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Characterization of a manganese-dependent regulatory protein, TroR, from *Treponema pallidum*

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ABSTRACT **Genome sequence analysis of *Treponema pallidum,* the causative agent of syphilis, suggests that this bacterium has a limited iron requirement with few, if any, proteins that require iron. Instead, *T. pallidum* may use manganese-dependent enzymes for metabolic pathways. This strategy apparently alleviates the necessity of *T. pallidum* to acquire iron from the host, thus overcoming iron limitation, which is a primary host defense. Interestingly, a putative metal-dependent regulatory protein, TroR, which has homology with the diphtheria toxin regulatory protein, DtxR, from *Corynebacterium diphtheriae* was identified from *T. pallidum*. We describe here the characterization of TroR, a regulatory protein. Mobility-shift DNA binding and DNase I footprint assays indicated that purified TroR bound to a 22-nt region of dyad symmetry that overlaps the −10 region of the promoter of the *tro* operon, which contains the genes for a putative metal transport system, the glycolytic enzyme phosphoglycerate mutase, and TroR. Unlike other metal-dependent regulatory proteins like diphtheria toxin regulatory protein and the ferric ion uptake regulator, Fur, which can be activated by divalent metals such as Fe^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+}, TroR is activated only by Mn^{2+}. The TroR-Mn^{2+} complex binds its target sequence and blocks transcription of the *tro*PO/*lacZ* fusion, suggesting that TroR acts as a metal-dependent repressor *in vivo*. In addition, TroR exists as a dimer in both its inactive (metal free) and active states as indicated by chemical crosslinking experiments. Based on these data, we propose that TroR represents a unique regulatory system for controlling gene expression in *T. pallidum* in response to Mn^{2+}.**

Bacteria sense and adapt to changes in their environment by coordinately altering the expression of essential genes. Likewise, pathogenic bacteria are able to monitor changing conditions and correspondingly modulate gene expression to enhance survival and cause disease in the host. Pathogens regulate virulence factors in response to pH, oxidative stress, temperature, nutrient availability, osmolarity, CO_2, and/or ions such as phosphate, calcium, or iron (1, 2). In particular, most pathogenic bacteria respond directly to iron limitation within the host and use this as a signal to coordinately regulate the expression of key virulence factors (3). The regulation occurs through an interaction of Fe^{2+} and a metal-dependent regulatory protein. Two of the best characterized metal-dependent regulatory proteins are the ferric ion uptake regulatory protein (Fur) from *Escherichia coli* and diphtheria toxin regulatory protein (DtxR) from *Corynebacterium diphtheriae.* Binding of Fe^{2+} to the metal-free forms of these proteins permits them to bind to palindromic sequences within the promoter region and repress transcription of the genes they regulate. Decreased intracellular iron concentration as a consequence of host limitation results in the inactivation of the regulatory proteins, leading to a derepression of genes important in virulence (e.g., toxins, adhesins, iron transport systems, etc.).

The lack of *in vitro* culture techniques and genetic systems for *Treponema pallidum* has made it extremely difficult to identify virulence or regulatory factors involved in the pathogenesis of syphilis. The recently completed genome sequence project revealed that *T. pallidum* has a small genome (1.3 Mb) and limited metabolic capabilities, with no tricarboxylic acid (TCA) cycle, no cytochromes, and no pathways for the biosynthesis of lipid, amino acid, nucleotide, lipopolysaccharide, or cell wall precursors. *T. pallidum* lacks a respiratory electron transport chain and must hydrolyze ATP to generate a proton motive force to drive transport and motility. Interestingly, few, if any, ORFs encoding putative iron-containing proteins such as superoxide dismutase (Sod), peroxidase, catalase, or cytochromes were identified (4). Taken together, these data suggest that *T. pallidum* has a low requirement for iron.

Despite its limited metal requirement, analysis of the *T. pallidum* genome revealed a putative metal-dependent regulatory protein, TroR. The gene encoding TroR is part of the transport-related operon (*tro*), which contains six genes. The first four genes in the operon encode a putative ABC metal transport system (*troA-D*), the fifth encodes TroR (*troR*), whereas the sixth gene encodes a glycolytic enzyme, phosphoglycerate mutase (*gpm*) (Fig. 1*A*). We report here on the metal dependence of TroR and its effect on gene expression. Mobility-shift DNA binding and DNase I footprinting assays indicated that TroR is activated only by Mn^{2+} as a cofactor. These data suggest that metal regulation, which is based on Mn^{2+} instead of Fe^{2+}, plays a pivotal role in the pathogenesis of *T. pallidum*.

MATERIALS AND METHODS

Bacterial Strains. *T. pallidum* was cultured by intratesticular infection of adult male New Zealand white rabbits, and bacterial cells were purified from host tissue as described (5). *E. coli* strains XL1-Blue MRF' (Stratagene) and TOP10 (Invitrogen) were grown at 37°C in LB broth supplemented with ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), isopropyl β-D-thiogalactopyranoside (IPTG) (1.0 mM or 0.01 mM), or 2′-2 dipyridyl (300 μM) as needed. All chemicals were purchased from Sigma, and DNA restriction or modifying enzymes were purchased from Promega unless stated otherwise.

