 (▲), and in the MC4100 *cya* (×) and *crp* mutant strains (●) grown in LB medium

(D) β-Galactosidase activity of the *traJ-lacZ* fusion in the w. t. strain MC4100 grown in LB medium (▲), and in MC4100 *cya* grown in LB medium with (◆) and without (×) 10 mM cAMP.

(E) β-Galactosidase activity in strain MC4100 carrying the wild type *traJ-lacZ* fusion (w. t. PtraJ, ▲) or the *traJ-lacZ* fusion in which the left (ML PtraJ, ◇) or right (MR PtraJ, ○) part of the CRP binding domain has been altered.

Experiments were carried out 3-4 times and representative results are shown.

The cAMP-CRP complex regulates the level of the *traJ* transcript

To ascertain that the cAMP-CRP complex influences the level of the *traJ* transcript, we compared the amounts of *traJ-lacZ* mRNA in the *cya* and *crp* mutant with that in the parent strain after 2 h of growth in LB medium using a competitive RT-PCR (12). The strategy used for quantification involved co-amplification of a competitive template, competing for the same oligonucleotide primers as the target RNA, but which, after amplification, could be distinguished from the target by a difference in size. Since the FinOP system reduced *traJ* mRNA levels below the level of the reliable detection by RT-PCR (data not shown), we used pTJ1 derived *traJ-lacZ* mRNA as the target and the Δ*lacZ* RNA as a competitive template. In these experiments semi-quantitative results were obtained by comparing the intensity of the DNA bands due to *traJ-lacZ* mRNA and the Δ*lacZ* RNA that were added to the sample (Fig. 3).

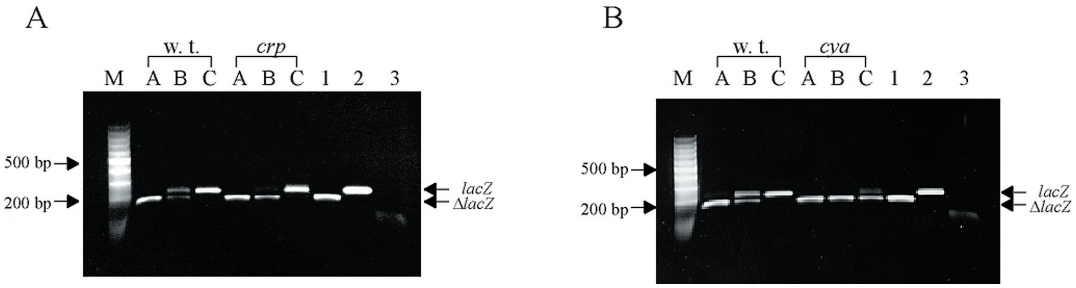

***Fig. 3.* Semi-quantitative determination of *traJ-lacZ* mRNA in the wild type and *crp* and *cya* mutant strains with competitive RT-PCR**

RT-PCR was performed on RNA isolated from the wild type MC4100 (w. t.) and *crp* mutant (*crp*) (panel A) and from the wild type MC4100 (w. t.) and *cya* mutant (*cya*) (panel B) in the presence of 50 pg (lanes A), 5 pg (lanes B) or 0.5 pg (lanes C) of competitive Δ*lacZ* RNA. Plasmid pINP2 carrying Δ*lacZ* (lane 1) and plasmid pTJ1 (lane 2) carrying the intact *lacZ* fragment, served as positive controls; RT-PCR with water instead of reverse transcriptase served as negative control (lane 3). M – 100 bp DNA ladder (MBI Fermentas).

Application of this approach to the wild type, *cya* and *crp* mutant strains carrying pTJ1 revealed that in the mutant strains much less *traJ-lacZ* mRNA was present than in the wild type strain (Fig. 3 panel A – lane B, Fig. 3 panel B - lanes B and C). This finding explains the observed reduced β-galactosidase activity in the mutant strains and suggests that this activity is due to the cAMP-CRP complex-dependent stimulation of transcription initiation.

