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Molecular Mechanism of Tryptophan-Dependent Transcriptional Regulation in *Chlamydia trachomatis*

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Tryptophan is an essential amino acid that is required for normal development in *Chlamydia* species, and tryptophan metabolism has been implicated in chlamydial persistence and tissue tropism. The ability to synthesize tryptophan is not universal among the *Chlamydiaceae*, but species that have a predicted tryptophan biosynthetic pathway also encode an ortholog of TrpR, a regulator of tryptophan metabolism in many gram-negative bacteria. We show that in *Chlamydia trachomatis* serovar D, TrpR regulates its own gene and *trpB* and *trpA*, the genes for the two subunits of tryptophan synthase. These three genes form an operon that is transcribed by the major form of chlamydial RNA polymerase. TrpR acts as a tryptophan-dependent aporepressor that binds specifically to operator sequences upstream of the *trpRBA* operon. We also found that TrpR repressed in vitro transcription of *trpRBA* in a promoter-specific manner, and the level of repression was dependent upon the concentrations of TrpR and tryptophan. Our findings provide a mechanism for chlamydiae to sense changes in tryptophan levels and to respond by modulating expression of the tryptophan biosynthesis genes, and we present a unified model that shows how *C. trachomatis* can combine transcriptional repression and attenuation to regulate intrachlamydial tryptophan levels. In the face of host defense mechanisms that limit tryptophan availability from the infected cell, the ability to maintain homeostatic control of intrachlamydial tryptophan levels is likely to play an important role in chlamydial pathogenesis.

There is accumulating evidence that tryptophan plays a pivotal role in intracellular chlamydial growth and pathogenesis, which has led to interest in the mechanisms that regulate intrachlamydial tryptophan levels. It is known that infected host cells can limit the supply of tryptophan through a gamma interferon (IFN-γ)-mediated mechanism (23). IFN-γ transcriptionally activates a cellular enzyme, indoleamine-2-3-dioxygenase, which degrades the intracellular tryptophan pool, leading to inhibition of chlamydial growth and replication (31). In tissue culture, low tryptophan levels cause chlamydiae to enter a persistent state that can last for months, with altered growth characterized by large aberrant organisms and a failure to produce infectious progeny (2). Chlamydial gene regulation has been shown to be dysregulated during this persistent cell culture state (1, 3, 8, 9). In particular, there is transcriptional upregulation of the tryptophan biosynthesis genes (35, 36), indicating a compensatory response by chlamydiae to increase tryptophan biosynthesis in the face of decreased availability of this essential amino acid from the host cell.

In *Escherichia coli*, the tryptophan biosynthesis genes are regulated by an aporepressor, TrpR, and tryptophan is required as a corepressor (22). The role of tryptophan is to induce a conformational change in the helix-turn-helix DNA binding domain of TrpR, which enables TrpR dimers to bind the cognate operator upstream of the *trp* operon and repress transcription (42). In contrast, when tryptophan levels are low, TrpR is unable to bind the operator, and expression of *trp* genes is increased through derepression. This control mechanism allows the bacterium to sense and respond to intracellular tryptophan levels.

Homologs of TrpR have been predicted in some species of *Chlamydia*, suggesting that this tryptophan-responsive mechanism of gene regulation may be utilized to homeostatically regulate intrachlamydial tryptophan levels. Intriguingly, only the chlamydial species predicted to encode TrpR also encode components of a tryptophan biosynthetic pathway (21, 27). For example, *Chlamydia caviae* (formerly known as *Chlamydia psittaci* strain GPIC) has an almost complete tryptophan biosynthetic pathway and a TrpR homolog (21). In *Chlamydia trachomatis*, the pathway is incomplete, but this species includes genes encoding both tryptophan synthase, the last enzyme in the pathway, and TrpR (7, 27). In contrast, *Chlamydia pneumoniae* has neither genes for tryptophan biosynthesis nor an identifiable TrpR homolog (20). These observations have led to the hypothesis that TrpR is the regulator of the tryptophan biosynthesis genes in *Chlamydia* (27, 35, 39).

In this study, we provide functional evidence that TrpR is an aporepressor that regulates tryptophan biosynthesis genes in *C. trachomatis*. We have identified a TrpR operator upstream of an operon containing *trpR* and *trpBA*. Recombinant TrpR was able to bind this operator, but binding was observed only when tryptophan was present. In addition, we show that TrpR is able to repress transcription of *trpRBA* in a promoter-specific and tryptophan-dependent manner.

MATERIALS AND METHODS

Reverse transcriptase PCR (RT-PCR). HeLa cell monolayers were pretreated with 5 ng/ml human IFN-γ and then infected with *C. trachomatis* serovar D at a multiplicity of infection of 15. Infected cells were incubated for 48 h at 37°C in RPMI 1640 supplemented with 5 ng/ml IFN-γ, and total RNA was prepared using RNA STAT-60 (Tel-Test, Friendswood, Tex.). Ten micrograms of RNA

