Published in final edited form as:
Mol Microbiol. 2009 June ; 72(5): 1087–1099. doi:10.1111/j.1365-2958.2009.06712.x.

TP0262 is a modulator of promoter activity of *tpr* Subfamily II genes of *Treponema pallidum* subsp. *pallidum*

Lorenzo Giacani[*], **Charmie Godornes**[*], **Maritza Puray-Chavez**[†], **Cristina Guerra-Giraldez**[‡], **Martin Tompa**[§], **Sheila A. Lukehart**[*], and **Arturo Centurion-Lara**[*,¶]

[*]Department of Medicine, University of Washington, Seattle (WA) 98104, USA

[†]Asociación Civil Selva Amazonica, Iquitos, Peru

[‡]Department of Microbiology, Universidad Peruana Cayetano Heredia, Lima 31, Peru

[§]Department of Computer Science and Engineering and Department of Genome Sciences, University of Washington, Seattle (WA) 98195, USA

Abstract

Transcriptional regulation in *Treponema pallidum* subsp. *pallidum* is poorly understood, primarily because this organism cannot be cultivated *in vitro* or genetically manipulated. We have recently shown a phase variation mechanism controlling transcription initiation of Subfamily II *tpr* (*T. pallidum* repeat) genes (*tprE*, *tprG*, and *tprJ*), a group of virulence factor candidates. Furthermore, the same study suggested that additional mechanisms might influence the level of transcription of these *tprs*. The *T. pallidum* genome sequence has revealed a few open reading frames (ORFs) with similarity to known bacterial transcription factors (TFs), including four catabolite activator protein (CAP) homologs. In this work, sequences matching the *E. coli* cAMP receptor protein (CRP) binding motif were identified *in silico* upstream of *tprE*, *tprG*, and *tprJ*. Using elecrophoretic mobility shift assay (EMSA) and DNaseI footprinting assay, recombinant TP0262, a *T. pallidum* CRP homolog, was shown to bind specifically to amplicons obtained from the *tpr* promoters containing putative CRP binding motifs. Using a heterologous reporter system, binding of TP0262 to these promoters was shown to either increase (*tprE* and *tprJ*) or decrease (*tprG*) *tpr* promoter activity. This is the first characterization of a *T. pallidum* transcriptional modulator which influences *tpr* promoter activity.

Keywords

Treponema pallidum; TP0262; CRP; *tpr* genes; transcriptional modulation

Introduction

Treponema pallidum, the causative agent of syphilis, is an obligate human pathogen unable to survive outside of its host. It cannot be genetically manipulated and no culture system has proven effective in supporting its long-term growth *in vitro* (Norris & Edmondson, 1986, Cox *et al.*, 1990). In an infected individual, the course of syphilis alternates between episodes of active clinical disease (primary, secondary, and tertiary stages) separated by periods of asymptomatic, latent infection. Primary and secondary syphilis are characterized by the appearance of typical lesions (a primary chancre, which marks the site of

[¶]Corresponding author: Mailing address: Department of Medicine, Box 359779; Harborview Medical Center, 325 Ninth Avenue, Seattle (WA) 98104-2499; Phone: 206 897-5364; Fax: 206 897-5363; Email: acentur@u.washington.edu.

transmission, followed by disseminated maculopapular skin lesions in the secondary stage) containing elevated numbers of treponemes (reviewed by Radolf & Lukehart, 2006). During early syphilis, a strong humoral and cellular host immune response eliminates the great majority of treponemes, leading to spontaneous lesion resolution; nevertheless, a small number of organisms escape immune clearance and cause chronic infection (Radolf & Lukehart, 2006). Unraveling the complex biology of this spirochete has been greatly helped by analysis of the *T. pallidum* (Nichols strain) genome sequence (Fraser *et al.*, 1998, Weinstock *et al*., 1998), which has revealed a lack of genes coding for many metabolic and biosynthetic pathways, perhaps explaining the absolute dependence of *T. pallidum* on its host to maintain viability and virulence. Genome analysis has also aided in the identification of putative virulence factors, such as the *tpr* (*T. pallidum* repeat) genes, whose role during infection has been actively investigated since their description.

The 12 members of the *tpr* gene family are homologous to the major sheath protein gene (*msp*) of *Treponema denticola*; *msp* is reported to encode a surface exposed protein with porin and adhesin properties (Fenno *et al.*, 1996). Most Tpr antigens are targets of a strong humoral and cellular host immune responses during experimental syphilis, and immunization with some recombinant Tpr antigens significantly attenuates lesion development after infectious challenge (Centurion-Lara *et al.*, 1999, Morgan *et al.*, 2002b, Morgan *et al.*, 2002a, Morgan *et al*., 2003, Leader *et al*., 2003, Sun *et al.*, 2004, Giacani *et al.*, 2005b), suggesting an important role for these antigens during infection. These genes are differentially transcribed within and among *T. pallidum* strains during experimental infection (Hazlett *et al.*, 2001, Smajs *et al.*, 2005, Giacani *et al.*, 2007b), supporting the hypothesis of regulation of expression among these genes. Regulation of expression of virulence traits by transcription factors is a common mechanism used by bacterial pathogens to adapt and persist in their hosts; bacterial invasion, multiplication, and dissemination in an infected individual often involve temporal and spatial control of gene expression in the pathogen (Kreft & Vasquez-Boland, 2001, Abdelrahman & Belland, 2005, Seshasayee *et al.*, 2006). Recently, the length of hypervariable homopolymeric guanosine (poly-G) tracts located upstream of Subfamily II *tpr* (*tprE*, *tprG*, and *tprJ*) transcriptional start sites (TSSs), was shown to control transcription (Giacani *et al.*, 2007a) of these genes. This finding does not fully explains the differential expression of these genes in that there is not an absolute correlation between *tpr* mRNA levels and the length of the poly-G sequences seen during experimental infection (Giacani et al., 2007a). This suggests the involvement of additional factors influencing their transcription.

Using a weight matrix generated from known binding sites for the *E. coli* cAMP receptor protein (CRP), a member of the *E. coli* catabolite activator protein (CAP) family, we identified instances of the *E. coli* CRP binding motif upstream of the experimentally determined TSSs of the *tprE*, *tprG*, and *tprJ* genes.

The *T. pallidum* genome sequence has revealed four CAP homologs (TP0098, TP0261, TP0262, and TP0995) (Fraser et al., 1998). Except for the TroR repressor protein (Posey *et al.*, 1999; Hazlett *et al.*, 2003,), the CAP homologs are the only annotated proteins likely to modulate gene expression at the transcription initiation step. Among the *T. pallidum* CAP homologs, TP0262 shows the highest sequence homology (41%) to *E. coli* CRP, and conservation of the DNA and cAMP binding domains (as described in Kolb *et al.*, 1993). Furthermore, prediction based on known crystal structures using the 3D-Jury consensus approach (http://meta.bioinfo.pl) identified TP0262 as a CRP structural homolog (data not shown). In *E. coli*, CRP is involved in controlling metabolic functions, but has also been reported to modulate expression of virulence factors, such as the genes encoded by the *pap* operon (Baga *et al*., 1985, Goransson *et al.*, 1989). Because of the paucity of transcription factors in *T. pallidum*, the very limited metabolic capabilities of this pathogen, and the

involvement of CRP in regulation of virulence genes in other systems, we began to explore the hypothesis that TP0262 modulates gene expression of important virulence factor candidates, such as the *tpr* genes.

Electrophoretic mobility shift assay (EMSA) and DNaseI footprinting analysis were combined to investigate recombinant TP0262's ability to specifically recognize amplicons with the predicted *T. pallidum* CRP binding sites upstream of these *tpr* genes; a heterologous reporter assay (Giacani et al., 2007a) was used to evaluate the effect *in vivo* of TP0262 expression on the activity of these *tpr* promoters. We demonstrated that TP0262 can bind these *T. pallidum* promoters and either up- or down-regulate their activity. This is the first characterization of a *T. pallidum* transcription factor that modulates transcription of *tpr* genes and might also function as a global regulator.

Results

TP0262 binds *tpr* Subfamily II promoters

Using a weight matrix generated from known binding sites for *E. coli* CRP (available at http://www.ccg.unam.mx/Computational_Genomics/tractorDB/) we identified instances of the *E. coli* CRP binding motif upstream of the experimentally determined TSSs of the *tprE* (−236.5), *tprG* (−388.5), and *tprJ* (−233.5) genes (Table I; Fig.1, in bold). EMSA was employed to investigate recombinant TP0262's ability to bind amplicons containing predicted *T. pallidum* CRP binding sites upstream of the *tpr E*, *G*, and *J* genes. EMSA using DNA fragments obtained from the *tprE*, *tprG*, and *tprJ* promoter regions, with increasing concentrations of recombinant TP0262, resulted in the shift in mobility of all fragments (Fig. 2A, and B). No shift was observed when the same fragments were incubated with identical concentrations of recombinant *E. coli* CRP (data not shown) or in absence of cAMP (Fig. 2A-C), showing that the shift induced by TP0262 depends on the presence of cAMP. Electrophoretic mobility of the amplicon containing the *E. coli lac* promoter, used as positive control for the assay, was appropriately retarded by recombinant *E. coli* CRP binding (Fig.2D).

Identification of TP0262 binding sites on *tpr* Subfamily II promoters

To confirm specific promoter binding and to precisely define TP0262 binding sites, DNaseI protection assays and automated fragment analysis were performed as described by Oyamada *et al.* (Oyamada *et al.*, 2007) on amplicons obtained from the *tprE*, *tprG*, *tprJ* upstream regions. Differences between the fluorescence intensity of the peaks between corresponding regions in protected and unprotected samples were statistically evaluated using paired Student's t-tests with significance set at $p<0.05$. By comparison with the size standard peaks in the same samples, protected sites were found to span the -253 to -228, -399 to -377, and -278 to -248 intervals for *tprE*, *tprG*, and *tprJ*, respectively, of the amplicons digested with DNaseI (Table I, right column; Fig.3A-C). The region of the *lac* promoter containing the TGTGA-6N-TCACA sequence, used as positive control for the footprinting assay, was protected by *E. coli* CRP binding from DNaseI digestion (Fig.3D).