Purified CRP protein binds to the *traJ* promoter region

To establish whether CRP regulates the level of *traJ* transcript by binding to the *traJ* promoter region electrophoretic mobility shift assays (EMSA) were performed. EMSA with a 226 bp PCR amplified and gel purified *ptraJ* fragment (containing the *traJ* promoter) and various concentrations of purified CRP showed that 5 nM of CRP was sufficient to cause a retardation of the migration of the *traJ* fragment (Fig. 4A).

The specificity of the CRP binding was confirmed in a competitive agarose gel shift assay with the DNA of a 100 bp ladder as a control. These experiments indicated that the addition of 0.5 μg CRP (1.1 μM) was already sufficient to cause a shift in the 226 bp *ptraJ* fragment, while much higher concentrations were needed to alter the migration of the "random" DNA of a 100 bp ladder (Fig. 4B). These results strongly suggest that CRP specifically bound to the *traJ* promoter region.

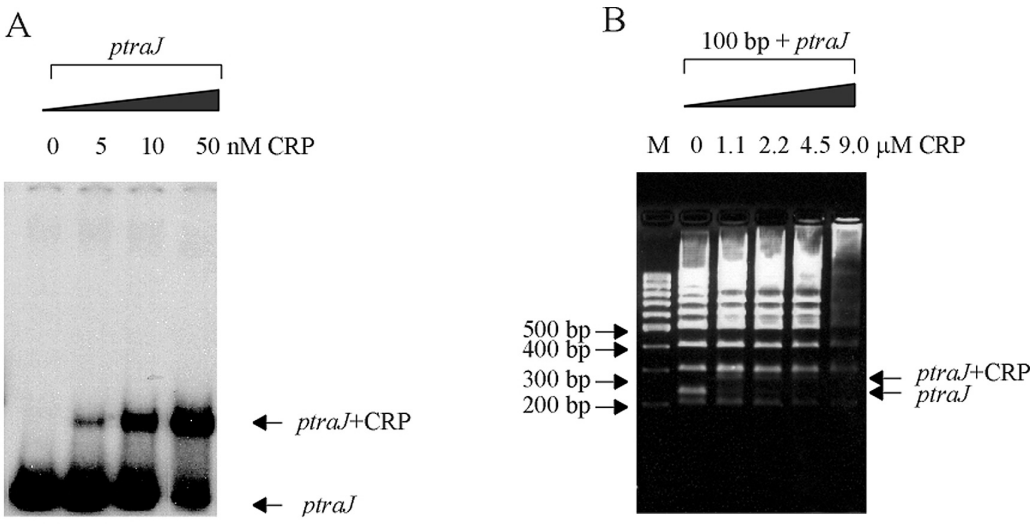

Fig. 4. **EMSA demonstrating the binding of purified CRP to the *traJ* promoter region**
(A) Migration of the ^{32}P-end labelled 226 bp PCR *traJ* promoter fragment (*ptraJ*) in the absence and presence of the indicated concentrations of purified CRP as determined by PAGE and autoradiography. The arrows indicate the *traJ* region and the shifted protein-DNA complex.
(B) Migration in an agarose gel of the 226 bp PCR *traJ* promoter fragment (*ptraJ*) after the addition of the indicated concentrations of CRP in the presence of 100 bp DNA ladder. Note the specificity of the shift of the *ptraJ* fragment (arrow) in the presence of CRP. M – 100 bp DNA marker.

Mapping of the CRP binding domain by DNaseI footprint experiments

In order to locate the CRP binding site on the *traJ* promoter region, we performed DNaseI protection assays. In these experiments, purified CRP was incubated with a radiolabelled *traJ* promoter fragment, which was then digested by DNaseI and subjected to gel electrophoresis and autoradiography. As shown in Fig. 5, CRP protected a distinct DNA region against DNaseI digestion in a dose-dependent fashion. Comparison with the nucleotide sequence of the pRK100 *traJ* regulatory region, run in parallel, indicated that the protected region extended from the position –57 to –78, relative to the transcription site. The corresponding nucleotide sequence 5'-aaaTTTGAct-tcgtTCAAAtat-3' strongly resembled the CRP consensus binding site 5'-aaaTGTGAtctagaTCACAttt-3' (31) (Fig. 6).

