JOURNAL OF BACTERIOLOGY, Nov. 2011, p. 6123–6131 Vol. 193, No. 22
0021-9193/11/$12.00 doi:10.1128/JB.05976-11

Identification and Functional Analysis of CT069 as a Novel Transcriptional Regulator in *Chlamydia*▽

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Received 11 August 2011/Accepted 30 August 2011

Only a small number of transcription factors have been predicted in *Chlamydia* spp., which are obligate intracellular bacteria that include a number of important human pathogens. We used a bioinformatics strategy to identify novel transcriptional regulators from the *Chlamydia trachomatis* genome by predicting proteins with the general structure and characteristic functional domains of a bacterial transcription factor. With this approach, we identified CT069 as a candidate transcription factor with sequence similarity at its C terminus to *Treponema pallidum* TroR. Like TroR, the gene for CT069 belongs to an operon that encodes components of a putative ABC transporter for importing divalent metal cations. However, CT069 has been annotated as YtgC because of sequence similarity at its N terminus to TroC, a transmembrane component of this metal ion transporter. Instead, CT069 appears to be a fusion protein composed of YtgC and a TroR ortholog that we have called YtgR. Although it has not been previously reported, a similar YtgC-YtgR fusion protein is predicted to be encoded by other *Chlamydia* spp. and several other bacteria, including *Bacillus subtilis*. We show that recombinant YtgR polypeptide bound specifically to an operator sequence upstream of the *ytg* operon and that binding was enhanced by Zn^{2+}. We also demonstrate that YtgR repressed transcription from the *ytg* promoter in a heterologous *in vivo* reporter assay. These results provide evidence that CT069 is a negative regulator of the *ytg* operon, which encodes a putative metal ion transporter in *C. trachomatis*.

Transcription factors allow bacteria to respond to intracellular and extracellular stimuli by altering gene expression at the transcriptional level. A transcription factor controls a target gene or operon by binding in a sequence-specific manner to a *cis*-acting DNA regulatory element that is typically located close to the promoter. This mechanism allows a transcription factor to coordinately regulate the expression of multiple genes that together make up a regulon. Some transcription factors require an allosteric cofactor, such as a corepressor or inducer, which provides an additional level of regulatory control. To illustrate these points, TrpR represses transcription of genes involved in tryptophan biosynthesis by binding the *trp* operator when its corepressor tryptophan is present (1, 40). If the levels of this essential amino acid are low, however, the tryptophan biosynthesis genes are derepressed, leading to tryptophan production in a simple, yet elegant, homeostatic mechanism.

The number of transcription factors varies widely among different bacteria. Some bacteria dedicate up to 10% of their genome to genes encoding transcription factors (26, 54). For instance, the soil microbe *Pseudomonas fluorescens* is estimated to utilize nearly 500 transcription factors to regulate its genes (54). *Escherichia coli* and *Bacillus subtilis* are predicted to have 314 and 237 transcription factors, respectively (31, 33). These bacteria with hundreds of transcription factors are exposed to many different environments and need to be able to respond to diverse stimuli. At the other end of the spectrum, obligate intracellular bacteria have far fewer transcription factors, presumably because they encounter a more controlled environment inside an infected cell. For example, *Coxiella burnetii* is proposed to have 29 transcription factors, and only 15 transcriptional regulators have been predicted in *Rickettsia rickettsii* (54).

The *Chlamydiaceae* are a large family of obligate intracellular bacteria that appear to control their gene expression with a small number of conserved transcription factors. On the basis of sequence conservation to known bacterial transcription factors, only eight transcription factors have been recognized in the chlamydial genome. There is functional data thus far to support seven of these chlamydial transcription factors, Euo, DcrA, HrcA, TrpR, ChxR, ArgR, and NrdR (1, 6, 6a, 25, 41, 52, 55, 56). An eighth predicted chlamydial transcription factor, CtcC, is a putative activator of σ^{54} RNA polymerase, but its function has not been verified in the absence of a chlamydial σ^{54}-dependent transcription assay (24). Even though the actual number of transcription factors in *Chlamydia* is likely to be low, we hypothesize that additional transcription factors remain to be identified because of limitations in the methods used to annotate the sequenced *Chlamydia* genomes (4, 17, 23, 30, 38, 39, 45, 50). The primary method for gene identification has been to perform an individual BLAST search for each predicted amino acid sequence to identify its best match to known bacterial proteins. However, it has not been possible to extrapolate the function for about a quarter of the genes in the chlamydial genome because they have no obvious orthologs in other bacterial genomes (45).