TP0262 binds the oligonucleotide DNA regions identified by footprinting assay

To further confirm the DNaseI protection assay results, EMSA was employed again to test recombinant TP0262's ability to recognize the binding sites experimentally identified by footprinting assay upstream of the *tprE*, *G*, and *J* genes. Double stranded DNA oligonucleotides (Table II) corresponding to the regions protected by TP0262 (Table I, right column; Fig.3A-D) were synthesized and used in the EMSA assay described above as competitor unlabelled DNA. In presence of competitor DNA, no shift of the labeled DNA amplicons containing the putative *tprE*, *G*, and *J* TP0262 binding sites was observed (Fig.

4A-B, lanes 1-3). A 22 bp oligonucleotide with the *E. coli* CRP binding site was also shown to prevent the shift of the labeled *lac* promoter amplicon containing the same sequence (Fig. 4B, lanes 4-6).

TP0262 influences *tpr* Subfamily II promoter activity

A heterologous green fluorescent protein (GFP) reporter assay (Giacani et al., 2007a) was used to determine the effect of TP0262 on each of the *tpr* promoters. In this assay, *tprE*, *tprG*, and *tprJ* promoters (reproduced in Fig.1) with and without the region containing the putative TP0262 binding sites were first cloned into a plasmid upstream of the *gfp* reporter gene, and then used to transform a *crp*-deficient *E. coli* strain (G1278, Δ*crp::cm*). Activity of the promoters was measured as fluorescence emission in G1278 *E. coli* cultures expressing TP0262. G1278 cultures not expressing TP0262 were included as controls. TP0262 mRNA was also determined by qualitative RT-PCR in all *E. coli* transformants carrying the TP0262 gene in the reporter vector (not shown). GFP fluorescence for the *tprE* and *tprJ* promoters containing the putative CRP binding sites was found to be significantly increased by TP0262 expression with respect to the same cells in the absence of TP0262 expression (Fig.5A, B); in these constructs, omission of the regions containing the TP0262 binding site reduced the level of fluorescence regardless of TP0262 expression (Fig.5A, B), indicating a specific interaction between the putative TP0262 binding sites and TP0262 which causes an increase in promoter activity. Conversely, the activity of the *tprG* promoter containing the putative TP0262 binding site was found to be decreased in cells expressing TP0262 (Fig.5C). Also for the *tprG* promoter; no effect was observed in the absence of either TP0262 expression or the putative binding sites (Fig.5C).

Analysis of the OD_{600} values at the time of fluorescence measurements showed that all cultures were still within the logarithmic phase of growth at the last measurement. Fluorescence signal induced by the positive control (*E. coli* cells transformed with a *lac* promoter- *gfp* construct, with the vector also carrying the *E. coli crp* gene) was always ≥10,000 Ar.U/OD_{600} (data not shown). As previously reported, equal low background fluorescence was seen in cultures carrying negative control plasmid (reporter vector carrying a fragment of the *tpN47* coding sequence, hence without promoter and RBS upstream of the *gfp* gene), and in untransformed *E. coli* cells, indicating absence of expression in the negative control samples (Giacani et al., 2007a).

The same heterologous reporter system was used to investigate TP0262 dependence on cAMP binding to influence promoter activity using the *tprJ* promoter as an example. In this experiment, the *tprJ* promoter-containing plasmid constructs were used to transform a *crp* and *cya*-deficient *E. coli* strain (G1044, Δ*crp::cm::cya*$^-$, unable to synthesize cAMP). Fluorescence measurements of these cells showed that the positive regulatory effect exerted by TP0262 on the *tprJ* promoter (containing the region with the putative CRP binding site) was absent unless the culture media was supplemented with cAMP (Fig. 6). This further confirms the role of Tp0262 as a CRP functional homolog.

Discussion

This study reports the identification of a *T. pallidum* CRP homolog that modulates the activity of the promoters of three *tpr* genes (*tprE*, *G*, and *J*) believed to be involved in syphilis pathogenesis. Although the characterization of elements involved in regulation of gene expression in *T. pallidum* is particularly challenging due to the inability to grow *T. pallidum in vitro*, it is plausible that transcriptional regulation plays a pivotal role in the pathogenesis of a disease that alternates periods of clinically active illness (characterized by more rapid replication of the pathogen) and periods of latent infection in which *T. pallidum* is rarely detected (Radolf & Lukehart, 2006). Furthermore, because *T. pallidum* can infect

virtually every organ of the human body, modulation of gene expression in different tissues may be a mechanism to respond and adapt to these varied microenvironments.

For the *tprE, G* and *J* genes, TP0262 is not the only modulating factor, however, given the effect of the poly-G sequences (Giacani et al., 2007a) on their transcription. In several bacterial pathogens, hypervariable homopolymeric repeats located within or near promoter regions have been shown to regulate a variety of virulence factors and surface antigens through a phase variation mechanism (van der Ende *et al.*, 1995, Saunders *et al.*, 1998, van der Woude & Baumler, 2004); similarly, in *T. pallidum*, poly-G sequences of ≤8 G's located upstream of *tprE, G*, and *J* were shown to be permissive for transcription, while the presence of ≥9 G's locks these genes in an near-OFF state (Giacani et al., 2007a). Although the effect of TP0262 on *tprE, G*, and *J* promoter activity reported here was determined using promoters with 8G-repeats, the same promoters with longer poly-G tracts were also evaluated, but no fluorescence above background was ever obtained (data not shown). Thus, TP0262 likely has a fine tuning effect in expression of these genes when the length of the poly-G tract allows their transcription.

Previous studies on gene organization and transcriptional analysis of the *tpr* genes showed that *tprG* and *tprJ* are the first ORFs of two larger operons containing other *tpr* genes. *tprG* and *tprJ* precede and are co-transcribed along with the *tprF* and *tprI* genes (members of *tpr* Subfamily I), respectively (Giacani *et al.*, 2005a). Transcription of both *tprF* and *I*, however, can also be driven by weak sub-operonic promoters located upstream of the *tprF* and *tprI* ORFs, respectively (Giacani et al., 2005a). Sequences homologous to the *E. coli* CRP binding sites were also identified upstream of both *tprF* and *I* ORFs (data not shown). The results of EMSAs and heterologous reporter assay experiments (data not shown) performed on amplicons containing these additional TP0262 binding sites showed, respectively, that 1) the DNA regions upstream of *tprF* and *I* were recognized by TP0262 when EMSA were performed; but 2) no significant difference in the level of GFP fluorescence was induced by the activity of these promoters in presence of absence of TP0262 expression when the heterologous reporter gene assay was applied to these promoters. There is no clear explanation yet for the lack of responsiveness exhibited by both *tprF* and *tprI* promoters to TP0262 expression (despite the presence of TP0262 binding sites), but it appears likely that transcription of the *tprG-F* and *tprJ-I* operons is mainly driven by the upstream *tprG* and *J* promoters. The lack of responsiveness may be also due to the binding of TP0262 under non-activating conditions or to the overriding effect of another TF that is present in the *E. coli* reporter system (Grainger *et al.*, 2007, Grainger *et al.*, 2004). It is also possible that TP0262 binding to the *tprF* and *I* promoters has no functional importance. It has recently proposed that the *E. coli* CRP also acts as a chromatin-shaping protein (Grainger *et al.*, 2005). By analogy, binding of TP0262 to the *tprF* and *tprI* promoters might result in a higher hierarchy of chromosomal organization.

The *E. coli* CRP binding site in the *lac* promoter is located at nucleotide position -62.5, which allows direct interaction between the transcription factor and the carboxyl-terminal domain of the α subunits of the *E.coli* RNA polymerase. It is worth noting that the TP0262 binding sites identified upstream of the *tprE, G*, and *J* genes are too far away from the putative or experimentally determined TSSs to allow a similar interaction between TP0262 and the *T. pallidum* RNA polymerase. In these promoters, TP0262 could potentially work in concert with still unidentified transcription factors that could bind the region downstream of the TP0262 binding motifs.

Although TP0262 putative binding sites were identified by computer analyses based on the consensus sequences for the *E. coli* CRP binding motifs, it is interesting that neither did recombinant *E. coli* CRP bind the *tpr* DNA fragments containing the TP0262 binding sites

nor was TP0262 able to bind the *lac* promoter or rescue lactose fermentation in *crp*-deficient *E. coli* mutants (Supplemental Information). One likely explanation is that species-specific promoter upstream and downstream regions of the binding motifs might play a role in this context. Also, evolutionary differences between these transcription modulators and their targets may be responsible for this lack of complementation between the two systems

We acknowledge the limitations of using the heterologous *E. coli* GFP reporter system (Giacani et al., 2007a) to study transcriptional regulation in *T. pallidum*. Sohaskey *et al.* (Sohaskey *et al.*, 1997), for example, showed that expression driven by the *ospA, ospC, flaD*, and *flaB* promoters of *Borrelia burgdorferi* (the spirochetal agent of Lyme disease) varies between *B. burgdorferi* and *E. coli* when these promoters are analyzed using a transient chloramphenicol acetyltransferase (CAT) expression system. The inability to grow *T. pallidum in vitro* precludes transcriptional regulation studies in the bacterium itself. Nonetheless, the specific application of the *E. coli* GFP reporter system to the *tpr* promoters is supported by our previous findings that *tprG* and *J* promoters are recognized in vitro by the *E. coli* RNA polymerase-σ70 complex, and that the TSSs are identical in both the *E. coli* system and in RNA extracted directly from *T. pallidum* cells (Giacani et al., 2005a). A combination of tools such as this heterologous system with *T. pallidum*-specific in vitro transcription systems (which could be developed by reconstituting the *T. pallidum* RNA polymerase holoenzyme from individual recombinant peptides) will greatly enhance our ability to study transcriptional regulation for this intractable spirochete.

Ongoing genome-wide chromatin immunoprecipitation studies in our laboratories will confirm the binding specificities of TP0262 to *tpr* promoters and will determine the TP0262 regulon. The identification of the TP0262 regulon along with the genome-wide distribution of the *T. pallidum* RNA polymerase will further define the role of TP0262 as a transcriptional regulator during the pathogenesis of syphilis. In parallel, other studies will focus on the expression of the TP0485 gene that encodes for the *T. pallidum* adenylate cyclase. Transcription of molecules associated with stress responses, such as chaperones (*dnaJ, dnaK, grpE, mopA*, and *mopB*) and several ATP-dependent proteases (*clpP, hflB, hslU, hslV*) have also been shown to be regulated by catabolite repression (Gosset *et al.*, 2004). *T. pallidum* possesses ORFs homologous to most of these genes, and preliminary *in silico* genome-wide analysis has revealed the presence of putative CRP binding sites in more than 200 upstream regions of the 1041 annotated coding sequences, including approximately 40% of the genes predicted to be associated with stress responses (data not shown). This supports involvement of TP0262 in mediating *T. pallidum*'s ability to adapt its physiology to environmental changes in the host.