Mutagenesis of the identified CRP binding site

To unequivocally demonstrate that the identified CRP binding sequence acts in the regulation of the *traJ* promoter, we performed site-directed mutagenesis. Mutations in the left (TTTGA → GTC-GA) and right (TCAAA → ATCGA) part of the identified CRP-binding site (Fig. 6) were introduced by PCR. The mutated *traJ-lacZ* fusions were cloned onto pCB267, which was then transferred to *E. coli* strain MC4100. As illustrated in Fig. 2E, mutagenesis of each of the domains of the CRP binding site led to a less profound increase in enzyme activity upon prolonged growth than observed for the *traJ-lacZ* fusion carrying the intact CRP binding site.

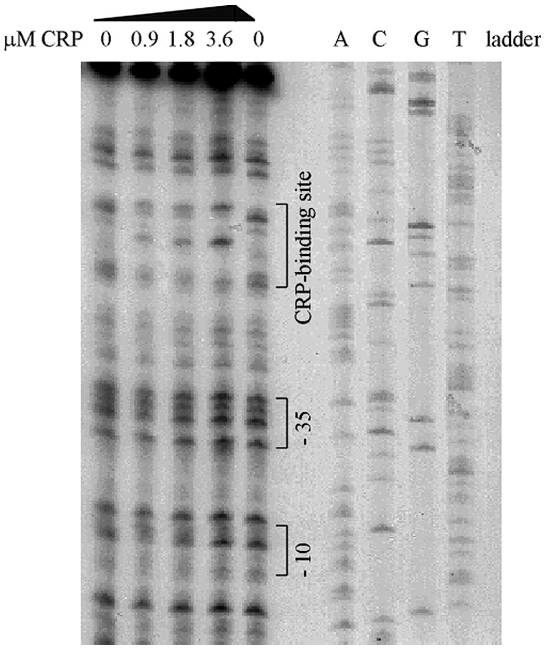

Fig. 5. **Mapping of the CRP binding site in the *traJ* promoter region as determined by DNaseI protection assay**
A 226 bp PCR ^{32}P labelled *traJ* promoter fragment was incubated with the indicated amounts of purified CRP, treated with DNaseI, and separated on 6% sequence gels. A nucleotide (A, C, G and T) sequencing reaction was run in parallel. The CRP-binding site, and the –10 and –35 promoter region are indicated.

Mutations in *cya* and *crp* inhibit conjugal transfer of pRK100

In an attempt to position our findings in a more natural setting, we investigated the apparent regulatory role of the cAMP-CRP complex in *traJ* transcription by measuring the efficiency of conjugal transfer of pRK100 for the wild type and *cya* and *crp* mutant strains. Conjugation experiments in which *E. coli* DH5α served as recipient strain demonstrated that the wild type strain MC4100 mated up to 100-fold more efficiently than the *cya* and *crp* mutant (Table 2). Addition of cAMP (10 mM) to the conjugative mixture considerably increased the mating efficiency of the *cya* mutant (Table 2) fully in line with the results with the *traJ-lacZ* fusion. Although the control of conjugation of F-like plasmids is very complex, these data support the idea that the cAMP-CRP complex influences *traJ* transcription in the natural pRK100 environment.

Table 2. **Conjugal transfer frequencies of plasmid pRK100**
Conjugal transfer of pRK100 was performed from the wild type (w. t.), *crp* and *cya* mutant strains into *E. coli* strain DH5α (2 h of conjugation) in the absence and presence of 10 mM cAMP.