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Abbreviations: Fur, ferric ion uptake regulatory protein; DtxR, diphtheria toxin regulatory protein; TCA, tricarboxylic acid; *tro*, transport-related operon; IPTG, isopropyl β-D-thiogalactopyranoside; fnr, fumarate nitrate reduction.
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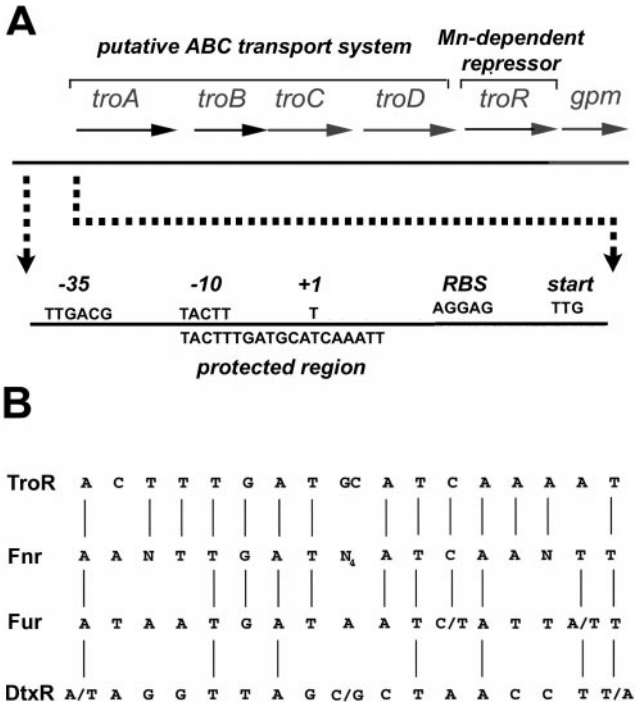

FIG. 1. A schematic diagram of the *tro* operon from *T. pallidum*. (*A*) Shown is the relative position of the promoter (−10 and −35), the putative ribosome binding site (RBS), the 5′ end of the mRNA (+1), the putative translational start site (start), and the region protected from DNase I digestion by purified TroR (protected region). The nucleotide sequence of each region is shown below the designated site. (*B*) Comparison of the binding site of TroR to the consensus binding sequences of Fnr, Fur, and DtxR

DNA Manipulations and Sequencing. *T. pallidum* chromosomal DNA was isolated from purified organisms as described by Saito and Miura (6). Oligonucleotide primers were synthesized by the Molecular Genetics Instrumentation Facility, University of Georgia, Athens. Primers TroR1 (CCGTGTAGGCTGAGAATTCGATGTCCTTAG) and TroR2 (GACAAAAGGCGAATTCCTCACCCCATAC) were designed to incorporate *Eco*RI sites into the PCR product and used to amplify *troR* from 10 ng of *T. pallidum* chromosomal DNA by PCR using Pfu polymerase (Stratagene) in a PTC-100 thermal cycler (MJ Research, Watertown, MA) (1 cycle for 2 min at 94°C and 40 cycles of 40 sec at 94°C (denaturation), 30 sec at 50°C (annealing), and 40 sec at 72°C (elongation). The PCR product was digested with *Eco*RI and ligated into the *Eco*RI site of the expression vector pTrcHisC (Invitrogen) generating pJEP1. This PCR product also was ligated into the *Eco*RI site of pKK223–3 (Amersham Pharmacia) generating pJEP2.

Primers TroP/O1 (GGAACAGGTGATCTAAGCTTTCGTTG) and TroP/O2 (CGAACTCTGCTTCCTCAAGAGAAAGACG) were used to amplify the *tro* promoter/operator region from 100 ng of *T. pallidum* chromosomal DNA by PCR using the Thermalase kit (Amresco, Solon, OH) (1 cycle for 2 min at 94°C and 30 cycles of 40 sec at 92°C, 40 sec at 60°C, and 60 sec at 72°C. An adenosine base was added to both 3′ ends of the PCR product by using 1 unit of *Taq* polymerase for 5 min at 72°C. The PCR product was extracted with Tris-saturated phenol, concentrated by precipitation with 100% ethanol, dried under vacuum, and suspended in 50 μl of sterile water. The fragment was ligated into pCR2.1 (Invitrogen) generating pJAM1. Plasmid pJAM1 was digested with *Xho*I and *Hinc*II, a 310-bp fragment was purified by using the Qiagen Gel Extraction Kit (Chatsworth, CA), and ligated into the *Xho*I and *Eco*RV restriction sites of pLitmus29 (New England BioLabs) generating pJAM2. To generate the *tro*PO/*lacZ* trancriptional fusion, pKOK6 was digested with *Bam*HI, and the 4.7-kb fragment that contained the promoterless *lacZ* was purified by using the Qiagen Gel Extraction Kit. This fragment was ligated into the *Bam*HI site of pJAM2 to generate pJAM3. This construct was digested with *Xba*I, filled in by using Klenow, and then treated with *Bgl*II. The 5-kb fragment that contained *tro*PO/*lacZ* was purified as described above and ligated into the *Bam*HI and *Eco*RV restriction sites of pACYC184 generating pJAM4. All constructs were sequenced at the Molecular Genetics Instrumentation Facility, University of Georgia to confirm that the insert was in the correct orientation and no errors had been introduced by PCR.