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treated with DNase I (Ambion, Austin, Tex.) was used for cDNA synthesis with avian myeloblastosis virus reverse transcriptase (Fisher Scientific, Pittsburgh, Pa.) and a specific 3′ primer that anneals to sequences within the open reading frame regions of *trpA*, T796 (5′-CACCATTTATTCCCGCGTCT). Negative control reactions were performed in the absence of reverse transcriptase. A *trpR* cDNA product was amplified by PCR with primers within *trpR*, T264 (5′-AAA AATCAAGAGGAGTCTGGCT) and T263 (5′-GCGGTACCTCAGATCTCT TTTTGTAAAAACTCTTTAAA). A *trpB* cDNA product was amplified with primers within *trpB*, T328 (5′-TGGGCAACAACACACTCATT) and T329 (5′-GC TCGTAACGCCTCTTCATC). A *trpA* cDNA product was amplified with primers within *trpA*, T795 (5′-GCGGCAAAAGCTCTGATTCAA) and T796. A *trpR-B* cDNA product was amplified by PCR with a 5′ primer within *trpR*, T264, and a 3′-primer within *trpB*, T330 (5′-CCCCCAAAGGATGTTTTATG). A *trpBA* cDNA product was amplified with a 5′ primer within *trpB*, T328, and a 3′ primer within *trpA*, T796.

Primer extension. Primer extension was performed as described previously (6) using primer T450 (5′-GTAAAAACTTTTCTTTTTGCATTTTAG), specific for the 5′ end of the *trpR* open reading frame, and end labeled with 3,000 Ci/mmol [α-^{32}P]ATP (MP Biomedical, Irvine, Calif.). The radiolabeled primer was annealed to 10 μg of chlamydial RNA, and cDNA was synthesized with avian myeloblastosis virus reverse transcriptase. The reaction mixture was incubated at 42°C for 50 min, and the cDNA products were electrophoresed on a 6% polyacrylamide-urea gel. The size of the primer extension product was determined by comparison to an M13 DNA sequencing ladder primed with the M13 forward primer.

Cloning of chlamydial *trpR*. *C. trachomatis trpR* was cloned into the expression vector pRSET-C (Invitrogen, Carlsbad, Calif.) to produce plasmid pMT1182, which expressed full-length TrpR (except for the ATG start codon) with an N-terminal six-histidine tag. *trpR* was amplified by PCR with *Tgo* DNA polymerase (Roche Diagnostics, Indianapolis, Ind.) using *C. trachomatis* serovar D genomic DNA and primers T264 and T263. The PCR product was digested with KpnI and cloned into pRSET-C between KpnI and blunted BamHI sites. pMT1182 was sequenced to ensure that the coding region of TrpR matched the published nucleotide sequence (27).

Overexpression and purification of TrpR protein. His_6-TrpR was overexpressed in *E. coli* BL21(DE3) (Stratagene, La Jolla, Calif.) freshly transformed with pMT1182. Two hundred fifty milliliters of cells was grown at 37°C to an optical density at 600 nm of 0.5 and induced with 1 mM isopropyl-β-D-thiogalactosidase (IPTG). After 2.5 h, cells were collected by centrifugation; resuspended in 10 ml buffer N (10 mM Tris-HCl [pH 8.0], 300 mM NaCl, 10 mM 2-mercaptoethanol) containing 50 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml of pepstatin A; and disrupted with a Branson Sonifier 450 (30 s for three times) in the presence of 0.2% Sarkosyl. Soluble protein was separated from cell debris by centrifugation at 10,000 × *g* for 10 min at 4°C (Beckman JA-17 rotor), and 2% Triton X-100 was added to sequester the Sarkosyl. The lysate was then loaded onto a 1-ml Ni^{2+}-charged HiTrap chelating column (Amersham Biosciences, Piscataway, N.J.). The column was washed with 40 ml of buffer N containing 100 mM imidazole. His-tagged protein was eluted with 5 ml buffer N containing 150 mM imidazole in 1-ml fractions. Fractions 3 to 5 were pooled, and the volume was brought up to 10 ml with buffer N to prevent precipitation during dialysis against storage buffer (10 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 100 μM EDTA, 10 mM 2-mercaptoethanol, 100 mM NaCl, 30% glycerol) overnight and again for 4 h. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by silver staining and Western blot analysis using anti-RGS-His_6 antibody (QIAGEN, Valencia, Calif.). The protein concentration was determined using the Bio-Rad protein assay (Hercules, Calif.).

Production of polyclonal anti-TrpR antibodies. Recombinant TrpR protein was gel purified by SDS-PAGE and used to generate rabbit polyclonal antibodies (Harlan Bioproducts for Science, Madison, Wis.). Antibodies were purified with a protein A agarose column according to the manufacturer's instructions (Bio-Rad).

DNA templates for the EMSA. A 110-bp restriction fragment containing the *trpRBA* promoter region and a putative *trp* operator was used as a probe for electrophoretic mobility shift assay (EMSA). This fragment was amplified by PCR from *C. trachomatis* serovar D genomic DNA with primers T566 (5′-GA GGGGAGAATTCTAAGAAAAGA) and T567 (5′-GCCAGCCAGACTCCT CTT) and cloned into the SmaI site of pGEM-7ZF(+) (Promega Biotech, Madison, Wis.) to produce pMT1283. The EMSA restriction fragment template was digested from pMT1283 with EcoRI and BamHI, followed by gel purification from a 2% agarose gel. A 156-bp *C. pneumoniae glnPQ* fragment was used as a nonspecific competitor fragment (24). Double-stranded oligonucleotide competitors were prepared as previously described by annealing two complementary primers (32). Primers T699 (5′-AATTCTTATGAAATGTTGTAATATTATAG CATTACAAAAAGGTGCGA) and T700 (5′-GATCCTCGCACCTTTTTGTA ATGCTATAATATTACAACATTTCATAA) were annealed to produce a specific oligonucleotide competitor containing the *C. trachomatis trp* operator. T690 (5′-GATCCTAATTGCATAAATATGATTTCATTATAAATAAATATGCAT AAG) and T691 (5′-CTAGACTTATGCATATTTATTTATAATGAAATCAT ATTTATGCAATTA) were annealed to produce a nonspecific oligonucleotide containing tandem *C. pneumoniae* ArgR operators (24).