In conclusion, this work provides an important insight into the role of modulators of gene transcription in *T. pallidum*, a field neglected in syphilis research, and sets the stage for further studies to identify the stimuli controlling TP0262 activity, and the involvement of other factors in transcriptional modulation of these *tpr* genes.

Experimental Procedures

T. pallidum and *E. coli* strain propagation, nucleic acid extraction and preparation of competent *E. coli* cells

T. pallidum subsp. *pallidum*, Nichols strains, originally provided by James N. Miller (University of California, Los Angeles), was propagated in New Zealand white rabbits as previously reported (Lukehart *et al.*, 1980). Briefly, rabbits were infected with 5×10^7 *T. pallidum* cells per testis, and treponemes were harvested at peak orchitis (day ~10 postinfection). Collected organisms were separated from host cellular gross debris by low speed centrifugation (250 × g for 10 min at room temperature), and the supernatants were

spun in a microcentrifuge for 30 min at 12,000 × g at 4°C. Bacterial pellets were resuspended in 200 μl of 1× lysis buffer (10mM Tris, pH 8.0; 0.1M EDTA; 0.5% sodium dodecyl sulfate) for DNA isolation. DNA extraction was performed as previously reported (Giacani *et al.*, 2004) using the QIAamp DNA Mini Kit (Qiagen Inc., Chatsworth, CA). DNA samples were stored at -20°C until use in amplification reactions.

E. coli MG1655-K12 (wild type), G1278 (Δ*crp::cm*), and G1044 (Δ*crp::cm:;cya*$^-$) strains, both derived from the K12 wild type, were kindly provided by Drs. Susan Garges, Sankar Adhya (Laboratory of Molecular Biology, NCI, NIH, Bethesda, MD), and Ding J. Jin (Gene Regulation and Chromosome Biology Laboratory, NCI, NIH, Frederick, MD) and propagated in Luria-Bertani (LB) plates supplemented with 25μg/ml chloramphenicol. DNA extraction from the MG1655-K12 strain was performed as described above for *T. pallidum* cells. RNA extraction to assess mRNA levels as surrogate marker for TP0262 expression in G1278 and G1044 cells transformed with TP0262-encoding plasmids used in this study was performed from cell pellets lysed in 400 μl of Ultraspec buffer (Biotecx Laboratories Inc., Houston, TX). Protocols for RNA extraction and DNaseI treatment to obtain DNA-free RNA samples were previously reported in detail (Giacani et al., 2005a). RT-PCR protocols were also reported in detail (Giacani et al., 2007b) and are only briefly described here (see GFP reporter assay). Preparation of competent G1278 and G1044 cells was performed according to Inoue *et al.* (Inoue H, 1990). Competent cells were transformed with approximately 300 ng of plasmid DNA. Transformants were plated on LB plates containing chloramphenicol (25μg/ml) and ampicillin (100 μg/ml) and grown overnight to be used the following day in the GFP reporter assay (see below).

Amplification and cloning

All primers in Table II were designed using the Primer3 software (http://fokker.wi.mit.edu/primer3/input.htm). All PCR amplifications were performed in 50 μl reactions containing 200 μM each dNTP, 20 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 50 mM KCl, 400 nM of each primer, and 1.0 U of Taq DNA Polymerase (Promega, Madison, WI) with approximately 100 ng of DNA template in each reaction. Cycling conditions were denaturation for 5 min at 95°C, followed by 1 min at 95°C, annealing for 1 min at 60°C and extension for 1 min at 72°C for a total of 45 cycles. Final extension was 10 min at 72°C. Amplicons for the *tprE*, *tprG*, and *tprJ* promoters, as well as for the *E. coli lac* promoter (used as positive control in the GFP reporter assay) were directly cloned into the pGlow-TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and for each promoter, insert-containing plasmids were extracted using the Qiagen Plasmid Mini Kit (Qiagen) and sequenced in both strands with the dye terminator kit (Applied Biosystems, Foster City, CA) to ensure the correct orientation of the insert with respect to the gfp gene and the absence of amplification errors. Because the poly-G repeats of varying length located upstream of the *tprE, tprG,* and *tprJ* TSSs (Giacani et al., 2005a,Giacani et al., 2007a) influence promoter strength (Giacani et al., 2007a), sequencing was also used to evaluate the number of G residues contained in these poly-G tracts. The *T. pallidum* TP0262 and *E. coli crp* genes were amplified and cloned into the AvrII restriction site of the pGlow-TOPO vector to enable the *crp*-deficient G1287 and G1044 *E. coli* strains to express these genes when required in the GFP reporter assay. Standard cloning procedures (Maniatis *et al.*, 1989) were adopted to insert the TP0262 and *E. coli crp* genes into the pGlow-TOPO vector in the opposite orientation with respect to the *gfp* coding sequence.

Recombinant peptides

To obtain recombinant 6X-His-tagged TP0262 and *E coli* CRP proteins for the EMSA and footprinting assays, amplicons with the two ORFs (primers in Table II) were first cloned into the TOPO-TA cloning vector (Invitrogen), checked for sequence accuracy, excised and

inserted into the pQE30 expression vector (Qiagen) between the KpnI and HindIII restriction sites. Recombinant proteins were expressed in M15 (pREP4) cells (Qiagen) according to the manufacturer's guidelines. Briefly cultures were induced with 1.0 mM IPTG when OD_{600} reached 0.4-0.6 Absorbance Units and harvested after 4 hour incubation at 37°C. Cell pellets were resuspended in 1X lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1 mg/ml lysozyme, pH 8.0) with EDTA-free protease inhibitor cocktail tablets (Complete; Roche, Basel, Switzerland), subjected to 12 rounds of sonication in ice for a total of 60 seconds, and centrifuged at 15,000 rpm for 30 min. Supernatants were filtered through a 0.45 μm filter prior to purification by nickel affinity chromatography. Wash and elution buffers were identical to the lysis buffer except for the increased concentration of imidazole (20 and 250 mM, respectively) and for the absence of lysozyme and protease inhibitors. Elution products were tested for size and purity by SDS-PAGE and quantified using a Bicinchoninic Acid Assay kit (Pierce, Rockford, IL). More than 95% of the protein was found in the expected size of the rTP0262 (~26 kDa). Protein reactivity with anti 6xHis antibody (Sigma, St. Louis, MI) was tested according to the antibody manufacturer's instructions. Purified proteins were dialyzed against phosphate buffered saline (PBS)-10% glycerol and stored in aliquots at -20°C until use.

EMSA

Amplicons of the upstream regions of *tprE*, *tprG*, and *tprJ* containing the putative TP0262 binding sites were obtained using the primers listed in Table II; primer pairs were designed in order to obtain short amplicons (116-163 bp, Table II) with the putative TP0262 binding site equally spaced from the 3′ and 5′ ends. Amplicons were gel-purified and concentrations measured spectrophotometrically using a ND-1000 instrument (NanoDrop Technologies, Wilmington, DE). Fragments were labeled with the Biotin 3′ End DNA Labeling Kit, suitable for labeling ds DNA with 3′ overhangs (Pierce). Labeling efficiency was determined following the manufacturer's protocol. Labeled fragments were then purified using the QIAquick PCR purification kit (Qiagen) and stored at -20°C until use. Twenty microliter reactions containing 0.5 fmol of DNA and increasing concentrations of purified recombinant TP0262 and *E. coli* CRP (10 μM, 20 μM, and 30 μM) in reaction buffer [10 mM Tris, 50 mM KCl, 10 mM DTT, pH7.5, 5 mM $MgCl_2$, 0.1 mM EDTA, 50 ng/μl Poly(dI·dC), 0.05% NP-40, and 2.5 % glycerol] were incubated at room temperature for 20 min. cAMP (Sigma) was added to all reactions to the final concentration of 0.1 mM, except to investigate binding activity in absence of cAMP. After incubation, reactions were mixed with 5μl of 5× DNA loading buffer and separated by electrophoresis in 4% polyacrylamide gels in 0.5X TBE buffer supplemented with 0.1 mM cAMP at 30 V/gel for 45 min. Gels were transferred to nylon membranes in a Mini Trans-Blot Cell (Bio-Rad, Hercules, CA) in 0.5X TBE at 200 mA for 30 min. After UV-crosslinking DNA to membranes for 10 minutes on a transilluminator equipped with 312 nm bulbs, biotin-labeled DNA was detected by using the LightShift Chemiluminescent EMSA Kit (Pierce) following the manufacturer's instruction. The streptavidin-HRP conjugate was used at 1:2000 dilution. As a positive control, the *lac* promoter of *E. coli* was also amplified (primers in Table II), labeled and analyzed for electrophoretic mobility retardation in presence of recombinant *E. coli* CRP, with and without cAMP.

The same protocol was applied when unlabelled competitor oligonucleotides were used investigate TP0262 interaction with the sequences experimentally identified by DNaseI footprinting assay (protocol below). In these experiments, the final concentration of the competitor DNA was approximately ~100 fold higher (50 fmol) than the labeled probe.