In the present study, we provide evidence from a bioinformatics analysis and functional studies that *ct069* (originally annotated as *ytgC*) (45) encodes a putative transcription factor in *C. trachomatis*. We searched the *C. trachomatis* genome for

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▽ Published ahead of print on 9 September 2011.

encoded proteins with the general structure, and functional domains such as a helix-turn-helix motif, that are characteristic of bacterial transcription factors. Although CT069 was one of our top candidates, it has been annotated as a membrane transporter because of sequence similarity at its N terminus to *Treponema pallidum* TroC (19, 45). We found, however, that there was also conservation between sequences in the C terminus of CT069 and the *T. pallidum* transcriptional repressor TroR, which was overlooked in the original annotation. CT069, thus, appears to be a fusion protein of TroC and TroR orthologs, and we propose that it is more accurate to refer to it as YtgC-YtgR to reflect this dual origin. We were unable to perform functional studies with full-length YtgC-YtgR because it is toxic to *E. coli*. As an alternative, we examined whether the YtgR C-terminal region of CT069 that is conserved with TroR is able to regulate the *ytg* operon. We found that YtgR bound a predicted operator upstream of the *C. trachomatis ytg* operon and repressed transcription of this operon in a promoter-specific manner, providing experimental support for CT069 as a novel chlamydial transcription factor.

MATERIALS AND METHODS

Identifying candidate transcriptional regulators. A total of 240 open reading frames (ORFs) in the *C. trachomatis* serovar D genome were analyzed with the Structure Prediction Meta Server (http://meta.bioinfo.pl) (13). ORFs were selected because they encode proteins whose functions have not been established. For validation purposes, ORFs encoding proteins predicted to function in transcriptional regulation were also included. To identify chlamydial proteins with predicted structural domains characteristic of transcription factors, the results were screened with a keyword filter for the following terms: transcription factor, transcriptional regulator, and DNA-binding domain. The proteins were then ranked according to the number of prediction servers that returned a result containing at least one of the keyword terms.

5′RACE. HeLa cell monolayers were infected with *C. trachomatis* serovar D/UW-3/CX at a multiplicity of infection of 15. Infected cells were incubated for 24 h at 37°C in RPMI 1640. Reticulate bodies were harvested as previously described (47), and RNA was prepared using RNA STAT-60 (Tel-Test). For each 5′RACE (5′ rapid amplification of cDNA ends) reaction, 10 μg of RNA was treated with DNase I (Ambion) and Terminator 5′-phosphate-dependent exonuclease (Epicentre) to remove processed transcripts, as previously described (44). cDNA synthesis was carried out with AMV reverse transcriptase (Fisher Scientific) and a specific *ytgA* primer T776 (5′-GTCTTCATCCCCCTTCACCA). A poly(dC) tail was added to the 5′ end of the cDNA using terminal deoxynucleotidyltransferase (New England Biolabs). The RACE products were amplified by PCR with an adapter primer T602 (5′-CGCGAATTCCTCTTCTAG ATGGGIIGGGIIGGGIIG), which annealed to the poly(dC) tail of the cDNA, and primer T777 (5′-ATCAGCGGGTTGATTTCCTC). A second round of PCR was performed with primers T541 (5′-CGCGAATTCCTCTTCTAGA TGG) and T777. 5′RACE PCR products were digested with EcoRI and subcloned into pGEM-7ZF(+) (Promega Biotech) between the EcoRI and SmaI sites. Plasmids were extracted and sequenced (Genewiz) to determine the 5′ end of the *troA* transcript.