EMSA. The DNA restriction fragment containing the putative *trp* operator was labeled with [α-^{32}P]dATP using the Klenow fragment of *E. coli* DNA polymerase. Free nucleotides were removed using a Mini Quick Spin DNA column (Roche Diagnostics), and the activity of the probe was quantified using a scintillation counter. The EMSA analysis was based on the method of Carey (4). An 0.5 nM concentration of labeled probe was mixed with His_6-TrpR in buffer containing 12.5 mM sodium phosphate buffer (pH 6.8), 25 mM NaCl, 1 mM EDTA, 1 mg/ml bovine serum albumin, 10% (vol/vol) glycerol, and 4 mM L-tryptophan and incubated at room temperature for 20 min. A range of concentrations of His_6-TrpR from 0 to 10 nM (monomer) was tested, with details provided in the legend to Fig. 3. Some reaction mixtures also included unlabeled competitor DNA fragments or oligonucleotide or polyclonal anti-TrpR antibodies. Samples were loaded under tension onto a 10% polyacrylamide gel, containing 0.1 mM sodium phosphate buffer (pH 6.8) and 1 mM L-tryptophan that had been prerun at 220 V for 1 h at 4°C. The gel was electrophoresed at 300 V for 5 h at 4°C in TrpR EMSA buffer (0.1 mM sodium phosphate [pH 6.8] and 1 mM L-tryptophan) with recirculation. After electrophoresis, the gel was dried down and exposed to a phosphorimager screen. The screen was scanned with a Bio-Rad Personal FX scanner, and the data were analyzed with Quantity One software (Bio-Rad). The dissociation constant (K_d) was calculated using KaleidaGraph software (Synergy Software, Reading, Pa.).

Construction of transcription plasmids. Plasmid pMT1250 contains the promoter region of *trpRBA* (+152 to −10), which was amplified from *C. trachomatis* serovar D genomic DNA by PCR with primers T521 (5′-GCGAGAACGAATT TATGGGTTTTAGAT) and T522 (5′-TTTTTGTAATGCTATAATATTACA ACATTTCA). The *trpRBA* promoter insert was cloned upstream of a promoterless G-less cassette transcription template in pMT1125 (32). Construction of control plasmid pMT1198 containing the *C. trachomatis omcB* promoter has been previously described (24).

In vitro transcription. Transcription reactions were performed with heparin-agarose-purified *C. trachomatis* RNA polymerase as previously described (28) and 25 nM (each) transcription plasmid. In some reactions, His_6-TrpR (over a range of concentrations from 0 to 500 nM) and/or L-tryptophan (from 0 to 50 μM) was added, with more details given in the legend to Fig. 5.

RESULTS

Characterization of the *trp* operon in *C. trachomatis* serovar D. As *trpR* has an unusual location in the *C. trachomatis* serovar D genome adjacent to the tryptophan biosynthesis genes, *trpB* and *trpA* (38), we examined the transcriptional organization to determine if these genes are part of an operon. Using RT-PCR, we synthesized a cDNA fragment using a primer in *trpA* and then individually amplified cDNA from *trpR*, *trpB*, and *trpA* and the overlapping regions between *trpR-trpB* and *trpB-trpA* (Fig. 1A and B). These results indicate that *trpR*, *trpB*, and *trpA* are cotranscribed as a polycistronic message and establish these genes as part of an operon.

To study the regulation of this tryptophan biosynthesis operon, we mapped the transcription start site of *trpRBA* by primer extension. The single primer extension product mapped to an adenine located 18 nucleotides upstream of the predicted translation start site for *trpR* (data not shown). Immediately upstream of this start site, we located a candidate promoter containing −35 and −10 elements (TAGCAT and TAATAT spaced 17 nucleotides apart; Fig. 1C) with a 2/6 and a 5/6 match to the respective optimal promoter elements recognized by *C. trachomatis* σ^{66} RNA polymerase (25, 29). We tested this predicted *trpRBA* promoter with our *C. trachomatis* in vitro