The β-galactosidase activity from cells harboring transcriptional fusions were performed as described by Miller (7). *E. coli* cells harboring pKK223–3, pJEP2, and/or pJAM4 were grown in LB, LB + IPTG (10 μM), and LB + IPTG + 2′-2 dipyridyl (300 μM) for 12 h at 37°C. Cells were harvested by centrifugation (8,000 × *g*, 5 min, 4°C) and assayed for enzyme activity. Enzymatic activity was determined from three separate experiments, and assays were performed in triplicate.

Electrophoresis and Immunoblotting. Proteins were separated by using SDS/PAGE) as described (8) and visualized with Coomassie brilliant blue R-250. Protein standards were purchased from Bio-Rad. For immunoblots, proteins were transferred to nitrocellulose (0.45 mM Protran membrane, Schleicher & Schleicher) by using a Bio-Rad Trans Blot Cell (30 V, 1.5 h, 4°C) (9). After transfer, the proteins were visualized with Ponceau red stain so that the protein standards could be marked. The immunoblots were probed with anti-TroR serum (diluted 1:1,000) and washed, and immunoreactive protein were visualized as described (10).

Purification of HisTroR and Recombinant TroR. His-TroR fusion was expressed in *E. coli* TOP10 harboring pJEP1 by growing the cells in 1 liter of LB at 37°C with vigorous shaking. When the cells reached an A_{600} of 0.8, IPTG was added to the culture to a final concentration of 1 mM and incubation was continued for an additional 3 h. After induction, the cells were harvested by centrifugation (12,000 × *g*, 20 min, 4°C) and stored at −20°C until needed. The his-TroR fusion was purified by using Ni-nitrilotriacetic acid resin (Qiagen) following the manufacturer's instructions for the denaturing purification of insoluble proteins. One-milliliter fractions were collected and analyzed by SDS/PAGE, and those that contained purified his-TroR were pooled and the protein was stored at −20°C. Purified his-TroR was used to raise polyconal antiserum in a female New Zealand White rabbit at Cocalico (Reamstown, PA).

For the purification of native TroR, *E. coli* XL1-Blue MRF' cells harboring pJEP2 were grown in 100 ml of LB at 37°C with shaking, and expression of TroR was induced as described above. After induction, the cells were harvested by centrifugation (12,000 × *g*, 20 min, 4°C) and stored at −20°C until needed. Under these conditions, TroR localized to inclusion bodies as determined by centrifugation (30,000 × *g*, 30 min, 4°C) and SDS/PAGE. Based on these results, TroR was purified from inclusion bodies by using the method of Nguyen *et al.* (11) with minor modifications. After harvesting, the cells were suspended in 20 ml of lysis buffer (0.1 mM EDTA/50 mM NaCl/0.1 mM DTT/5% glycerol in 50 mM Tris·HCl, pH 7.0) that contained deoxycholate (0.2% wt/vol) and lysozyme (200 μg/ml), and incubated for 30 min at 4°C. The cells were lysed by two passages through a cold French Press cell at 8,000 psi. The inclusion bodies were harvested by centrifugation (12,000 × *g*, 30 min, 4°C), suspended in lysis buffer containing 2% deoxycholate, and incubated with gentle agitation for 60 min at 4°C. The inclusion bodies were harvested by centrifugation (30,000 × *g*, 30 min, 4°C) and washed a second time. The inclusion bodies were suspended in 25 ml of lysis buffer (without EDTA) that contained 0.25% Sarkosyl and incubated for 1 h at 4°C with gentle agitation. The insoluble material was

removed by centrifugation (12,000 × *g*, 30 min, 4°C), and the supernatant was dialyzed against 4 liters of dialysis buffer (0.1 mM DTT/5% glycerol in 50 mM Tris·HCl, pH 7.6) for 12 h at 4°C. The extract was applied to a 5-ml HiTrap SP column (Amersham Pharmacia) that was equilibrated with dialysis buffer, and proteins were eluted with a linear 0–1.0 M NaCl gradient. One-milliliter fractions were collected and analyzed by SDS/PAGE and immunoblot. The fractions that contained TroR were pooled and dialyzed against 4 liters of dialysis buffer for 12 h at 4°C, and the purified protein was stored at −80°C. The concentration of metal(s) associated with purified TroR was determined by using inductively coupled plasma-emission MS as described (12).