RT-PCR. cDNA for the *ytg* operon was synthesized from 10 μg of DNase I-treated *C. trachomatis* RNA using Superscript II reverse transcriptase (RT; Invitrogen) and a specific 3′ primer T1324 (5′-CGATGCTGATCCACAAGA GC). T1324 anneals to sequences within the open reading frame of *yaeM*, which is predicted to be the fifth gene in this operon (see Fig. 3A). Each of the predicted genes of the *ytg* operon was then amplified by PCR using this cDNA as the template and a pair of gene-specific primers: *ytgA*, T774 (5′-AAGGGGGA TGAAGACCGAAT) and T775 (5′-GCCGCAGCCATCTCTTTTAC); *ytgB*, T1347 (5′-TCTGGCCAAAGCTTGTTCTT) and T1348 (5′-CAAACCAACCC GCTCTAAAA); *ytgC* region of CT069, T1069 (5′- ATGCTGAGTTGTATAT TTCAGGACACTATCT) and T1176 (5′- CACTACAGTCTGACAAGTGAAA GCTAC); *ytgR* region of CT069, T1071 (5′- ATGGGAGTGCTAGTTTTCTTA TGTTTAATTTTTTCA) and T1070 (5′-GCAACCATCCGACTTCCTTGT); *ytgD*, T1684 (5′-AAGATGGCTATGTATGCGAATG) and T1323 (5′-TGACA AGCTCGGTCCCTACA); and *yaeM*, T1685 (5′-CGCACTTTTCCAGTGCT TAG) and T1325 (5′-AGGATGGATCACCGCCTCTA). For each gene, a negative control reaction was performed in which RT was omitted from the cDNA synthesis.

Cloning of *C. trachomatis* YtgR. To express the predicted YtgR region of YtgC-YtgR fusion, together with a C-terminal six-histidine and myc tag, sequences from amino acids (aa) 281 to 451 of CT069 were cloned into an arabinose-inducible expression vector pBAD-ITO to produce plasmid pMT1476. pBAD-ITO was derived from pBAD-gIII (Invitrogen) by removal of the gene III secretion signal. This *ytgR* insert was amplified from *C. trachomatis* serovar D genomic DNA by PCR with *Tgo* DNA polymerase (Roche Diagnostics) and the primers T1071 and T1070. The PCR product was cloned into pBAD-ITO between blunted NdeI and BamHI sites. pMT1476 was sequenced to ensure that the coding region matched the published nucleotide sequence (45).

A second, codon-optimized version of YtgR from *C. trachomatis* serovar D was cloned for improved expression in an *E. coli* luciferase reporter assay. First, the codon-optimized sequence for the full-length CT069 gene was generated (Verdezyne) and used as a template to amplify the YtgR region by PCR with the primers T1438 (5′- ATGGGTGTTTTGGTGTTCCTGTGCCTGAT) and T1453 (5′-CTAGCAACCGTCAGATTTGCGGGTAC). This codon-optimized *ytgR* insert was ligated into pGEX-4TI (GE Healthcare) to add sequences for an N-terminal glutathione *S*-transferase (GST) tag. The GST-*ytgR* insert was then amplified by PCR with the primers T1561 (5′- ATGTCCCCTATACTAGGTT ATTGGAAAATTAAGGGC) and T1437 (5′-GCAACCGTCAGATTTGCGG GTAC) and ligated into blunted NdeI and BamHI sites of pBAD-ITO to produce pMT1603.

Overexpression and purification of YtgR protein. Recombinant YtgR was overexpressed in *E. coli* TOP10 (Invitrogen) freshly transformed with pMT1476. A total of 5 liters of cells were grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.5 and induced with 0.02% L-arabinose. After 4 h, cells were collected by centrifugation, resuspended in 50 ml of buffer B (6 M guanidine hydrochloride, 20 mM Tris-HCl [pH 8.0], 500 mM NaCl) containing 10 mM imidazole and disrupted with a Branson 250D digital sonifier (30 s, three times). Denatured protein was separated from cell debris by centrifugation at 10,000 × *g* for 20 min at 4°C (Beckman JA-17 rotor) and allowed to bind to 5 ml of Co^{2+}-charged Talon resin (BD Biosciences) for 20 min. Bound proteins were washed with buffer B containing 10 mM imidazole and eluted with 15 ml of buffer B containing 150 mM imidazole. The eluate was concentrated with a Centricon centrifugal filter (Millipore) and allowed to refold during dialysis against storage buffer (10 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 100 mM NaCl, 30% glycerol). Aliquots of YtgR were stored at −80°C. Protein concentration was determined using the Bio-Rad protein assay.

DNA templates for EMSA. A 135-bp restriction fragment containing the *ytg* promoter region and the predicted operator was used as a probe for electrophoretic mobility shift assay (EMSA). This sequence was first amplified by PCR from *C. trachomatis* serovar D genomic DNA with the primers T1117 (5′-AGAATT GTCTCTTCGAGATGAGAA) and T1118 (5′-ACCTCGCTTATCGCAAAGA TAC) and cloned into the SmaI site of pGEM-7ZF(+) to produce pMT1479. pMT1479 was digested with EcoRI and BamHI, and the EMSA fragment was recovered by gel purification from a 2% agarose gel. A 78-bp double-stranded oligonucleotide containing the *C. trachomatis nrdAB* promoter region (6a) and a 110-bp restriction fragment containing the *C. trachomatis dnaK* promoter and its CIRCE operator (52) were used as nonspecific DNA probes.