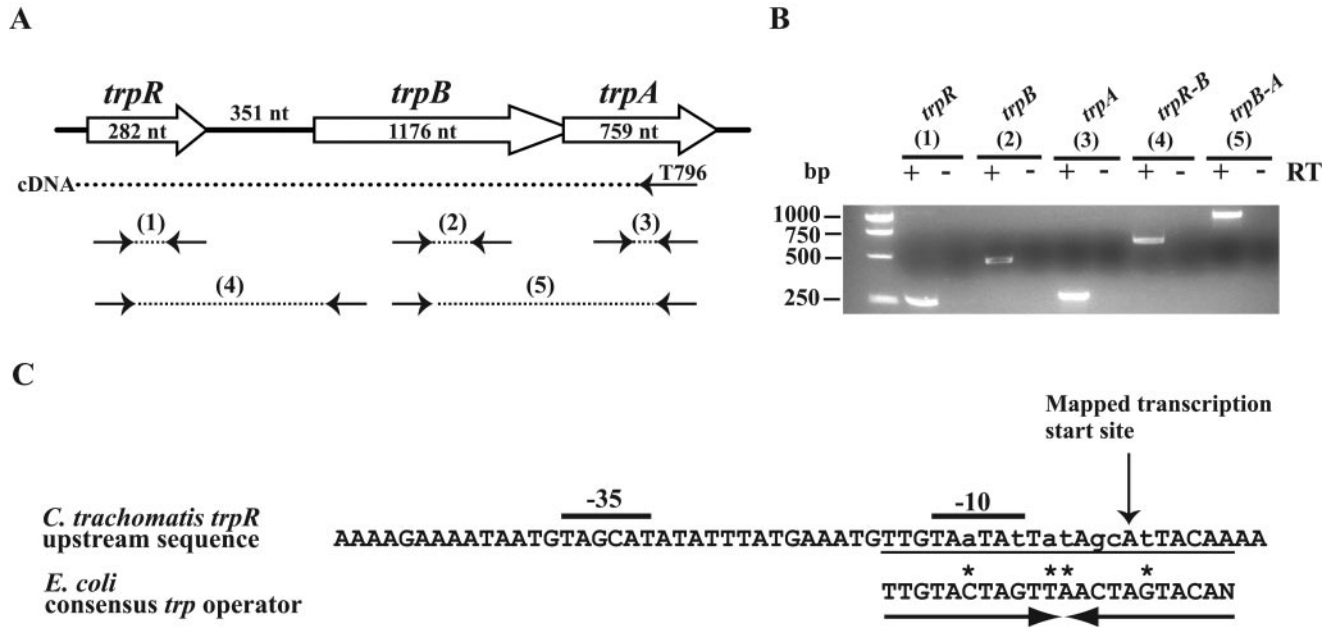

FIG. 1. Transcriptional organization of tryptophan biosynthesis genes in *C. trachomatis*. (A) Schematic diagram of genes involved in tryptophan biosynthesis showing predicted RT-PCR products. nt, nucleotides. (B) RT-PCR analysis. cDNA synthesized using a primer that annealed in *trpA* (T796) was amplified using primers for *trpR* (product 1, shown in part A), *trpB* (product 2), *trpA* (product 3), *trpR-trpB* (product 4), and *trpB-trpA* (product 5). A no-RT control is included for each reaction. (C) Sequence upstream of *trpR*. The transcription start site determined by primer extension is marked by a vertical arrow. Predicted σ^{66} −35 and −10 promoter elements are marked. A putative *trp* operator overlapping the −10 promoter element and the transcription start site is underlined and aligned with the *E. coli* consensus *trp* operator. The operator forms an inverted repeat as shown by a pair of arrows. Lowercase letters indicate mismatches with the consensus, and an asterisk marks a position in the chlamydial sequence where the mismatch is accompanied by a complementary change in the inverted repeat.

transcription assay and found that it was transcribed by σ^{66} RNA polymerase (see Fig. 5A, lane 1).

By sequence inspection, we also identified a 22-bp inverted repeat that overlapped the −10 promoter element and the transcription start site of *trpRBA* (Fig. 1C). This predicted *trp* operator matched the *E. coli* consensus *trp* operator at 15 out of 22 positions (18). Two of the mismatches in the first repeat were accompanied by compensatory base substitutions in the inverted repeat sequence.

Purification of chlamydial TrpR. We purified *C. trachomatis* TrpR as a recombinant protein with an N-terminal six-histidine tag. The *trpR* coding sequence was cloned into the *E. coli* protein expression vector pRSET and overexpressed in *E. coli* strain BL21(DE3). Soluble His_6-TrpR was purified by nickel affinity chromatography in a one-step purification to near-homogeneity (Fig. 2) at a concentration of 9.8 μM.

TrpR binds to the upstream region of *C. trachomatis trpR* in vitro. We used an EMSA to determine if TrpR can bind to the predicted *trp* operator located upstream of *trpR*. EMSA conditions were adapted from previous studies with *E. coli* TrpR (4), but the pH of the electrophoresis buffer had to be increased from pH 6.0 to pH 6.8 to allow the TrpR-DNA complex to enter the gel. As L-tryptophan has been shown to be a necessary cofactor for TrpR in other bacteria (22), we performed the EMSA reactions in the presence and absence of L-tryptophan. When L-tryptophan was present, TrpR bound the *trpR* probe in a concentration-dependent manner, with complete binding of probe by 10 nM TrpR (Fig. 3A, lanes 1 to 8). The gel shift complex consisted of at least four lower-mobility bands, which is consistent with the observation in *E. coli* that there is oligomerization of TrpR dimers at the *trp* operator (4, 15, 40). Addition of polyclonal anti-TrpR antibodies produced a supershift, indicating that the binding was due to TrpR (Fig. 3A, lane 9). There was no gel shift with antibodies alone (Fig. 3A, lane 10) or in the absence of L-tryptophan (data not shown). To measure the kinetics of binding, EMSA