Mobility-Shift DNA Binding. Mobility-shift DNA binding were performed as described by de Lorenzo *et al.* (13) with minor modifications. To generate a ^{32}P-labeled DNA fragment that contained the *tro*P/O target sequence, pJAM1 was treated with *Xho*I and *Hin*cII and labeled with [a^{32}P]dATP by using Klenow, and a 310-bp DNA fragment was isolated after gel electrophoresis (14). The binding of TroR to the target sequence was assayed in a 20-μl reaction mixture that contained 5 mM MgCl, 40 mM KCl, 1 mM DTT, 5% glycerol, 20 mM Tris·HCl (pH 7.6), 5 μg of BSA, 1 μg of sonicated calf thymus DNA, purified TroR, and 20,000 cpm of target sequence. Stock solutions of metals ($MnCl_2$, $FeSO_4$, $ZnCl_2$, $CoCl_2$, or $NiCl_2$) were made fresh before each assay and used at a final concentration of 125 μM. The reaction was incubated for 15 min at 30°C, and 10 μl of the reaction mixture was applied to a 5% nondenaturing polyacrylamide gel that contained 2.5% glycerol in 40 mM Bis-Tris borate (pH 7.6). The samples were separated on a 5% polyacrylamide gel (200 V constant voltage, 3 h) and analyzed by autoradiography.

DNase I Footprint. The target sequence for this assay was generated by treating pJAM2 with *Bgl*II and end-labeling with [g^{32}P]dATP by using T4 polynucleotide kinase. The plasmid then was treated with *Bam*HI, and the 310-bp fragment that contained the promoter region was isolated from a 5% nondenaturing polyacrylamide gel. A 19-μl reaction that contained 20 mM Tris·HCl (pH 7.6), 40 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT, 5% glycerol, 2 mg/ml sonicated calf thymus DNA, 1 mg BSA, target sequence DNA (20,000 cpm), and purified TroR with or without the divalent metals was incubated for 15 min at 30°C. Then 1 μl of DNase I (0.1 unit) was added, and the incubation was continued for an additional 1 min. The reaction was stopped by adding 20 μl of 5 M ammonium acetate, 50 mM EDTA, and 50 mg/ml tRNA (stop buffer). Cold absolute ethanol (120 μl) was added, and fragments were precipitated for 12 h at −20°C. The DNA was harvested by centrifugation (12,000 × *g*, 30 min, 25°C), washed with 200 μl of 70% ethanol, and dried under a vacuum. The fragments were suspended in 4 μl of formamide/tracking dye solution, heated for 3 min at 95°C, and then placed immediately on ice. The DNA fragments were separated on a 6% denaturing polyacrylamide gel and analyzed by autoradiography.

Chemical Crosslinking of TroR. To determine whether TroR is in the dimer or monomer form before and after activation by Mn, chemical crosslinking assays were performed as described with slight modifications (15). Purified TroR was dialyzed against 2 liters of 2 mM DTT in 20 mM Hepes buffer (pH 7.6) at 4°C for 4 h. A 90-μl reaction mixture containing 2 μg/ml of purified TroR in crosslinking buffer (20 mM NaCl/10 mM KCl/2 mM DTT in 20 mM Hepes, pH 7.6) and $MnCl_2$ or target DNA were incubated for 10 min at 30°C. Glutaraldehyde then was added to a final concentration of 0.1%, and the reaction mixture was incubated for 60 sec at 30°C. Reactions were stopped by adding 30 μl of SDS/PAGE sample buffer, and the samples were heated at 95°C for 5 min and analyzed by immunoblot as described above.

RESULTS

Purification of TroR. Comparisons of the deduced amino acid sequences of the ORFs in the *tro* operon (Fig. 1*A*) indicated that one ORF (designated as *troR*) encodes a hypothetical protein with homology to iron-activated repressor proteins such as DtxR from *C. diphtheriae* (16). Because iron-dependent regulation plays a critical role in gene expression in several bacterial pathogens, we analyzed the role of TroR in regulation in *T. pallidum.* The ORF encoding TroR was amplified by PCR from *T. pallidum* chromosomal DNA by using primers TroR1 plus TroR2, and the product was introduced into the *Eco*RI site of expression vector pTrcHisC generating pJEP1. *E. coli* cells harboring pJEP1 overexpressed a 25-kDa his-TroR fusion protein, and it localized to the insoluble fraction of the cell lysate. To purify this his-TroR fusion protein, cell lysate was treated with 8 M urea and applied to a Ni-nitrilotriacetic acid column. The protein was eluted with 250 mM imidazole in 8 M urea, fractions containing his-TroR were pooled, and purified protein was stored at −70°C. Polyclonal sera was generated against the purified fusion protein and used to monitor the purification of the native TroR, overexpressed in *E. coli*.

The *troR* gene was introduced into the *Eco*RI site of expression vector pKK223–3 generating pJEP2. Localization experiments indicated the induction of TroR in cells harboring pJEP2 resulted in >90% of TroR being localized in inclusion bodies whereas <10% was located in the soluble fraction as determined by SDS/PAGE and immunoblots (data not shown). Inclusion bodies were harvested by centrifugation and washed with 2% deoxycholate. TroR was solublized with 0.25% Sarkosyl and then subjected to ion-exchange chromatography, which resulted in the purification of TroR to apparent homogeneity as determined by SDS/PAGE (data not shown). The estimated molecular mass of the purified protein was 19 kDa, which was similar to the predicted size of the protein (17.1 kDa).