DNaseI footprinting assay

Footprinting analysis was performed using a modified protocol published by Oyamada *et al.* (Oyamada et al., 2007). Probes containing the *tprE*, *tprG*, and *tprJ* putative TP0262 binding sites, and a control probe with the *E. coli lac* promoter containing the *E. coli* CRP binding site were obtained by PCR amplification from a plasmid template in which these regions were previously cloned. Amplification was carried out using the conditions described above and primers listed in Table II. A common sense primer, which is labeled with the fluorescent dye 6-FAM, anneals to the plasmid (pGlow-TOPO) and can be used on multiple templates in combination with a specific antisense primer (Table II). Amplification reaction products were run in 1% agarose gels and amplicons were gel-purified to eliminate excess primers. Concentration was determined using a ND-1000 spectrophotometer (NanoDrop Technologies). Approximately 2 pmol of DNA per fragment were digested with 3 U of DNaseI (Promega) for 1 min at room temperature in 100 μl of binding buffer (25 mM Tris-HCL, pH 8.0; 50 mM KCl, 6.25 mM MgCl2; 0.5 mM EDTA; 10% glycerol, and 1 mM DTT) in presence of 1 mM cAMP and 25 pmol of recombinant TP0262 (*tprE*, *tprG*, and *tprJ*) or recombinant *E. coli* CRP (*lac* promoter). Reactions were stopped by adding 90 μl of stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS, and 100 μg/ml yeast DNA), phenol-chloroform purified, precipitated with 3 volumes of 100% ethanol, and washed with 70% ethanol. Samples were then vacuum dried and resuspended in 17 μl Hi-Di formamide (Applied Biosystems). Digestions were then mixed with 0.5 μl of ROX-labeled DNA ladder (MapMarker400, Bioventures, Murfreesboro, TN) and run in an ABI3100 Genetic Analyzer (Applied Biosystems). After fragment separation, fluorescence intensity and peak areas were analyzed with the GeneMapper 3.5 software (Applied Biosystems). The identification of the DNA regions protected by TP0262 or *E. coli* CRP binding was determined by comparison of both fluorescence intensity and peak areas between samples digested in presence or absence of recombinant TP0262 or *E. coli* CRP using the paired Student's t-test with significance set at $p<0.05$.

GFP reporter assay

PCR products for the *tprE*, *tprG*, and *tprJ* promoters were obtained from *T. pallidum* DNA using primers (Table II) specifically designed for expression of a GFP fusion protein using the pGlow-TOPO system (Invitrogen). Amplicons were gel purified using the QIAquick gel extraction kit (Qiagen) and cloned into the pGlow-TOPO vector according to manufacturer's instructions. For each *tpr* promoter, the amplicon included the predicted TP0262 binding sites, the predicted *tpr* gene RBS (-AGGAG-), and the GTG start codon in frame with the *gfp* coding sequence in the vector (Fig. 1A-C). The start codons of these *tpr* genes have not yet been experimentally identified, and, as previously reported (Giacani et al., 2007a), we selected RBSs and start codons for the pGlow-TOPO constructs separated 8 to 12 nucleotides from each other in order to provide *E. coli* with the optimal translational spacing. To facilitate recognition of the *tpr* start codons (GTG) by *E. coli*, the GTG triplet was mutated to an ATG in all clones (Fig.1A-C) by site directed mutagenesis. Expression of GFP from these constructs resulted in the addition of nine extra amino acids to the actual GFP peptide, encoded by the *tpr* start codon and eight additional, vector-encoded aminoacids (Fig.1A-C). In total, four different constructs were obtained for both *tprE* and *J* promoters (with poly-G repeats 8-11 G nucleotides long), and three constructs for *tprG* (with 8-10 G's) (Fig.1). Identical *tpr* promoter-pGlow TOPO plasmids with the same poly-G variants but without the DNA region containing the putative CRP binding sites were already available from previous studies (Giacani et al., 2007a). As mentioned above, when expression of TP0262 in the G1278 *E. coli* cells was required, the DNA fragment containing the TP0262 ORF and ~150 bp of the 5′- and 3′- flanking regions was cloned into the AvrII site of the pGlow-TOPO vector, in opposite orientation to the *gfp* coding sequence. A construct containing both the *lac* promoter and the operator upstream of the *gfp* gene was

used as a positive control; because transcription of the *lac* promoter does not occur in a *crp*-deficient *E. coli* strain, the *E. coli crp* gene was also cloned into the vector (in the AvrII site, in opposite orientation to the *gfp* gene). As a negative control, to determine background fluorescence, a ~300 bp fragment of the *tpN47* (TP0574) coding sequence with no RBS and no promoter activity was inserted out-of-frame with the *gfp* coding sequence into the pGlow-TOPO vector. All constructs were sequenced in both directions to verify correct orientation, continuity in the reading frame, and absence of mutations, as well as to ensure that the length of the poly-G repeats did not change after amplification and propagation in *E. coli*. Constructs were subsequently used to transform G1278 *E. coli* cells. For GFP fluorescence measurements, cells were inoculated from a Petri dish into 4 ml of LB medium at room temperature containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol, and grown at 37°C for 4 hrs. Cultures with optical densities (OD_{600}) of ~ 0.5 Absorbance units (AU) were used for fluorescence measurements in quadruplicate every 30 min until cultures reached OD_{600} of ~2 AU. Briefly, 400 μl of culture were spun for 4 minutes at 12,000 × g and resuspended in an equal volume of phosphate buffered saline (PBS); cells were then divided in four wells (100 μl/well) of a black OptiPlate-96F (Perkin Elmer, Boston MA) for top fluorescence reading. Excitation and emission wavelength were 405 and 505 nm, respectively, and readings were performed in a Fusion Universal Microplate Analyzer (Perkin Elmer). Before each fluorescence readings, OD_{600} of the cultures was recorded again for normalization of the fluorescence measurements (expressed in Arbitrary Units, Ar.U) to the optical density of the culture. Background values obtained from each experiment (using *E. coli* cells transformed with the *tpN47*-pGlow TOPO vector) were subtracted from the sample values. Differences between levels of fluorescence were compared using Student's t-test, with significance set at $p<0.05$.

To confirm TP0262 expression in G1278 cells carrying the TP0262 gene in the pGLow-TOPO vector, we used RT-PCR analysis to determine TP0262 mRNA levels as a surrogate marker. 400 μl of each *E. coli* culture was spun immediately after fluorescence measurements and cells resuspended in an equal volume of Ultraspec buffer (Biotecx Laboratories) for RNA isolation. Extraction was performed according to the manufacturer's guidelines and followed by RNA treatment with DNaseI (Turbo-DNase, Ambion, Austin, TX) according to the provided protocol. DNase-treated RNA was tested for residual plasmid DNA contamination by qualitative amplification using *gfp* -specific primers (*gfp*-S and *gfp*-As primers, Table II) and the amplification conditions described above. DNA-free RNA was stored in aliquots at -80°C until use. Reverse transcription of total RNA was performed using the Superscript II First Strand Synthesis Kit (Invitrogen) with the TP0262-As primer (Table II) according to the provided protocol. All cDNA samples were then tested for TP0262 specific message using qualitative conventional PCR with the TP0262-S and -As primers (Table II) and the amplification conditions described above.

Identical procedures were applied when G1044 *E. coli* cells were used to evaluate TP0262 dependency on cAMP (co-effector) to exert its modulatory effect on the *tprJ* promoter. In this experiment, cAMP effect was evaluated by supplementing the growth medium with a final concentration of 0.75 mM cAMP (Sigma), and comparing fluorescence measurements of cultures grown in absence or presence of cAMP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are grateful to Dr. Susan Garges (National Institutes of Health) for her valuable advice and suggestions; to Drs. Susan Garges, Sankar Adhya, and Ding J. Jin (National Institutes of Health) for providing the *E. coli* strains; Nan Li (University of Washington) for help with the *in silico* identification of the TP0262 binding sites; to Drs. Julio Cabrera, Dale Lewis (National Institutes of Health), Mofang Liu (Shanghai Institutes for Biological Sciences, China) and Elisabetta Marotti (University of Ancona, Italy) for their invaluable suggestions; Bruce Godfrey (Comparative Genomics Center at the University of Washington) for assistance with the footprinting experiments, and to Miko Robertson for manuscript preparation. This work was supported by NIH grants AI42143 and AI63940 (to SAL and ACL), and GM076222 (to MT).

References

Abdelrahman YM, Belland RJ. The chlamydial developmental cycle. FEMS Microbiol Rev. 2005; 29:949–959. [PubMed: 16043254]

Baga M, Goransson M, Normark S, Uhlin BE. Transcriptional activation of a pap pilus virulence operon from uropathogenic Escherichia coli. Embo J. 1985; 4:3887–3893. [PubMed: 2868893]

Centurion-Lara A, Castro C, Barrett L, Cameron C, Mostowfi M, Van Voorhis WC, Lukehart SA. *Treponema pallidum* major sheath protein homologue Tpr K is a target of opsonic antibody and the protective immune response. J Exp Med. 1999; 189:647–656. [PubMed: 9989979]

Cox DL, Riley B, Chang P, Sayahtaheri S, Tassell S, Hevelone J. Effects of molecular oxygen, oxidation-reduction potential, and antioxidants upon in vitro replication of *Treponema pallidum* subsp. *pallidum*. Appl Environ Microbiol. 1990; 56:3063–3072. [PubMed: 2285317]

Fenno JC, Muller KH, McBride BC. Sequence analysis, expression, and binding activity of recombinant major outer sheath protein (Msp) of *Treponema denticola*. J Bacteriol. 1996; 178:2489–2497. [PubMed: 8626313]

Fraser CM, Norris SJ, Weinstock GM, White O, Sutton GG, Dodson R, Gwinn M, Hickey EK, Clayton R, Ketchum KA, Sodergren E, Hardham JM, McLeod MP, Salzberg S, Peterson J, Khalak H, Richardson D, Howell JK, Chidambaram M, Utterback T, McDonald L, Artiach P, Bowman C, Cotton MD, Venter JC. Complete genome sequence of *Treponema pallidum*, the syphilis spirochete. Science. 1998; 281:375–388. [PubMed: 9665876]

Giacani L, Hevner K, Centurion-Lara A. Gene organization and transcriptional analysis of the *tprJ*, *tprI*, *tprG* and *tprF* loci in the Nichols and Sea 81-4 *Treponema pallidum* isolates. J Bacteriol. 2005a; 187:6084–6093. [PubMed: 16109950]

Giacani L, Lukehart S, Centurion-Lara A. Length of guanosine homopolymeric repeats modulates promoter activity of Subfamily II *tpr* genes of *Treponema pallidum* ssp. *pallidum*. FEMS Immunol Med Microbiol. 2007a; 51:289–301. [PubMed: 17683506]

Giacani L, Molini B, Godornes C, Barrett L, Van Voorhis WC, Centurion-Lara A, Lukehart SA. Quantitative analysis of *tpr* gene expression in *Treponema pallidum* isolates: differences among isolates and correlation with T-cell responsiveness in experimental syphilis. Infect Immun. 2007b; 75:104–112. [PubMed: 17030565]