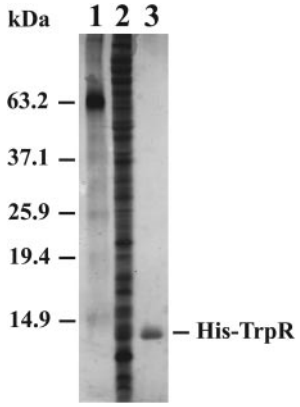

FIG. 2. Silver stain of SDS-PAGE gel showing purification of recombinant His-TrpR. Lane 1, molecular mass markers; lane 2, lysate of *E. coli* overexpressing *C. trachomatis* TrpR; lane 3, TrpR after purification with a Ni column.

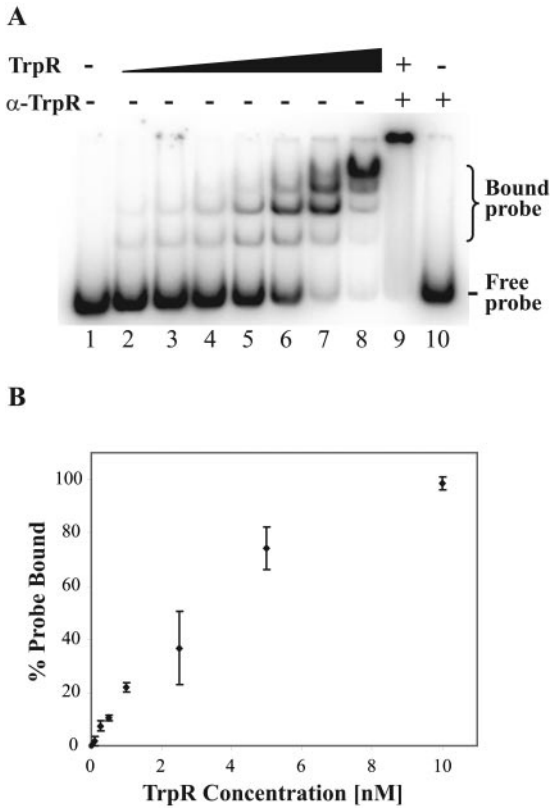

FIG. 3. EMSA with *C. trachomatis trpR* probe titrated with recombinant TrpR. EMSA reactions and electrophoresis were performed in the presence of L-tryptophan. (A) The positions of bound and free probe are indicated. Lane 1, labeled probe alone; lanes 2 to 8, addition of increasing concentrations of TrpR (0.1, 0.25, 0.5, 1, 2.5, 5, and 10 nM, respectively); lane 9, addition of 10 nM TrpR and polyclonal anti-TrpR antibody; lane 10, addition of polyclonal anti-TrpR antibody alone. (B) Quantification of EMSA. EMSA reactions with various concentrations of recombinant TrpR were performed in triplicate and quantified by phosphorimager analysis. Standard deviations are marked by error bars.

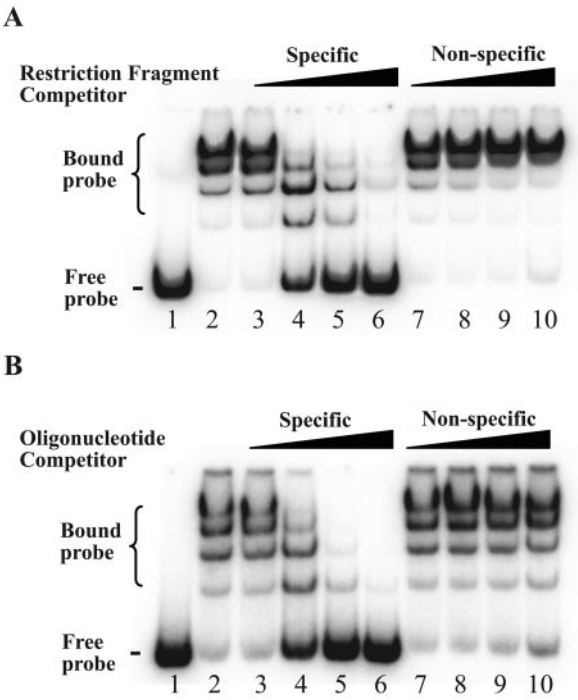

FIG. 4. Competitive EMSA with *trpR* probe and recombinant TrpR. All reactions, except those with probe alone, were performed with 10 nM TrpR. (A) Competition with DNA fragment containing *trp* operator. Lane 1, probe alone; lane 2, no competitor; lanes 3 to 6, increasing amounts of molar excess of specific competitor relative to labeled probe (1-, 4-, 16-, and 64-fold, respectively); lanes 7 to 10, molar excess of nonspecific competitor (1-, 4-, 16-, and 64-fold, respectively). (B) Competition with double-stranded DNA oligonucleotide containing *trp* operator. Lane 1, probe alone; lane 2, no competitor; lanes 3 to 6, increasing amount of molar excess of specific competitor (10-, 50-, 250-, and 1,250-fold, respectively); lanes 7 to 10, molar excess of nonspecific competitor (10-, 50-, 250-, and 1,250-fold, respectively).

reactions were performed over a range of TrpR concentrations (0.1, 0.25, 0.5, 1, 2.5, 5, and 10 nM) and an apparent dissociation constant (K_d) of 2 nM was calculated (Fig. 3B).