TroR Bound to the *tro* Promoter/Operator Region. Using primer extension analysis, Hardham *et al.* (16) identified the 5′ end of a transcript that originated 25 nt upstream from a putative translational start codon for *troA,* the first gene in the *tro* operon (Fig. 1*A*). Based on these data, a potential −10 promoter region was identified. This sequence was flanked by a small region of dyad symmetry that is 81% identical to the consensus binding sequence for fumarate nitrate reduction (Fnr) regulator (17) and 63% identical to Fur from *E. coli* (Fig. 1*B*) (18). In contrast, the sequence is only 41% identical to the consensus binding sequence recognized by DtxR (Fig. 1*B*) (19). Based on the proximity of a potential binding sequence to the −10 region and the sequence similarities between TroR and other metal-activated repressor proteins, we postulated that TroR binds to this target sequence and regulates the operon in a metal-dependent fashion. To test this possibility, TroR binding to this region was determined by using a mobility-shift DNA binding assay.

A 310-bp fragment that contained the promoter region of the *tro* operon was isolated from plasmid pJAM1 and labeled with [^{32}P]. This fragment was incubated with purified TroR in the presence or absence of divalent metal ions, and binding was assayed by using a mobility-shift DNA binding assay. In the absence of divalent metals, TroR bound to the probe very poorly and only at concentrations >1 μM (Fig. 2*A*, lanes 5 and 6). Inductively coupled plasma emission MS did not detect any Mn^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, or Cd^{2+} contamination of purified TroR. In the presence of 100 μM Mn^{2+}, however, >90% of the labeled probe was shifted with 10 nM TroR (Fig. 2*B*, lane 3). Other divalent metal ions such as Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, or Zn^{2+} (final concentrations of 125 μM) also were tested for the ability to activate TroR binding in this assay. None of these metals activated TroR, suggesting the protein is

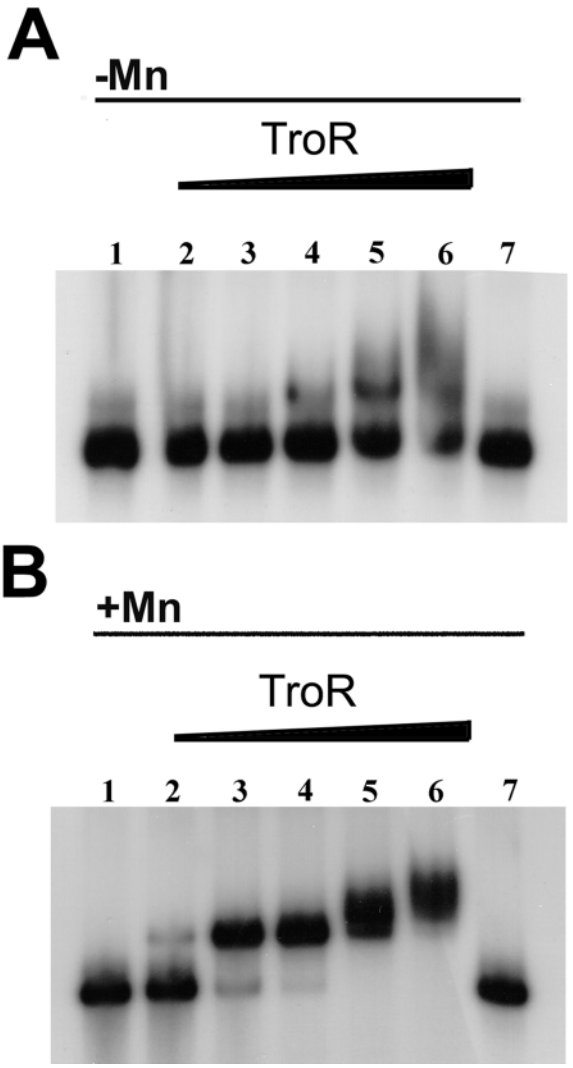

FIG. 2. TroR bound to a DNA fragment that contained the *tro* promoter. Five percent polyacrylamide gels showing the binding of purified TroR to the 310-bp ^{32}P-labeled fragment containing the *tro*PO. Increased concentrations of TroR (1, 10, 100, 1,000, and 2,000 nM; lanes 2–6, respectively) were incubated with radiolabeled probe in the absence of Mn (*A*, designated −Mn) or in the presence of 100 μM $MnCl_2$ (*B*, designated +Mn). Lanes 1 and 7 (in *A* and *B*) were reaction mixtures that contained no TroR

specific for Mn^{2+} (data not shown). This finding was quite unexpected because metal-activated repressors, like DtxR or Fur, can be activated by several divalent metal ions *in vitro* (18, 20, 21).