Giacani L, Sambri V, Marangoni A, Cavrini F, Storni E, Donati M, Corona S, Lanzarini P, Cevenini R. Immunological evaluation and cellular location analysis of the TprI antigen of *Treponema pallidum* subsp. *pallidum*. Infect Immun. 2005b; 73:3817–3822. [PubMed: 15908421]

Giacani L, Sun ES, Hevner K, Molini BJ, Van Voorhis WC, Lukehart SA, Centurion-Lara A. Tpr homologs in *Treponema paraluiscuniculi* Cuniculi A strain. Infect Immun. 2004; 72:6561–6576. [PubMed: 15501788]

Goransson M, Forsman P, Nilsson P, Uhlin BE. Upstream activating sequences that are shared by two divergently transcribed operons mediate cAMP-CRP regulation of pilus-adhesin in Escherichia coli. Mol Microbiol. 1989; 3:1557–1565. [PubMed: 2575704]

Gosset G, Zhang Z, Nayyar S, Cuevas WA, Saier MH Jr. Transcriptome analysis of Crp-dependent catabolite control of gene expression in *Escherichia coli*. J Bacteriol. 2004; 186:3516–3524. [PubMed: 15150239]

Grainger DC, Aiba H, Hurd D, Browning DF, Busby SJ. Transcription factor distribution in Escherichia coli: studies with FNR protein. Nucleic Acids Res. 2007; 35:269–278. [PubMed: 17164287]

Grainger DC, Hurd D, Harrison M, Holdstock J, Busby SJ. Studies of the distribution of *Escherichia coli* cAMP receptor protein and RNA polymerase along the *E. coli* chromosome. Proc Natl Acad Sci U S A. 2005; 102:17693–17698. [PubMed: 16301522]

Grainger DC, Overton TW, Reppas N, Wade JT, Tamai E, Hobman JL, Constantinidou C, Struhl K, Church G, Busby SJ. Genomic studies with Escherichia coli MelR protein: applications of chromatin immunoprecipitation and microarrays. J Bacteriol. 2004; 186:6938–6943. [PubMed: 15466047]

Hazlett KR, Rusnak F, Kehres DG, Bearden SW, La Vake CJ, La Vake ME, Maguire ME, Perry RD, Radolf JD. The Treponema pallidum tro operon encodes a multiple metal transporter, a zinc-dependent transcriptional repressor, and a semi-autonomously expressed phosphoglycerate mutase. J Biol Chem. 2003; 278:20687–20694. [PubMed: 12668673]

Hazlett KRO, Sellati TJ, Nguyen TT, Cox DL, Clawson ML, Caimano MJ, Radolf JD. The TprK protein of *Treponema pallidum* is periplasmic and is not a target of opsonic antibody or protective immunity. J Exp Med. 2001; 193:1015–1026. [PubMed: 11342586]

Inoue H, N H, Okayama H. High efficiency transformation of Escherichia coli with plasmids. Gene. 1990; 96:23–28. [PubMed: 2265755]

Kolb A, Busby S, Buc H, Garges S, Adhya S. Transcriptional regulation by cAMP and its receptor protein. Annu Rev Biochem. 1993; 62:749–795. [PubMed: 8394684]

Kreft J, Vazquez-Boland JA. Regulation of virulence genes in Listeria. Int J Med Microbiol. 2001; 291:145–157. [PubMed: 11437337]

Leader BT, Hevner K, Molini BJ, Barrett LK, Van Voorhis WC, Lukehart SA. Antibody responses elicited against the *Treponema pallidum* repeat proteins differ during infection with different isolates of *Treponema pallidum* subsp. *pallidum*. Infect Immun. 2003; 71:6054–6057. [PubMed: 14500529]

Lukehart SA, Baker-Zander SA, Sell S. Characterization of lymphocyte responsiveness in early experimental syphilis. I. In vitro response to mitogens and *Treponema pallidum* antigens. J Immunol. 1980; 124:454–460. [PubMed: 6153102]

Maniatis, T.; Sambrook, J.; Fritsch, EF. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory; 1989.

Morgan CA, Lukehart SA, Van Voorhis WC. Immunization with the N-terminal portion of *Treponema pallidum* repeat protein K attenuates syphilitic lesion development in the rabbit model. Infect Immun. 2002a; 70:6811–6816. [PubMed: 12438357]

Morgan CA, Lukehart SA, Van Voorhis WC. Protection against syphilis correlates with specificity of antibodies to the variable regions of *Treponema pallidum* repeat protein K. Infect Immun. 2003; 71:5605–5612. [PubMed: 14500480]

Morgan CA, Molini BJ, Lukehart SA, Van Voorhis WC. Segregation of B and T cell epitopes of *Treponema pallidum* repeat protein K to variable and conserved regions during experimental syphilis infection. J Immunol. 2002b; 169:952–957. [PubMed: 12097401]

Norris SJ, Edmondson DG. Factors affecting the multiplication and subculture of *Treponema pallidum* subsp. *pallidum* in a tissue culture system. Infect Immun. 1986; 53:534–539. [PubMed: 3091504]

Oyamada T, Yokoyama K, Morinaga M, Suzuki M, Makino K. Expression of *Escherichia coli* DcuS-R two-component regulatory system is regulated by the secondary internal promoter which is activated by CRP-cAMP. J Microbiol. 2007; 45:234–240. [PubMed: 17618229]

Posey JE, Hardham JM, Norris SJ, Gherardini FC. Characterization of a manganese-dependent regulatory protein, TroR, from *Treponema pallidum*. Proc Natl Acad Sci U S A. 1999; 96:10887–10892. [PubMed: 10485921]

Radolf, JD.; Lukehart, SA. Pathogenic *Treponema*: Molecular and Cellular Biology. Norfolk, England: Caister Academic Press; 2006.

Saunders NJ, Peden JF, Hood DW, Moxon ER. Simple sequence repeats in the *Helicobacter pylori* genome. Mol Microbiol. 1998; 27:1091–1098. [PubMed: 9570395]

Seshasayee AS, Bertone P, Fraser GM, Luscombe NM. Transcriptional regulatory networks in bacteria: from input signals to output responses. Curr Opin Microbiol. 2006; 9:511–519. [PubMed: 16942903]

Smajs D, McKevitt M, Howell JK, Norris SJ, Cai WW, Palzkill T, Weinstock GM. Transcriptome of *Treponema pallidum*: Gene Expression Profile during Experimental Rabbit Infection. J Bacteriol. 2005; 187:1866–1874. [PubMed: 15716460]

Sohaskey CD, Arnold C, Barbour AG. Analysis of promoters in *Borrelia burgdorferi* by use of a transiently expressed reporter gene. J Bacteriol. 1997; 179:6837–6842. [PubMed: 9352937]

Sun ES, Molini BJ, Barrett LK, Centurion-Lara A, Lukehart SA, Van Voorhis WC. Subfamily I *Treponema pallidum* repeat protein family: sequence variation and immunity. Microbes Infect. 2004; 6:725–737. [PubMed: 15207819]

van der Ende A, Hopman CT, Zaat S, Essink BB, Berkhout B, Dankert J. Variable expression of class I outer membrane protein in *Neisseria meningitidis* is caused by variation in the spacing between the -10 and -35 regions of the promoter. J Bacteriol. 1995; 177:2475–2480. [PubMed: 7730280]

van der Woude MW, Baumler AJ. Phase and antigenic variation in bacteria. Clin Microbiol Rev. 2004; 17:581–611. [PubMed: 15258095]

Weinstock GM, Hardham JM, McLeod MP, Sodergren EJ, Norris SJ. The genome of *Treponema pallidum*: new light on the agent of syphilis. FEMS Microb Rev. 1998; 22:323–332.

FIG. 1.

tprE, *tprG*, *tprJ* promoter sequences cloned into the pGlow-TOPO vector and used for the GFP reporter assay. Underlines indicate the primers [→: sense primer(s), ←: antisense primer] used to amplify the promoter regions to obtain amplicons containing or lacking the putative TP0262 binding site. Regulatory elements are in bold. *tprG* and *J* TSSs (+1) have been experimentally demonstrated (Giacani et al., 2005a), *tprE* TSS (+1) is hypothetical but based on sequence homology with the other *tpr* genes. TP0262 bs: predicted TP0262 binding site; $[GGGGGGGGGG]_n$ indicates the hypervariable poly-G sequences upstream of the *tpr* promoters; +1: Transcriptional Start Site (TSS); RBS: ribosomal binding site; SC: start codon; GFP SC: green fluorescent protein start codon. The sequence Lys-Gly-Gln-Phe-

Cys-Arg-Ser-Arg between the putative Tpr SCs and the GFP ORF start are vector-encoded aminoacids. Shorter amplicons were obtained from these promoter regions for EMSA and footprinting analysis using primers reported in Table II.