Competitive EMSA experiments were performed to confirm that TrpR binding was sequence specific. A molar excess of unlabeled *trpR* DNA fragment was able to compete for TrpR binding in a concentration-dependent manner, with almost complete loss of the gel shift at a 64-fold excess of competitor (Fig. 4A, lanes 3 to 6). In contrast, a nonspecific DNA restriction fragment did not compete for TrpR binding (Fig. 4A, lanes 7 to 10).

We used double-stranded oligonucleotide competitors to narrow the site of binding to the predicted *trp* operator. A short, unlabeled oligonucleotide, containing the *trp* operator and 10 nucleotides of flanking DNA on each end, was able to compete with the labeled *trp* DNA fragment for TrpR binding (Fig. 4B, lanes 3 to 7). In contrast, a nonspecific double-stranded oligonucleotide competitor did not compete for TrpR binding (Fig. 4B, lanes 7 to 10). These results demonstrate that in *C. trachomatis*, the predicted *trp* operator of the *trpRBA* operon is a likely binding site for TrpR.

TrpR represses transcription of the *trpRBA* promoter in vitro. To determine if TrpR functions as a transcriptional repressor, we measured the effect of TrpR on transcription of the *trpRBA* promoter. In an in vitro assay, TrpR repressed transcription from the *trpRBA* promoter in the presence of L-tryptophan (Fig. 5A, lanes 4 to 7). Neither TrpR nor L-tryptophan alone had a significant effect on transcription (Fig. 5A, lanes 2 and 3). TrpR-mediated repression was concentration dependent with complete repression by 500 nM TrpR (Fig. 5B). This concentration of TrpR is 50 times higher than that required in the EMSA experiments, which correlates with the 50-fold-higher DNA concentration used in the transcription reactions. Transcription of the internal control *omcB* promoter was not affected (Fig. 5A), demonstrating that the activity of TrpR was promoter specific.

Next, we tested the role of L-tryptophan as a cofactor for TrpR-mediated repression. Keeping the concentration of TrpR constant, we titrated the amount of L-tryptophan used for transcription of the *trpRBA* promoter. We found that repression was dependent on the concentration of L-tryptophan and maximum repression was not reached until at least a 100:1

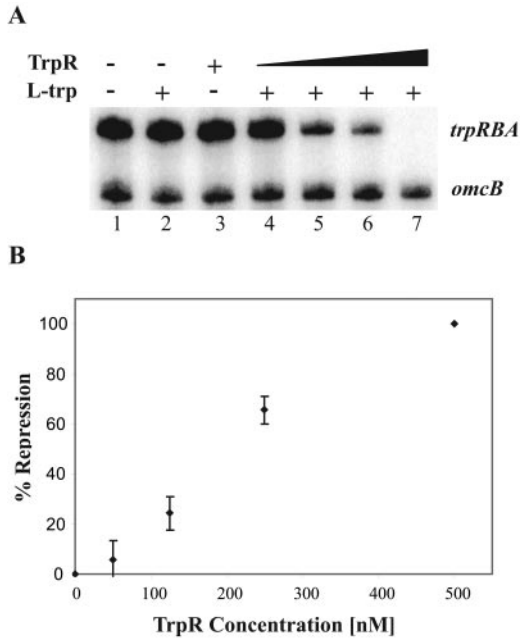

FIG. 5. In vitro transcription with *C. trachomatis* RNA polymerase and recombinant TrpR. The upper band is the transcript from the *trpRBA* promoter, and the lower band is the transcript from the *omcB* promoter. (A) Lane 1, no TrpR, no L-tryptophan; lane 2, no TrpR, 50 μM L-tryptophan; lane 3, 500 nM TrpR, no L-tryptophan; lanes 4 to 7, increasing concentrations of TrpR (50, 125, 250, and 500 nM, respectively), 50 μM L-tryptophan. (B) Quantification of TrpR-mediated transcriptional repression. Reactions were performed in triplicate and quantified by phosphorimager analysis. Standard deviations are marked by error bars. The degree of transcriptional repression was calculated by normalizing it to the amount of transcription in the absence of TrpR and the presence of L-tryptophan.

ratio of L-tryptophan to TrpR (Fig. 6). This finding is consistent with the observations in other bacteria that a large molar excess of the corepressor, L-tryptophan, is necessary for negative regulation by TrpR (10, 38). We also tested other representative amino acids but found that neither L-tyrosine, L-phenylalanine, L-alanine, L-arginine, L-serine, L-methionine, nor L-proline could function as a corepressor for TrpR (data not shown). Our results indicate that TrpR functions as an aporepressor that modulates *trpRBA* promoter activity in an L-tryptophan-dependent manner.

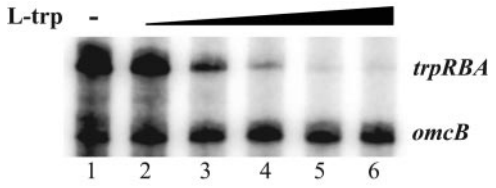

FIG. 6. In vitro transcription assaying the effect of L-tryptophan titration on TrpR-mediated repression. All reactions were performed with 250 nM recombinant TrpR. Lane 1, no L-tryptophan; lanes 2 to 6, increasing concentrations of L-tryptophan (1, 5, 10, 25, and 50 μM, respectively).