Donor strain	Conjugal frequency (transconjugants/donor cells)		Conjugal frequency (transconjugants/recipient cells)	
	– cAMP	+ cAMP	– cAMP	+ cAMP
MC4100 w. t.	3.0×10^{-5}	3.6×10^{-5}	1.1×10^{-3}	1.3×10^{-3}
MC4100 *cya*	2.9×10^{-7}	2.2×10^{-6}	4.1×10^{-6}	3.1×10^{-5}
MC4100 *crp*	7.6×10^{-7}	7.7×10^{-7}	5.6×10^{-6}	5.7×10^{-6}

4. DISCUSSION

Conjugation is an important bacterial mechanism to transfer genetic material to other microorganisms. This process is facilitated via a large array of genes that encode the sophisticated conjugation system. Synthesis of the conjugative apparatus represents a heavy metabolic load and, due to the presence of receptors on the surface of conjugative pili, makes the bacterium vulnerable to bacteriophage infection. Conjugation of F-like plasmids is therefore a tightly regulated process resulting in only few bacteria synthesising a conjugative apparatus. It has been well-documented that the TraJ protein is the central positive regulator of F-like plasmid conjugal transfer. The expression of this protein is normally suppressed by the effects of the FinP antisense RNA and the FinO protein (7, 10). In this work, we provide direct evidence that the global regulator CRP acts as a positive transcriptional regulator of *traJ* expression via direct binding to the *traJ* promoter region.

The first indication that CRP positively influences *traJ* promoter activity was the increase in β-galactosidase activity in bacteria carrying a *traJ-lacZ* fusion during a prolonged period of growth and, more specifically, in conditioned medium in the absence of bacterial growth. This pointed to a depletion of nutrients as a signal for enhanced *traJ-lacZ* activity. The finding that glucose was an important factor in the regulation led us to hypothesise that CRP may act as a putative positive regulator molecule. Glucose starvation results in a rapid stimulation of cAMP synthesis, which in turn leads to the formation of high intracellular levels of the cAMP-CRP complex. The significant lower β-galactosidase activity in a *cya* and *crp* mutant carrying the *traJ-lacZ* fusion, the partial restoration of the defect in the *cya* mutant by the addition of exogenous cAMP, and the reduced *traJ-lacZ* mRNA levels detected by competitive RT-PCR collectively indicated the involvement of cAMP-CRP complex in the regulation of *traJ-lacZ* activity. The proposed function of CRP may form the basis of the observed association between intracellular levels of cAMP and the expression of transfer-related activities from F-like plasmids (14, 19).

Direct evidence that CRP regulates *traJ* promoter activity via specific recognition of a nucleotide sequence in the *traJ* promoter region was obtained in series of experiments: (a) mobility shift experiments demonstrated direct binding of CRP to the *traJ* promoter region, (b) DNaseI footprinting experiments showed that binding of purified CRP to a fragment carrying the *traJ* promoter region provided protection against DNAse I activity, and (c) targeted mutagenesis of the putative CRP binding sites in the *traJ* promoter region reduced the activation of TraJ under conditions of starvation. Binding of CRP to target promoter sequences is known to regulate gene expression. On interaction with cAMP, CRP undergoes a conformational change that enables binding of the cAMP-CRP complex to a distinct 22 bp DNA consensus sequence. This binding and the protein-protein interaction between CRP and RNA polymerase enhance the intrinsic promoter activity (17).

Mapping of the CRP binding domain in the *traJ* promoter region by DNaseI footprinting and confirmed by targeted mutagenesis, revealed that the binding site was centred around position –67.5 upstream of the assumed transcriptional start site of *traJ* and that it had strong similarity with the CRP consensus sequence (31). The obtained location at –67.5 differs from the one that has been putatively assigned for the F-plasmid in the database (Genbank Accession number U01159). This possible CRP site, which does not constitute a CRP consensus sequence (31), was proposed to be located in the first short stem-loop in the *traJ* mRNA. Comparative sequence analysis indicated that the identified CRP binding site in the *traJ* promoter region of pRK100 is conserved among other F-like plasmids (Fig. 6).