EMSA. Each DNA probe 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 by using a scintillation counter. A 0.5 nM labeled probe was mixed with recombinant YtgR in buffer containing 40 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 70 mM KCl, 100 μM dithiothreitol, 7.5% glycerol, and 100 μM $ZnCl_2$, followed by incubation at room temperature for 20 min. Samples were loaded under tension onto a 6% polyacrylamide gel that had been pre-run at 100 V for 1 h at 4°C. Both the gel and the running buffer contained 0.5× Tris-borate buffer. Assays were performed in the presence or absence of metal ions as indicated in the text. After electrophoresis at 150 V for 4 h at 4°C, the gel was dried 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).

Construction of luciferase reporter plasmids. Reporter plasmids containing the promoter of the *C. trachomatis ytg* operon were cloned upstream of the *luxAB* genes from *Vibrio harveyi* in the vector pMT1597. pMT1597 was derived from pMT1580 (6a) by the addition of a tetracycline resistance cassette from PACYC184 that was digested with XbaI and BsoBI and inserted between blunted KpnI sites. The *ytg* promoter region from −82 to +5 was amplified from *C. trachomatis* serovar D genomic DNA by PCR with primers T1117 and T1060 (5′-ATTGTAAAGATACTTTTAACAGAATGAATTTATATCC) and cloned

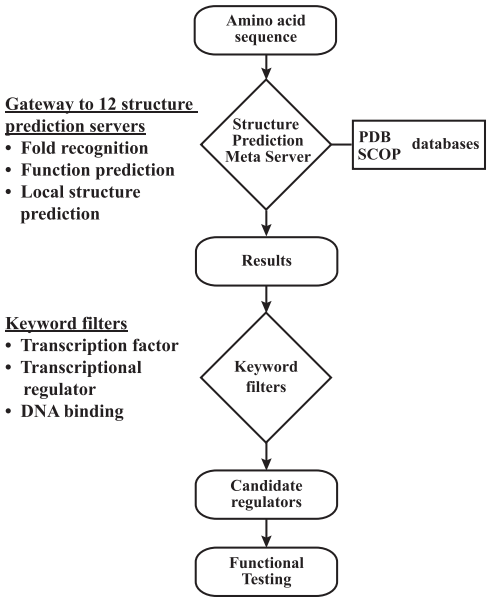

FIG. 1. Flow chart showing the scheme used to identify novel transcription factors in *C. trachomatis*. Amino acid sequences for 240 ORFs in the *C. trachomatis* genome were analyzed by the Structure Prediction Meta Server from BioInfoBank, which references protein databases such as the Protein Data Bank (PDB) and Structural Classification of Proteins (SCOP). Proteins with structural domains characteristic of bacterial transcription factors were identified and scored with a keyword filter. The 10 highest-scoring proteins are listed in Table 1.

into pMT1597 to generate pMT1623. The −60 to +5 promoter region in pMT1618 was produced by annealing the complementary oligonucleotides T1601 (5′- AAACGCGCTAGATTTTCCTTTTGGTTGAGGATATAAATTCATTCT GTTAAAAGTATCTTTACAAT) and T1602 (5′- ATTGTAAA GATACTTTTAACAGAATGAATTTATATCCTCAACCAAAAGGAAAATC TAGCGCGTTT) and ligated into pMT1597 at blunted XbaI sites. Similarly, the −46 to +5 promoter region in pMT1614 was obtained by annealing T1599 (5′- TTCCTTTTGGTTGAGGATATAAATTCATTCTGTTAAAAGTATCTT TACAAT) and T1600 (5′- ATTGTAAAGATACTTTTAACAGAATGAATTT ATATCCTCAACCAAAAGGAA) and ligated into pMT1597.