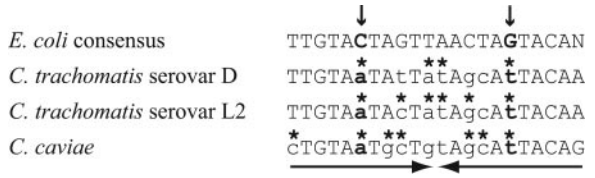

FIG. 7. Alignment of the *E. coli* consensus *trp* operator with the *C. trachomatis* serovar D *trp* operator and predicted *trp* operators from *C. trachomatis* serovar L2 and *C. caviae.* An inverted repeat within the operator is marked by a pair of arrows. Lowercase letters indicate mismatches with the consensus, and an asterisk marks a position in the chlamydial sequence where the mismatch is accompanied by a complementary change in the inverted repeat. Vertical arrows and boldface mark invariant positions in the *E. coli* consensus operator that are not conserved in the chlamydial *trp* operators.

DISCUSSION

In this report, we demonstrate that TrpR is an aporepressor that together with the corepressor L-tryptophan regulates transcription of tryptophan biosynthesis genes in *C. trachomatis*. Using in vitro assays, we have shown that *C. trachomatis* TrpR binds in the presence of tryptophan to its cognate operator upstream of *trpRBA* and represses transcription by the major form of chlamydial RNA polymerase. Our studies provide a mechanism for the homeostatic control of intrachlamydial tryptophan levels through transcriptional regulation of the tryptophan biosynthetic pathway.

We found that *trpR* and the adjacent tryptophan biosynthesis genes, *trpBA*, are cotranscribed as an operon in *C. trachomatis* serovar D. This is an unusual transcriptional organization in bacteria, and the more typical arrangement is seen in *E. coli* where *trpR* is physically separate from the operon containing the tryptophan biosynthesis genes, although both are regulated by TrpR (11, 22). The location of *trpR* adjacent to *trpBA* is conserved in all other serovars of *C. trachomatis* (7). One point of difference, though, is that *trpR* and *trpBA* were not found to be part of an operon in serovar L2 (35). However, in this serovar, *trpBA* is still induced at the transcriptional level by tryptophan limitation, and we infer that it is still under the control of a TrpR-dependent promoter. So far, there is no functional evidence of an independent *trpBA* promoter in serovar L2, and we have not been able to locate an operator for *trpBA* using TrpR binding studies (J. C. Akers and M. Tan, unpublished observation).

We predict that other chlamydial species can also sense tryptophan levels and regulate the expression of tryptophan biosynthesis genes through TrpR but that this mechanism is not conserved among all members of the family *Chlamydiaceae*. *C. caviae* includes a gene encoding a TrpR ortholog together with an almost complete tryptophan biosynthetic pathway in a single operon (36). Expression of this operon is induced in tissue culture by tryptophan limitation (36), providing support for tryptophan-dependent regulation by TrpR in this species. We have identified a candidate operator upstream of the *C. caviae trp* operon with sequence conservation to the *C. trachomatis* and consensus *E. coli trp* operators (Fig. 7). In contrast, *trpR* and *trpBA* have not been identified in the genomes of *C. pneumoniae*, *Chlamydia muridarum* (formerly known as *C. trachomatis* strain MoPn) (20), and *Parachlamydia*

strain UWE25 (14), a *Chlamydia*-like endosymbiont of free-living amoebae. These findings indicate that TrpR-mediated transcriptional repression is not uniformly conserved among chlamydial species.

While the chlamydial *trp* operator shows conservation with the consensus *E. coli trp* operator, there are nucleotide differences especially towards the center of the operator (Fig. 7). The internal symmetry of the chlamydial *trp* operator is preserved, however, as several nucleotide substitutions in the first repeat are accompanied by compensatory changes in the inverted repeat sequence. Of these differences between the chlamydial and *E. coli trp* operators, the most noteworthy are at positions 6 and 11, which are invariably a C and G, respectively, in *E. coli*, but replaced by an A and T, respectively, in the three chlamydial *trp* operators.

An interesting paradox is that TrpR appears to be present in all *C. trachomatis* serovars even when tryptophan synthase, the product of TrpR regulation, is enzymatically inactive (serovar B is an anomaly because it has a large deletion involving the *trpRBA* locus) (7). The serovars of *C. trachomatis* that cause ocular disease all have mutations in *trpA* or *trpB* which result in a nonfunctional tryptophan synthase. Surprisingly, the expression of *trpBA* in these ocular serovars is still regulated in response to tryptophan levels (35), and we can identify a well-conserved operator upstream of *trpRBA* for each serovar (Akers and Tan, unpublished). The obvious question is why these serovars would maintain a mechanism to regulate the expression of a nonfunctional enzyme. Perhaps tryptophan synthase in *C. trachomatis* has other functions besides tryptophan biosynthesis. It is also possible, however, that it is the homeostatic regulation of *trpR* expression in response to tryptophan levels that is being conserved, which would in turn suggest that TrpR regulates other target genes in *C. trachomatis*.