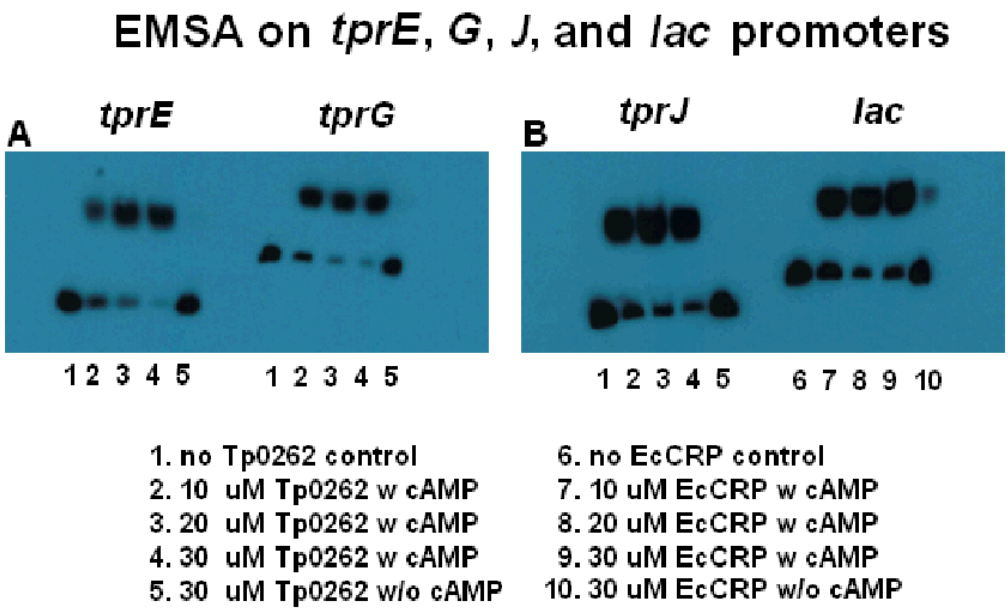
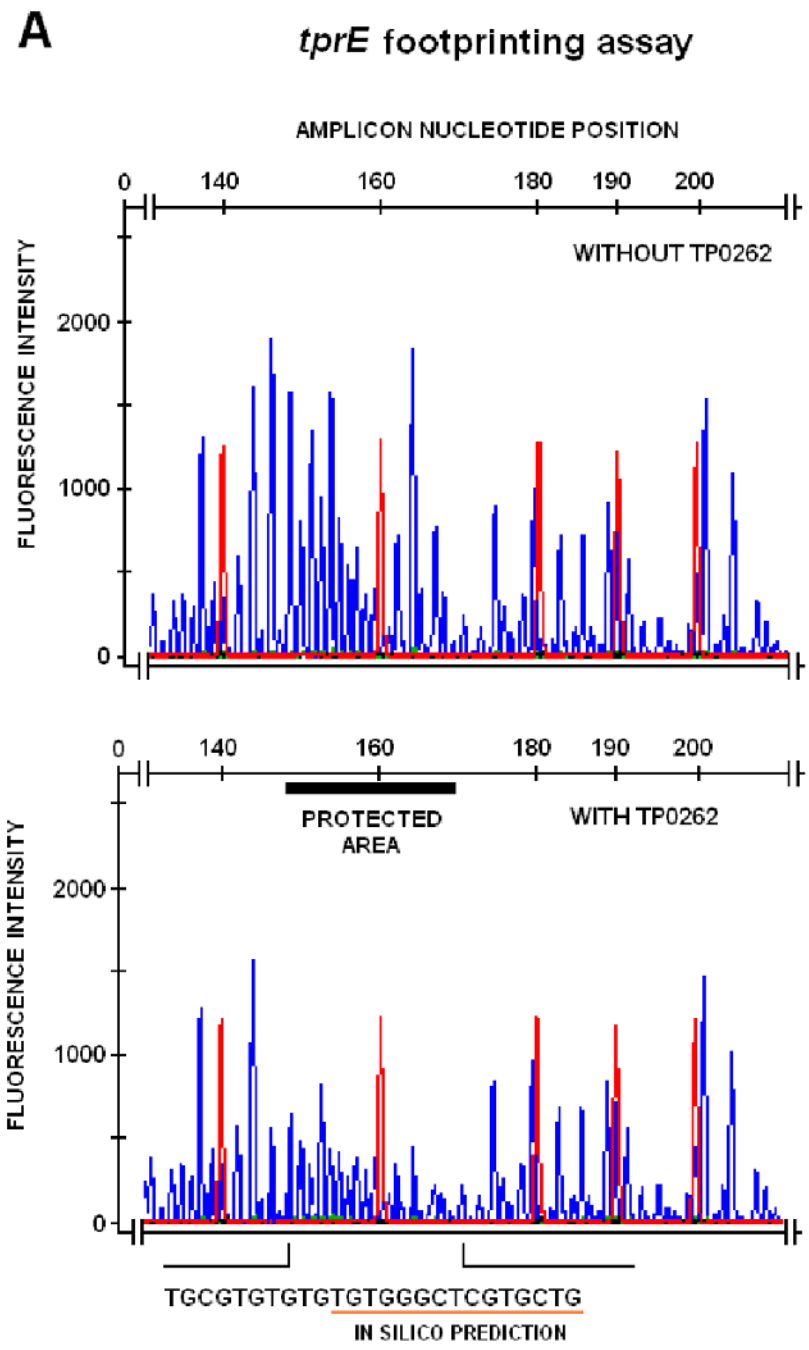
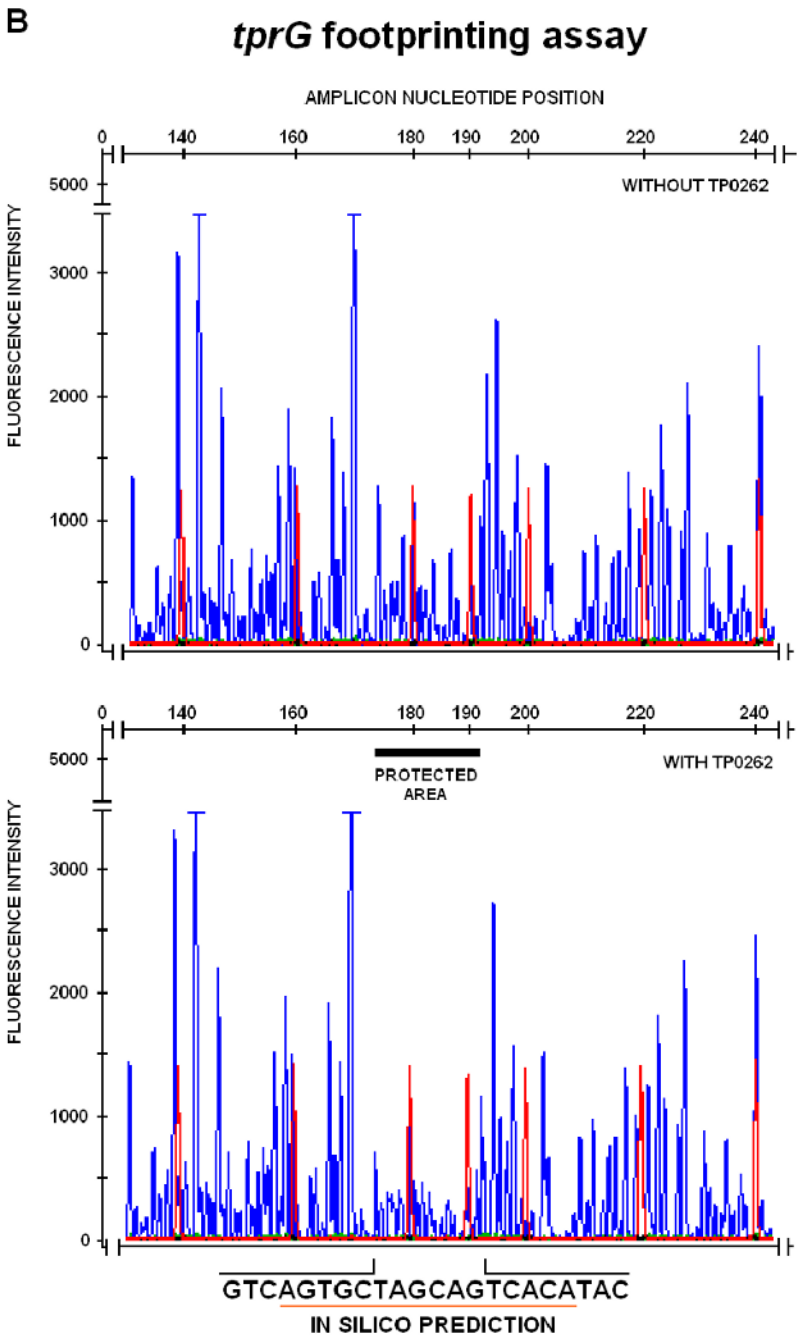
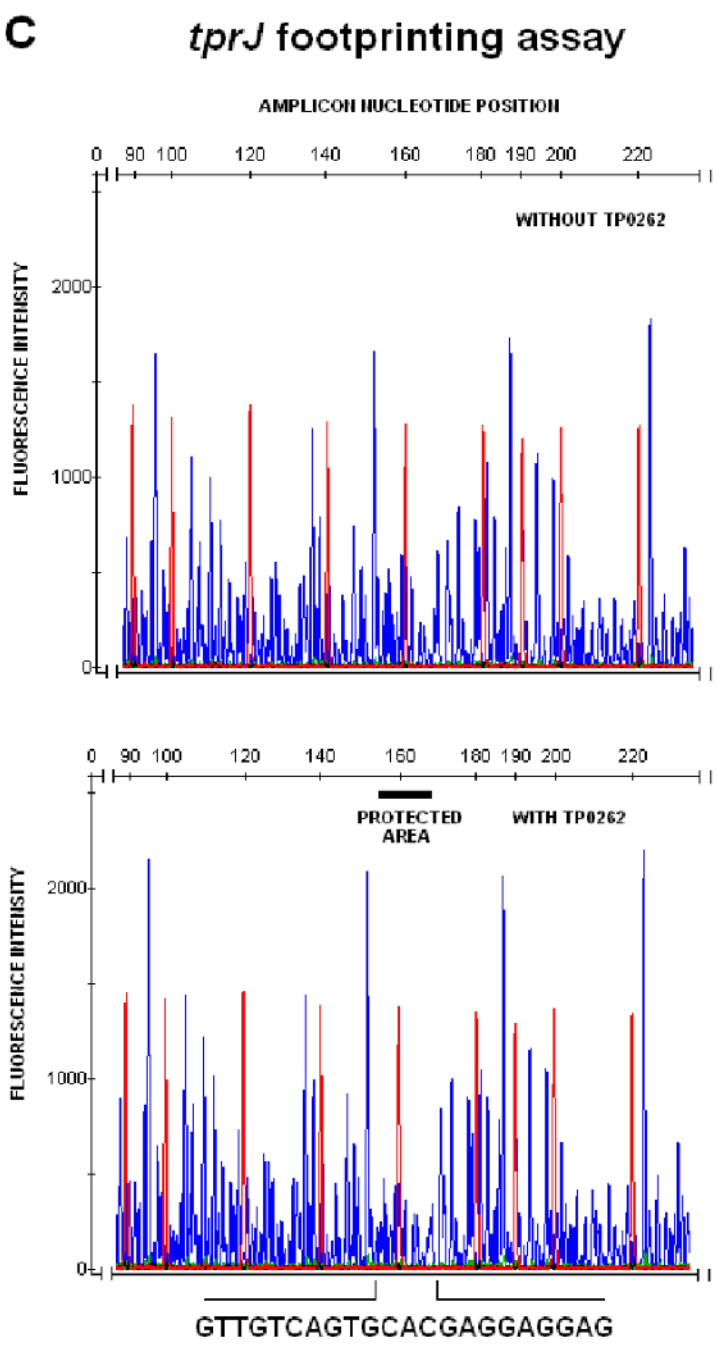


FIG. 2.