TroR Bound to a Region of Dyad Symmetry 5′ of the *tro* Operon. Mobility-shift DNA binding assays indicated that TroR was activated by Mn^{2+} and bound to a fragment of DNA that contained the *tro* promoter. To determine the sequence that was recognized by TroR within the *tro* promoter region, DNase I footprint analysis was performed. A 310-bp fragment that contained the −150 to +160 region of the *tro* operon was labeled on the coding strand for the DNase I footprint assays. Because the mobility-shift DNA binding assay indicated that TroR would not bind to the target sequence without Mn^{2+}, 100 μm $MnCl_2$ was included in the buffer for these assays. The results of a typical footprint are shown in Fig. 3. Complete protection of DNase I digestion was observed at concentrations of TroR as low as 40 nM (Fig. 3, lane 4). TroR protected a 22-nt area on the coding strand that included the region of dyad symmetry (Fig. 3, denoted by the arrows), the potential transcriptional start site (Fig. 3, denoted by the +1 and the

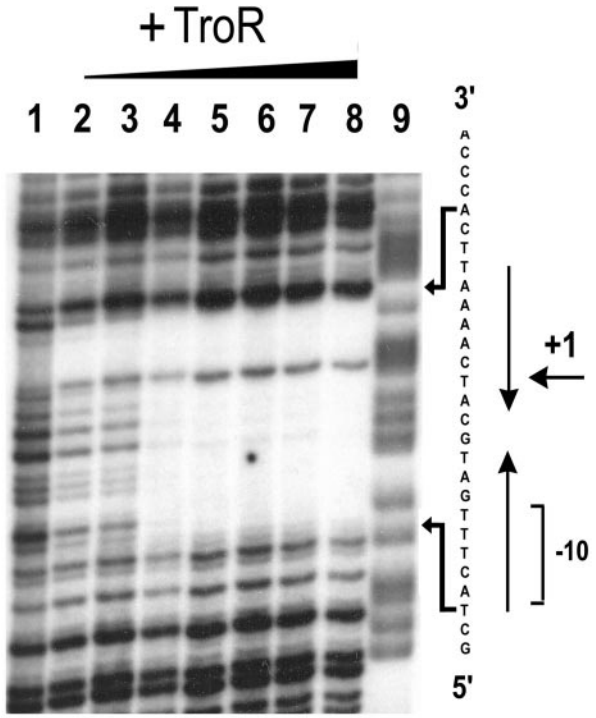

FIG. 3. DNase I footprint analysis of TroR. The autoradiograph of a 6% denaturing polyacrylamide gel showing the sequence of the *tro*PO protected from DNase I digestion by TroR. Lanes 1–8 show reactions that contained increasing concentrations of TroR (0, 10, 20, 40, 60, 80, and 100 nM, respectively) in the presence of 100 μM $MnCl_2$. G+A sequence ladder is shown in lane 9, and the sequence protected by TroR is shown on the right. The −10 region of the promoter is indicated on the far right and +1 indicates the 5′ end of the mRNA. The arrows denote region of dyad symmetry within the binding sequence.

arrow), and the −10 region of the putative promoter of the *tro* operon. Binding to the target sequence depended on TroR concentration and the protected palindromic sequence, TACTTTGATGCATCAAAATTCA, is 88% identical to the consensus binding sequence for Fnr from *E. coli* (17), 68% to that for Fur (18), and 44% to the consensus target sequence for DtxR (19). At this time, we have not tested the ability of TroR to recognize these other sequences *in vivo* or *in vitro*.

Titration of Mn^{2+} in the footprint assay indicated that maximum protection occurred at 80 μM Mn^{2+} (data not shown). In the absence of metals, TroR failed to protect the DNA from digestion (Fig. 4, lane −). TroR also failed to protect the target sequence from DNase I digestion in the presence of other metals tested, including Zn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, and Fe^{2+} at concentrations of 100 μM (Fig. 4) or as high as 1 mM (data not shown). These data confirmed the results from the mobility-shift DNA binding assays, which indicated that TroR was activated only by Mn^{2+}. This finding is in marked contrast to the activation profiles that have been reported for other metal-dependent repressor proteins, such as Fur or DtxR, which are activated by several different divalent heavy metals both *in vitro* and *in vivo* (18, 21). Comparison of the five key iron-binding residues in DtxR with comparable residues in TroR shows that two amino acids are different. Cys-102 and Met-10 in DtxR correspond to Glu-105 and Asp-10 in TroR whereas the other three metal ligands (Asp-4, Glu-105, and His-106) are the same (22). These differences may explain the observed variations in metal specificity between TroR and DtxR.

TroR Bound to the *tro* Promoter/Operator *in Vivo* to Repress Transcription. Having demonstrated that TroR bound to a 22-nt sequence within the −10 region of the