A number of candidate TrpR target genes in *Chlamydia* can be considered because they are homologs of genes that are regulated by TrpR in *E. coli*. The only other tryptophan biosynthesis gene in *C. trachomatis* is *trpC*, which encodes an enzyme upstream of tryptophan synthase in the tryptophan biosynthetic pathway (27). *trpC* is located at a separate site from *trpRBA* in the *C. trachomatis* genome. In *E. coli*, TrpR also regulates *aroH* and *aroL*, the genes for aromatic amino acid biosynthesis (12, 43), and *mtr*, which encodes a tryptophan transporter (13). Homologs of all three genes can be identified in *C. trachomatis*. The *mtr* homolog is called *tyrP*, and it is predicted to encode a tyrosine/tryptophan transporter. There has been particular interest in *tyrP* because the number of gene copies in *C. pneumoniae* has been shown to vary according to tissue tropism and disease manifestation (10). So far, however, there is little evidence to indicate that any of these genes are regulated by TrpR in *Chlamydia*. The expression of neither *C. trachomatis trpC*, *aroH*, *aroL*, *tyrP.1*, nor *tyrP.2* is induced by tryptophan limitation (35). In addition, we have not been able to identify sequences resembling a *trp* operator upstream of these genes in *C. trachomatis* (Akers and Tan, unpublished). One approach for recognizing additional TrpR-regulated genes would be to perform transcriptional profiling with a DNA microarray under specific conditions of tryptophan limitation to identify genes that are coregulated with the *trpRBA* operon.

It is also possible that the tryptophan biosynthesis operon is the only target of TrpR in *Chlamydia*. There is only one other example in eubacteria where *trpR* is adjacent to the tryptophan biosynthesis genes (38). Furthermore, TrpR is found only in the chlamydial species that have a tryptophan biosynthesis operon (20, 21, 27). An additional clue is provided by the location of *trpR* and the tryptophan biosynthesis genes in the plasticity zone, which is a region of genetic diversity among chlamydial species (20). It would appear that *trpR* and the tryptophan biosynthesis genes form a functional unit in *Chlamydia* that has been selectively acquired or lost during chlamydial evolution and divergence.

While TrpR provides a mechanism for regulation at the level of transcription initiation, there is evidence that the tryptophan biosynthesis genes in *Chlamydia* are also regulated by transcription attenuation, as is the case in *E. coli*. Using a bioinformatics approach, Merino and Yanofsky (17) have predicted an attenuator upstream of *C. trachomatis trpBA.* The presence of an attenuator could explain the relatively large intergenic region between *trpR* and *trpBA* even though they are part of an operon; intergenic regions are characteristically short in *Chlamydia*, especially for genes within an operon (16, 26, 30). We propose a unified model in which transcriptional repression and attenuation allow *C. trachomatis* to regulate intrachlamydial tryptophan levels. During chlamydial growth in the presence of sufficient tryptophan, the aporepressor, TrpR, forms a complex with the corepressor tryptophan and binds the cognate operator to repress transcription from the *trp* operon. In addition, formation of the transcription terminator structure in the *trpR-trpBA* intergenic region further reduces readthrough transcription of *trpBA*. However, in conditions of tryptophan limitation, such as during chlamydial persistence, TrpR is unable to bind its operator, leading to derepression of *trpRBA* expression. Low tryptophan levels would also decrease the availability of charged $tRNA^{Trp}$, favoring ribosome pausing and formation of the antiterminator structure, thus allowing RNA polymerase to read through and transcribe *trpBA*. The net effect would be that expression of *trpR* and that of *trpBA* would both be upregulated when tryptophan levels are low, allowing synthesis of tryptophan if the necessary substrate is available (7). This combination of control through transcriptional repression and attenuation may allow chlamydiae to respond quickly to changes in tryptophan availability by regulating tryptophan biosynthesis.

Even though chlamydiae are intracellular parasites that reside in what might be thought of as a relatively stable environment, it is clear that they maintain the capacity to respond to environmental stimuli by coordinately regulating gene expression. Among the recently described transcriptional regulators in *Chlamydia* that can sense and respond to environmental and metabolic conditions are a stress response repressor, HrcA (32–34); a metal-dependent repressor, DcrA (19, 37); and an arginine-responsive aporepressor, ArgR, that utilizes arginine as a corepressor to regulate expression of a putative arginine transporter (24). An alternative sigma factor, σ^{28}, has been shown to be a stage-specific transcriptional regulator (41), although the signals that it responds to are not known. Understanding the response to tryptophan levels is crucial because tryptophan depletion has been shown to be a consequence of the host IFN-γ response in infected cells and to result in chlamydial persistence. TrpR provides a mechanism that al-

lows chlamydiae to sense tryptophan limitation and to respond by upregulating tryptophan biosynthesis. It remains to be seen whether derepression of TrpR gene regulation is purely a marker of persistence or a clue to the underlying molecular mechanism of chlamydial persistence.

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ADDENDUM

While the manuscript was in review, a complementary study by McClarty and colleagues was published that provides elegant genetic evidence that TrpR is a regulator of *trp* genes in *C. trachomatis* (5). In addition, the study identified an operator upstream of *trpR* and predicts a second operator upstream of *trpBA*.

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