Electrophoretic mobility shift assay (EMSA) on *T. pallidum tprE* and *tprG* (Panel A), *tprJ* and *lac* (Panel B) promoters in presence of cAMP and increasing concentrations (0μM, 10 μM, 20μM, and 30 μM, respectively) of recombinant TP0262 (lanes 1-4 for the *tpr* genes) or *E. coli* CRP (lanes 6-9 for the *E. coli lac* promoter) proteins. TP0262 + cAMP bound the *T. pallidum tpr* promoters; *E. coli* CRP + cAMP failed to bind *tpr* promoters in this assay (data not shown). Recombinant *E. coli* CRP was shown to retard electrophoretic mobility of the *lac* promoter amplicon containing the TGTGA-6N-TCACA binding site. Without cAMP (lines 5 and 10), no binding was seen. 0.5 fmols of labeled probe were used in each experiment.







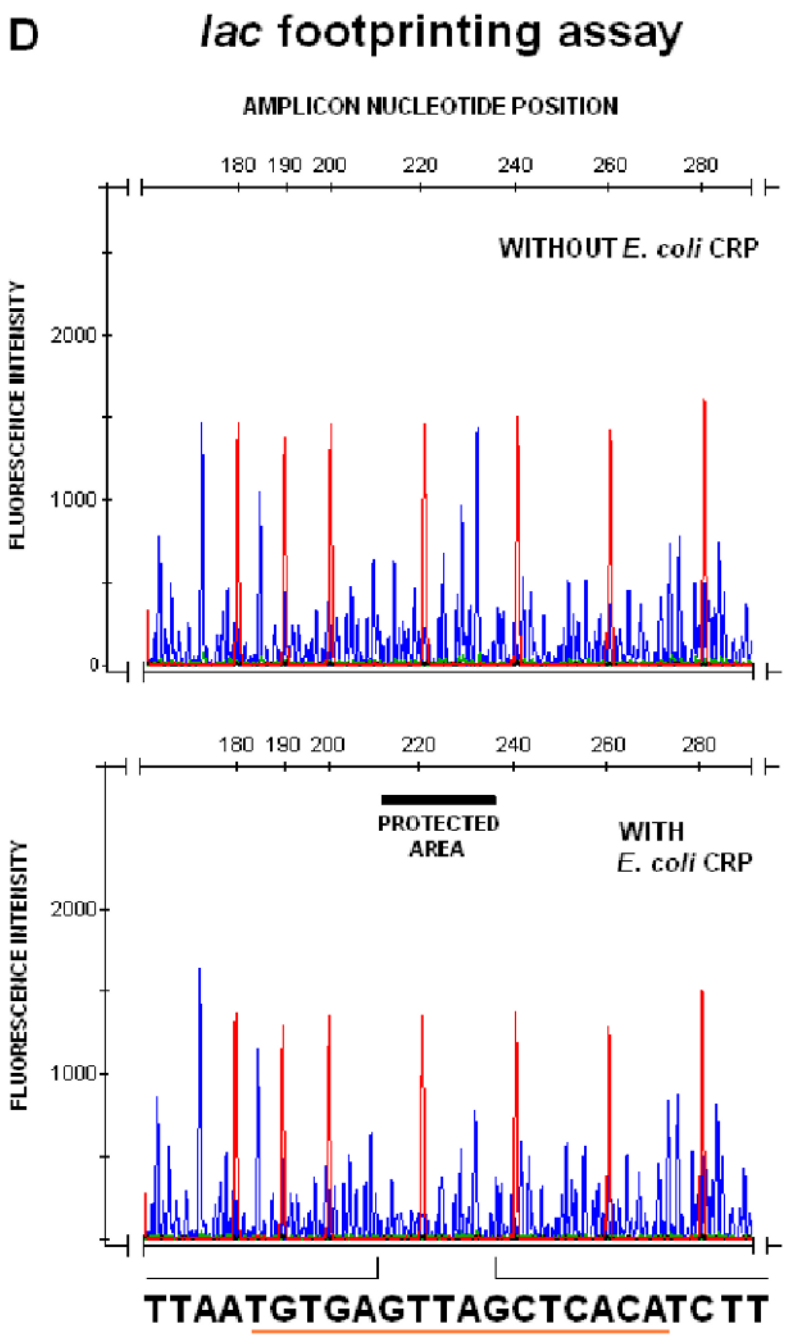


FIG. 3.

DNAseI footprinting assay on *tprE* (Panel A), *tprG* (Panel B), and *tprJ* (Panel C) promoters. Fluorescence intensity and peak size were evaluated using the GeneMapper 3.5 software. Signal from samples digested in presence or absence of recombinant TP0262 or *E. coli* CRP were statistically compared using the paired Student's t-test with significance set at $p<0.05$. Red peaks indicate the ROX-labeled DNA ladder, blue peaks represent fragments generated by DNaseI digestion. Sequences of the protected areas are represented at the bottom of each figure; underlined sequences represent the TP0262 binding site originally predicted *in silico*. In the positive control, the GTGTA-6N-TCACT sequence in the *E. coli lac* promoter was shown to be protected by recombinant *E. coli* CRP as expected (Panel D).

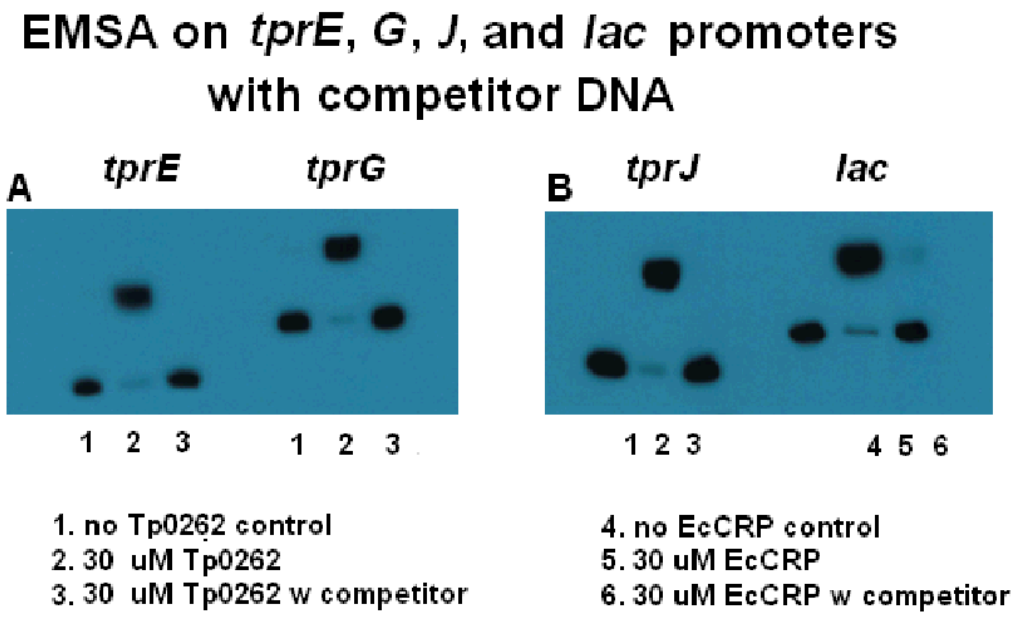


FIG. 4.

Recombinant TP0262 binds the DNA sequences identified by DNaseI footprinting assay. In presence of unlabelled competitor oligonucleotide fragments (Table II) containing the TP0262 *tprE*, *tprG* (Panel A), and *J* (Panel B) binding sites identified by footprinting assay, no shift of the labeled amplicons (containing the putative TP0262 binding sites) used in the previous EMSA experiments was observed. As positive control, a 22 bp unlabelled oligonucleotide (Table II) containing the *E. coli* CRP binding site prevented *E. coli* CRP from shifting the labeled *lac* promoter amplicon. In each experiment, the amount of the unlabelled fragments was 100 fold higher (50 fmol) than the labeled probe (0.5 fmol).

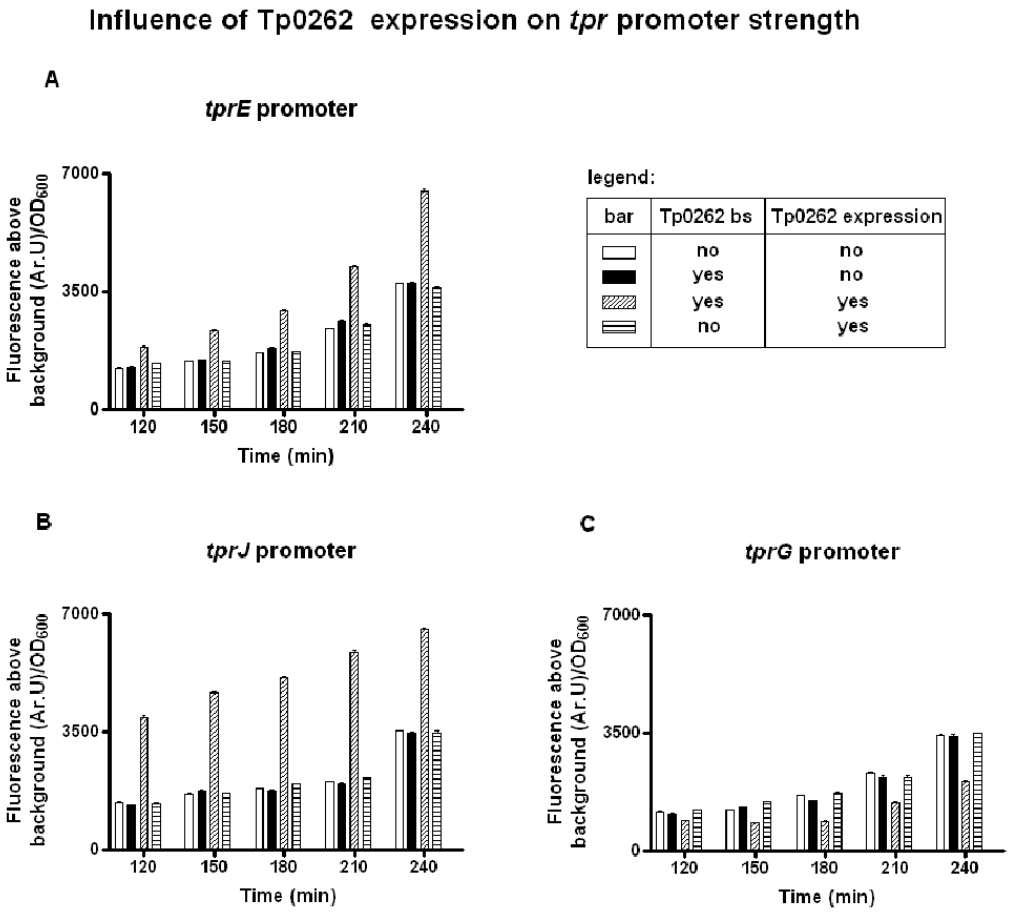


FIG. 5.