F-like plasmid	Sequence *traJ* promoter region
pRK100	gaataaaag-------**aaaTTTGA**ct**t**c**g**t**TCAAAt**at**cagagttttt**
pColV-K30	gaataaaag-------**aaaTTTGA**ct**t**c**g**t**TCAAAt**at**cagagttttt**
pSU316	gaataaaag-------**aaaTTTGA**ct**t**c**g**t**TCAAAt**at**cagagttttt**
F	gaataaacg-------**aaaTTTGA**ct**t**c**g**t**TCAAAt**at**cagagttttt**
pSU233	gaataaagggaaata-**aa**t**TTTGA**ct**t**t**g**t**TCAAAt**at**cagacttttt**
ColB4	gaataaatgaaaatg-**aa**g**TTTGA**gt**t**g**g**t**TCAAAt**at**aagaattttt**
P307	gaataaagggaaata-**aa**t**TTTGA**ct**t**t**g**t**TCAAAt**at**caggtttttt**
R100	gaataaaaaagaatta**aaa**GT**TGAt**t**t**act**TCAAAt**aa**caggattttg**
R1-19	gaataaaaaagaatta**aaa**GT**TGAt**t**t**gct**TCAAAt**aa**caggattttg**
CRP consensus	**aaaTGTGAtctagaTCACAttt**

***Fig. 6.* Sequence alignment of *traJ* promoter regions of different F-like plasmids**
Nucleotides that are identical to the CRP binding consensus sequence (31) are indicated in bold. The more conserved nucleotides are capitalised. Deviations from the CRP consensus sequence are grey-shaded.

Promoters activated by CRP have been grouped into three classes according to the location of the bound CRP. At class I CRP-dependent promoters the CRP binding sites are located at sites near positions –61.5, –71.5, –81.5 or –91.5 with respect to the transcription startpoint, whereas at class II CRP-dependent promoters the CRP-binding site is centred near position –41.5. In these cases, CRP and RNA polymerase are located at the same face of the DNA and CRP is thought to exert its effect by direct contact with the RNA polymerase (6, 23). Class III CRP-dependent promoters have the CRP-binding site situated further upstream and they require, besides the cAMP-CRP complex, also an additional regulator. Assuming that the transcription startpoint in the pRK100 *traJ* promoter is the same as in the related F-plasmid, the CRP binding site on pRK100 is located at position –67.5, which implies that CRP and the RNA polymerase bind at different faces of the DNA. This unusual position of a CRP binding site might be of particular interest, as it may indicate that besides the CRP-cAMP complex, an additional regulator binds to the DNA and assists CRP to establish contact with RNA polymerase. The pRK100 *traJ* promoter has many characteristics of a strong promoter. The putative –35 and –10 sequences exhibit strong similarity to the consensus sequence of *E. coli* promoters, and the distance between the –35 and –10 hexamers is 17 bp, which is optimal for promoter activity. Furthermore, a putative UP element is present upstream of the –35 region (Fig. 1) (26). This element is known to increase promoter activity. The regulation of the *traJ* promoter activity by the cAMP-CRP complex demonstrated in the present study, suggests that the initiation of transcription due to –35 and –10 sequences and the UP element may not be sufficient to increase *traJ* mRNA levels above those of FinP antisense RNA. The positive effect of cAMP-CRP complex on *traJ* transcription may be needed to derepress the transcription of the *tra* genes and to enable synthesis of the conjugation machinery. This scenario is particularly attractive as it relates conjugation activity to distinct environmental conditions (such as the limited availability of glucose) encountered by the bacteria. In combination with the strong negative regulation by the FinOP system, the effect of cAMP-CRP complex and perhaps additional regulatory intermediates, may enable fine tuning of the conjugation process, which is necessary to balance the efficient DNA transfer and the exposure of the required structural machinery to the environment. However, it should be noted that the control of conjugation is extremely complex and that much more work with intact conjugative plasmid is needed to firmly establish the role of the cAMP-CRP complex beyond the level of *traJ* transcription.

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