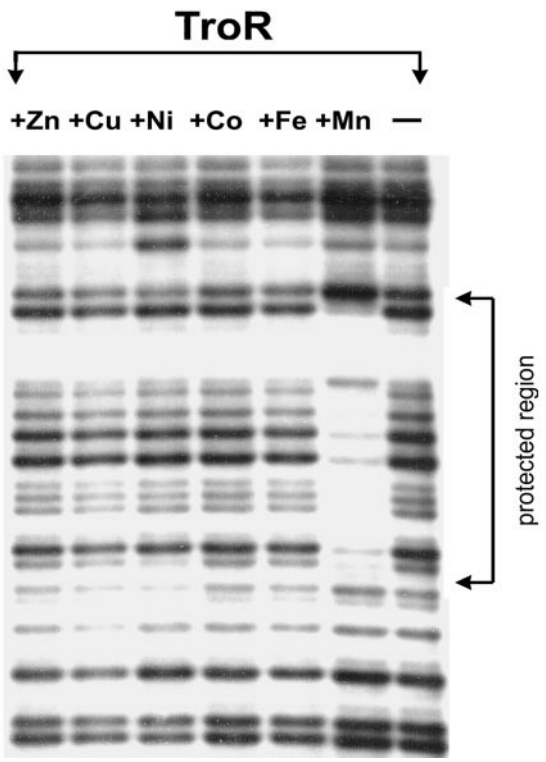

FIG. 4. DNase I footprint analysis of the activation of TroR by divalent heavy metal ions. An autoradiograph of a 6% denaturing polyacrylamide gel showing the effect of different divalent metal ions on the binding of TroR to the target sequence. The metal used in each reaction (final concentration of 100 μM) is shown at the top. The lane designated − contained no metal. The region protected from DNase I digestion is shown to the right.

promoter of the *tro* operon *in vitro*, we wanted to determine whether the binding of TroR affected transcription of the *tro* operon either positively or negatively. To determine whether TroR was acting as either a transcriptional activator or repressor of *tro* operon, we constructed a *tro*PO/*lacZ* transcriptional fusion, introduced it into *E. coli* harboring pJEP2 (*troR*), and measured β-galactosidase activity (Fig. 5). When TroR was expressed in *E. coli* cells harboring *troPO*/*lacZ*, little β-galactosidase activity was detected (Fig. 5, lane 2, gray bar). Without TroR, β-galactosidase activity was high (Fig. 5, lane 2, black bar), indicating that TroR exerted a negative regulatory effect

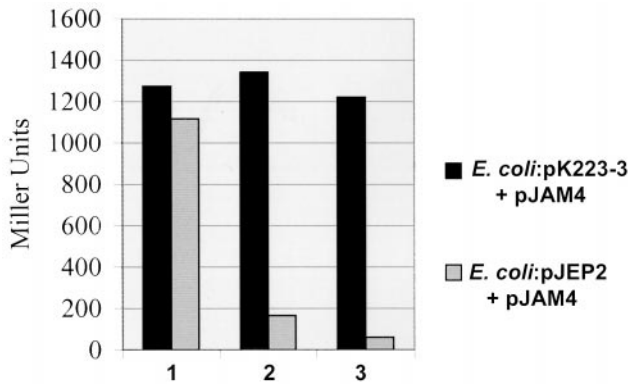

FIG. 5. TroR bound to the tro promoter and blocked transcription. β-galactosidase activity measured in *E. coli* cells harboring pJAM4 coresident with pKK223–3 (designated by black bar) or pJEP2 (pKK223–3-*troR*)(designated by gray bar). IPTG was added to induce the expression of TroR and 2′-2 dipyrdil (DIP) to chelate divalent metal ions. The strains were grown in LB (lane 1), LB and IPTG (lane 2), or LB, IPTG, and DIP (lane 3) and assayed as described above.

on the transcriptional fusion. When chelators, such as 2,2′ dipyridyl, EDTA, or desferrioxamine are added at sublethal concentrations to cells harboring these constructs, no increase in β-galactosidase activity was detected (Fig. 5, lane 3, gray bar). This finding suggested that the conditions used in these experiments did not decrease the intracellular Mn concentrations to levels that resulted in derepression of the *tro*PO/*lacZ* fusion. The data from the analysis of the transcriptional fusion suggest that TroR acts as a repressor of the *tro* operon.

Inactive and Activated TroR Exist as Dimers. Many metal-dependent regulatory proteins bind their target sequence as dimers. In some cases, repressor proteins such as DtxR (15) and Fnr (23) exist as monomers in the inactive state and dimerize in the presence of metals. Others, such as Fur, exist as dimers or multimers and bind target sequences as dimers after activation by Fe^{2+} (24–26). To determine whether TroR existed as a monomer or dimer in the inactive or active state, chemical crosslinking experiments were performed on purified TroR using glutaraldehyde, and reaction products were analyzed by using immunoblots probed with anti-his-TroR sera (Fig. 6). Analysis of reaction mixtures that contained purified TroR (Fig. 6, lane 1) or TroR + glutaraldehyde (Fig. 6, lane 2) indicated that TroR existed as a dimer in solution. The addition of Mn^{2+} and/or target sequence had no effect on dimerization (Fig. 6, lanes 3–5). Given that purified TroR failed to bind to the *tro* promoter/operator in the absence of Mn^{2+}, these data suggest that Mn^{2+} induces a conformational change in TroR that stimulates DNA binding activity.