TP0262 modulates *tprE*, *tprJ*, and *tprG*, promoter activity (Panels A, B, and C, respectively). Graph values for the *tprs* are for promoter sequences containing only poly-G tracts of 8G residues within the hypervariable poly-G tract. As addressed in the Discussion paragraphs, 8G's were previously shown to be permissive for transcription, while ≥9G's were shown to drastically reduce *tprE*, *G*, and *J* transcription (Giacani et al., 2007a). The GFP reporter assay showed that *tprE*, *G*, and *J* promoters containing ≥9 G residues did not induce fluorescence above background, even in presence TP0262 expression (data not shown). Fluorescence signal induced by the *E. coli lac* promoter (used as positive control) was always ≥10,000 Ar.U/OD_{600} (data not shown). Background fluorescence induced by the negative control plasmid was subtracted from the sample-induced fluorescence signals. Means ± SE of quadruplicate values are represented. TP0262 bs: binding site for TP0262.

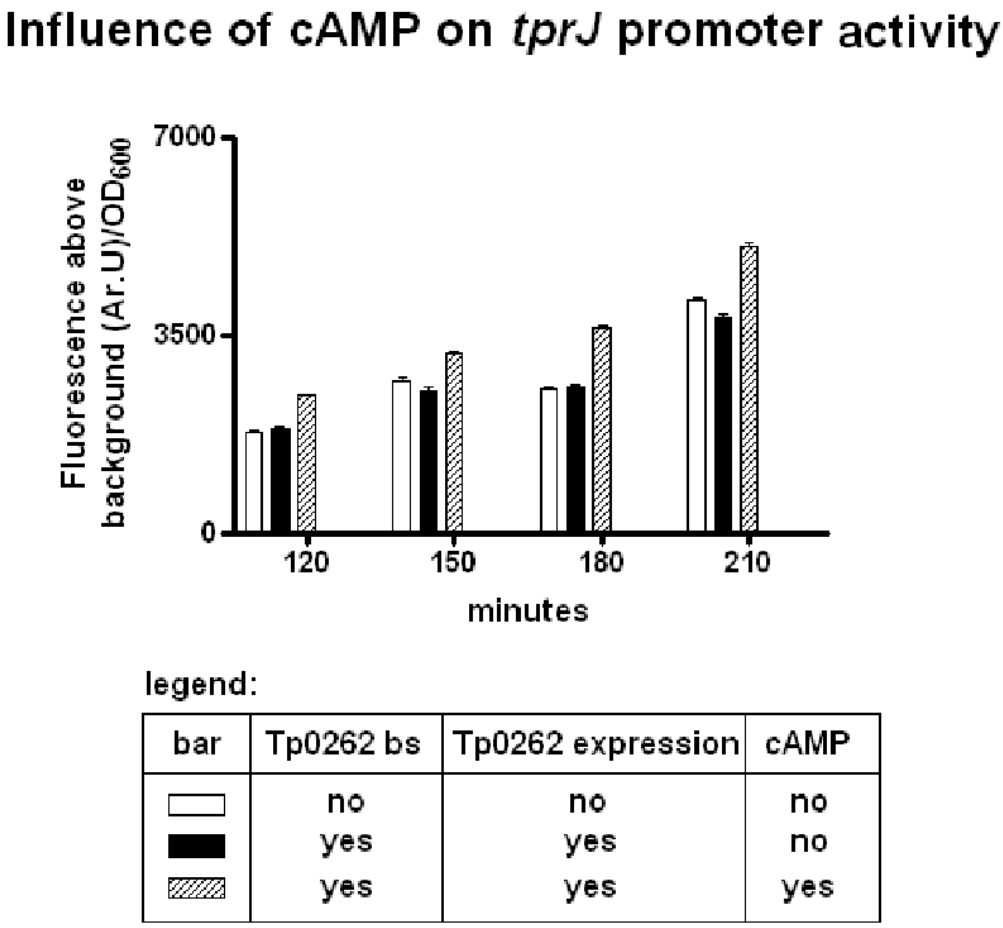


FIG. 6.

cAMP is required for TP0262 influence *tprJ* promoter activity. The constructs containing the *tprJ* promoters were used to transform a *crp* and *cya*-deficient *E. coli* strain (G1044, Δ*crp::cm::cya*$^-$, unable to synthesize cAMP). The positive regulatory effect exerted by TP0262 on the *tprJ* promoter (containing the region with the putative CRP binding site) was absent in culture media not supplemented with cAMP.

Table I

In silico predictions and experimentally determined TpCRP binding sites

Gene	TP0262 binding sites predicted *in silico*		TP0262 binding sites determined by DNaseI footprinting	
	Sequence	Location[1]	Sequence	Location[1]
tprE	TGTGGGCTCGTGCTGG	−236.5	TGCGTGTGTGTGTGGGCTCGTGCTG	-242.5
tprG	AGTGCTAGCAGTCACA	−388.5	GTCAGTGCTAGCAGTCACATAC	-388.5
tprJ	GTTTATTGCGTGCAAG	−233.5	GTTGTCAGTGCACGAGGAGGAG	-259.5

[1] location is expressed as distance (nucleotide position) from the experimentally predicted TSS (Giacani et al., 2005a). For *in silico* predictions, underlines indicate the predicted CRP binding motifs; for footprinting results, underlines represent the TP0262 binding sites originally identified *in silico*.

Table II

Primers used in this study

Purpose	Name[1]	Sequence (5′ to 3′)		Size (bp)[2]
DIRECT CLONING (into pGlow-TOPO vector)	*tprE*-Glow-F	catggtttcggttgtaccc		359
	tprG-Glow-F	taccacgagccccatattct		531
	tprJ-Glow-F	gtacgctgcggtcgatattt		382
	tprE/G/J-Glow-R	tcattcccccgctcgctcct	common	
	t47-Sense	cgtgtggtatcaactatgg		313
	t47-Antisense	tcaaccgtgtactcagtgc		
	*lac*Pro-Glow-F	gtgagcgcaacgcaattaat		213
	*lac*Pro-Glow-R	tcatagctgtttcctgtgtg		
CLONING into the AvrII site (cctagg, underlined) of pGlow-TOPO vector	TP0262-AvrIIF	aaacctagggccttctgcttttgctgagt		1311
	TP0262-AvrIIR	aaacctaggccaacaagggcgaatattgt		
	*Ecoli*CRP-AvrIIF	aaacctaggcactgcgtcaattttcctga		1046
	*Ecoli*CRP-AvrIIR	aaacctagggcgtttgtcgaagtgcatag		
SEQUENCING[3]	T7 promoter	taatacgactcactataggg		N.A.
	gfp-Rev	gggtaagctttccgtatgtacg		
	TP0262-S	ctggagttcaatcgggagaa		N.A
	TP0262-As	gggctatctcttcgatgctg		
	TP0262-F	gccttctgcttttgctgagt		
	TP0262-R	gccaacaagggcgaatattgt		
	*Ecoli*CRP-S	aaacagacccgactctcgaa		N.A.
	*Ecoli*CRP-As	tgtccgggtttacctgaatc		
	*Ecoli*CRP-F	gcactgcgtcaattttcctga		
	*Ecoli*CRP-R	gcgtttgtcgaagtgcatag		
EMSA (primers to generate *tpr* and *lac* promoter amplicons)	*tprE*-EMSA-F	catggtttcggttgtaccc		116
	tprE-EMSA-R	cgtaaactgatcctgcatcc		
	tprG-EMSA-F	taccacgagccccatattct		151
	tprG-EMSA-R	tgacaagtcctttaaccagca		
	tprJ-EMSA-F	gtacgctgcggtcgatattt		163
	tprJ-EMSA-R	gctttgcggaaatccttttt		
	lac-EMSA-F	gtgagcgcaacgcaattaat		213
	lac-EMSA-R	tcatagctgtttcctgtgtg		
EMSA (oligonucleotides) competitors)[4]	*tprE*	tgcgtgtgtgtgtgggctcgtgctg		25
	tprG	gtcagtgctagcagtcacatac		22

Purpose	Name[1]	Sequence (5′ to 3′)	Size (bp)[2]
	tprJ	gttgtcagtgcacgaggaggag	22
	lac	aaatgtgatctagatcacattt	22
FOOTPRINTING	pGLow-FAM-For	F-cggatcgggagatctaatacg[5] common	
	tprE/J-foot-R	ccagtctagtagccccctca	384(*E*); 407(*J*)
	tprG-foot-R	ctgctcatcggctgctcta	372
	lac-foot-R	gggtaagctttccgtatgtagc	483
RT-PCR[6]	*gfp*-S	ctacctgttccatggccaac	262
	gfp-As	tgtgtccgagaatgtttcca	
	TP0262-S	ctggagttcaatcgggagaa	241
	TP0262-As	gggctatctcttcgatgctg	

[1] When three or more primers are grouped together, a single reverse or forward primer (indicated as “common”) was used in different amplification reactions.

[2] The size of the amplicons relative to the *tpr* genes were calculated according to the genome sequence of the Nichols strain (Fraser et al., 1998), without taking into account variability within the poly-G repeats (Giacani et al., 2007a).

[3] The first group of primers was used to sequence the promoters cloned upstream of the *gfp* gene in the pGlow-TOPO vector (Invitrogen); the second and third groups were used to sequence TP0262 and *E. coli crp*, respectively, cloned into the AvrII site of the pGlow-TOPO vector.

[4] For simplicity only one strand of the oligonucleotide is represented.

[5] F indicates 5′-end labeling with the fluorophor 6′-FAM.

[6] The first primer pair was used to assess lack of DNA contamination in DNaseI-treated RNA samples; TP0262-As was used for both gene-specific reverse transcription and DNA amplification.

N.A.: Not Applicable.