DISCUSSION

Pathogenic bacteria are able to respond to different conditions they encounter at various sites within a host and alter gene expression to promote growth and survival and cause disease. Likewise, as *T. pallidum* is transmitted from human to human through sexual contact, it first must adapt to and then overcome the challenges presented at the mucosa and submucosa to begin colonization of the new host. As the infection progresses from primary to secondary syphilis, the bacterial cells disseminate to other sites within the body, including the skin, heart, joints, and central nervous system, which present a new set of conditions to which the bacterium must adapt (27). The inability to culture *T. pallidum in vitro* has made it difficult to identify or study the mechanisms that the bacterium uses to sense and correspondingly regulate gene expression in response to these changes.

We report here on the characterization of TroR and its interaction with the *tro*PO region. The most surprising feature of the TroR-DNA interaction was the metal specificity. Mobility-shift DNA binding and DNase I footprint assays demonstrated that only Mn^{2+} could activate purified TroR. This

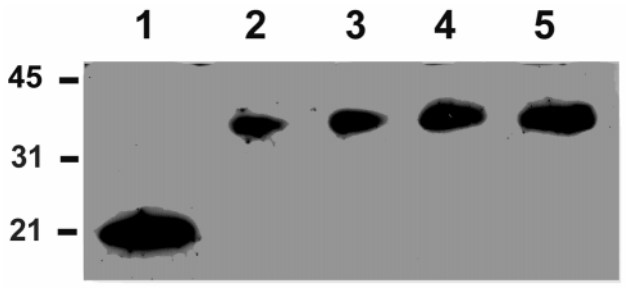

FIG. 6. TroR existed as a dimer in the absence of Mn. Immunoblot of purified TroR in chemical crosslinking assay: TroR (lane 1); TroR + 0.2% glutaraldehyde (lane 2); TroR + 0.2% glutaraldehyde + 100 μM $MnCl_2$ (lane 3); TroR + 0.2% gluteraldehyde + 0.5 μg of *tro*PO target DNA (lane 4); and TroR + 0.2% gluteraldehyde + 100 μM $MnCl_2$ + 0.5 μg of *tro*PO target DNA (lane 5). Molecular mass standards are shown on the left.

finding is in contrast to other metal-dependent repressor proteins such as Fur, DtxR, SirR from *Staphylococcus epidermidis,* DesR from *Streptomyces* species, and IdeR from *Mycobacterium tuberculosis,* which regulate gene expression in pathogenic bacteria by sensing intracellular levels of Fe^{2+}. Furthermore, these proteins are activated by a variety of divalent metal ions. For example, Co^{2+}, Ni^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+} promote the binding of DtxR to its target sequence (21), whereas Fur is activated by Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, or Mn^{2+} (13). DtxR is activated by these divalent metal ions *in vivo*, as addition of Co^{2+}, Ni^{2+}, Fe^{2+}, Mn^{2+}, or Cu^{2+} to the growth media of *C. diphtheriae* at sublethal concentrations inhibits diphtheria toxin production (20). We demonstrated that TroR repressed transcription of a *tro*PO/*lacZ* reporter gene in *E. coli*, and our *in vitro* data suggest that this repression depends on Mn^{2+}.

One major contributing factor to the survival of pathogenic bacteria within the host is iron availability. Although it is abundant in the host, the amount of free iron within tissues is tightly controlled because these ions are toxic at high concentrations. Levels are extremely limited by siderophilins, such as transferrin and lactoferrin, or storage proteins like ferritin. The challenge to invading bacteria is to acquire sufficient Fe^{2+} for growth, and the mechanisms for iron acquisition have been studied extensively (28, 29). Pathogenic bacteria also use iron limitation as a signal to alter gene expression of key virulence factors, which does not seem to be the case for *T. pallidum.* Analysis of the genome sequence revealed that *T. pallidum* has no identified iron transport system and few identified genes encoding proteins that would require Fe^{2+} as a cofactor. No genes were identified that encoded heme-containing proteins (e.g., catalase, cytochrome, cytochrome oxidase, or peroxidase), Fe-S proteins (e.g., aconitase, succinate dehydrogenase, or the large subunit of glutamate synthase) or non-Fe-S, nonheme proteins (e.g., superoxide dismutase) (4). Therefore, based on these data and the metal specificity of TroR reported here, we propose that Mn^{2+} availability rather than Fe^{2+} is important for metal-dependent gene regulation in *T. pallidum*.

Measured levels of Mn at various sites in the human show a very interesting pattern with extremely low levels (0.0025 μg/ml) in the skin, 30-fold higher in the blood (0.08–0.13 μg/ml), and $\geq$100-fold higher in the central nervous system (9–10 μg/ml) than in the blood (30–32). These higher levels in the central nervous system are required for normal neurological function by serving as a cofactor for essential enzymes such as glutamine synthetase, mitochondrial superoxide dismutase, and calmodulin-dependent phoshatase. These data indicate that *T. pallidum* encounters changing levels of Mn as it disseminates to various sites. It seems that one likely function of TroR is to prevent the accumulation of toxic levels of intracellular Mn as it encounters increased levels of Mn in the blood or central nervous system. Also, the presence in the *tro* operon of a gene encoding the glycolytic enzyme phosphoglycerate mutase suggest that TroR exerts a regulatory effect on central metabolism as well.

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