Luciferase reporter assays. *E. coli* strain GG48 (ΔZntA/ΔZit) (14, 15) was cotransformed with pMT1603, which expresses GST-YtgR, and one of the luciferase reporter plasmids (pMT1623, PMT1618, or pMT1614). An empty pBAD-ITO vector was used instead of pMT1603 in control experiments. An overnight culture was diluted 1:100 in LB medium containing ampicillin, tetracycline, and 0.2% L-arabinose and then incubated at 37°C with shaking until reaching an OD_{600} of ~0.75. Samples were then diluted to an OD_{600} of 0.1. Next, 25 μl of the diluted sample was added to 75 μl of luciferase reaction buffer (50 mM sodium phosphate buffer [pH 7], 50 mM 2-mercaptoethanol, 2% [wt/vol] bovine serum albumin, and *n*-decanal [1:2,000, final dilution]). After incubation at room temperature for 10 min in the dark, light production was measured over 10 s with a Sirius Luminometer v3.1 (Berthold Detection Systems). Measurements were performed in triplicate for each experiment. The results from three independent experiments are reported, with light production normalized to the vector only control. The expression of GST-YtgR was confirmed by Western blot analysis.

RESULTS

Bioinformatics search for transcriptional regulators in *C. trachomatis*. We used the Structure Prediction Meta Server from BioInfoBank (http://meta.bioinfo.pl) to identify candidate transcriptional regulators encoded by the *C. trachomatis* genome (Fig. 1). The Structure Prediction Meta Server acts as a gateway for 12 servers, or software applications, that predict the function of a protein based on its amino acid sequence. Each individual server has its own algorithm that utilizes different combinations of methods, including fold recognition, prediction of function and local structure, and comparison to protein databases such as the Protein Data Bank (PDB) and the Structural Classification of Proteins (SCOP). We used the Meta Server to analyze 240 ORFs in the *C. trachomatis* serovar D genome. Four of the ten highest-scoring genes encoded proteins with known roles in transcription. These include RNA polymerase subunits (encoded by *fliA, rpoN*, and *rpoA*) and a predicted transcription antiterminator encoded by *nusG* (Table 1). Five of the top ten genes encoded hypothetical proteins. Of these, only CT132, which belongs to the RNase BN family, has a predicted function. Two of the hypothetical proteins were *Chlamydia* specific, with no known orthologs in other bacterial species.

Identification of CT069 as a candidate transcriptional regulator in *Chlamydia*. One of the top-scoring proteins, CT069, has been annotated as a component of a putative metal ion transporter (45) and an "integral membrane protein" in the National Center for Biotechnology Information (NCBI) database but, on closer inspection, it also contains features of a transcription factor. The original annotation of CT069 as YtgC was based on sequence similarity to *Treponema pallidum* TroC (19) and other ATP-binding cassette (ABC) transporters. However, when we performed a BLAST search, we found that the C terminus of CT069 is predicted to contain a helix-turn-helix (HTH) DNA binding motif commonly found in the DtxR family of metal ion-dependent transcription factors (35). Intriguingly, the deduced sequence of this ~170-aa region of CT069 shares homology with *T. pallidum* TroR (Fig. 2A), an aporepressor that regulates transcription of the *tro* operon in the presence of a corepressor, Zn^{2+} or Mn^{2+} (20, 34). CLUSTAL W alignment revealed that the region from aa 1 to 313 of CT069 has 23.0% identity and 45.7% similarity with *T. pallidum* TroC, whereas aa 286 to 451 of CT069 have 21.8% identity and 28.2% similarity with *T. pallidum* TroR (Fig. 2B). In addition, bioinformatics analysis using SignalP (5, 32), PrediSi (21), and SPEPLip (11) predicted the presence of a signal sequence on the N-terminal end of CT069 for membrane targeting CT069 to the membrane. These findings suggest that

TABLE 1. Bioinformatics prediction of candidate transcriptional regulators in *C. trachomatis*

Rank	Gene	Predicted function
1	*yfgA* (*ct009*)	Conserved hypothetical protein
2	*ct132*	Conserved hypothetical protein; RNase BN family
3	*fliA* (*rpsD, ct061*)	RNA polymerase alternative sigma factor; sigma 28
4	*rpoN* (*ct609*)	RNA polymerase alternative sigma factor; sigma 54
5	*ytgC* (*ct069*)	Integral membrane protein; ABC transporter membrane protein
6	*rpoA* (*ct507*)	RNA polymerase alpha subunit
7	*ct676*	Hypothetical protein
8	*nusG* (*ct320*)	Transcription antiterminator
9	*ct645*	*Chlamydia*-specific hypothetical protein
10	*ct391*	*Chlamydia*-specific hypothetical protein

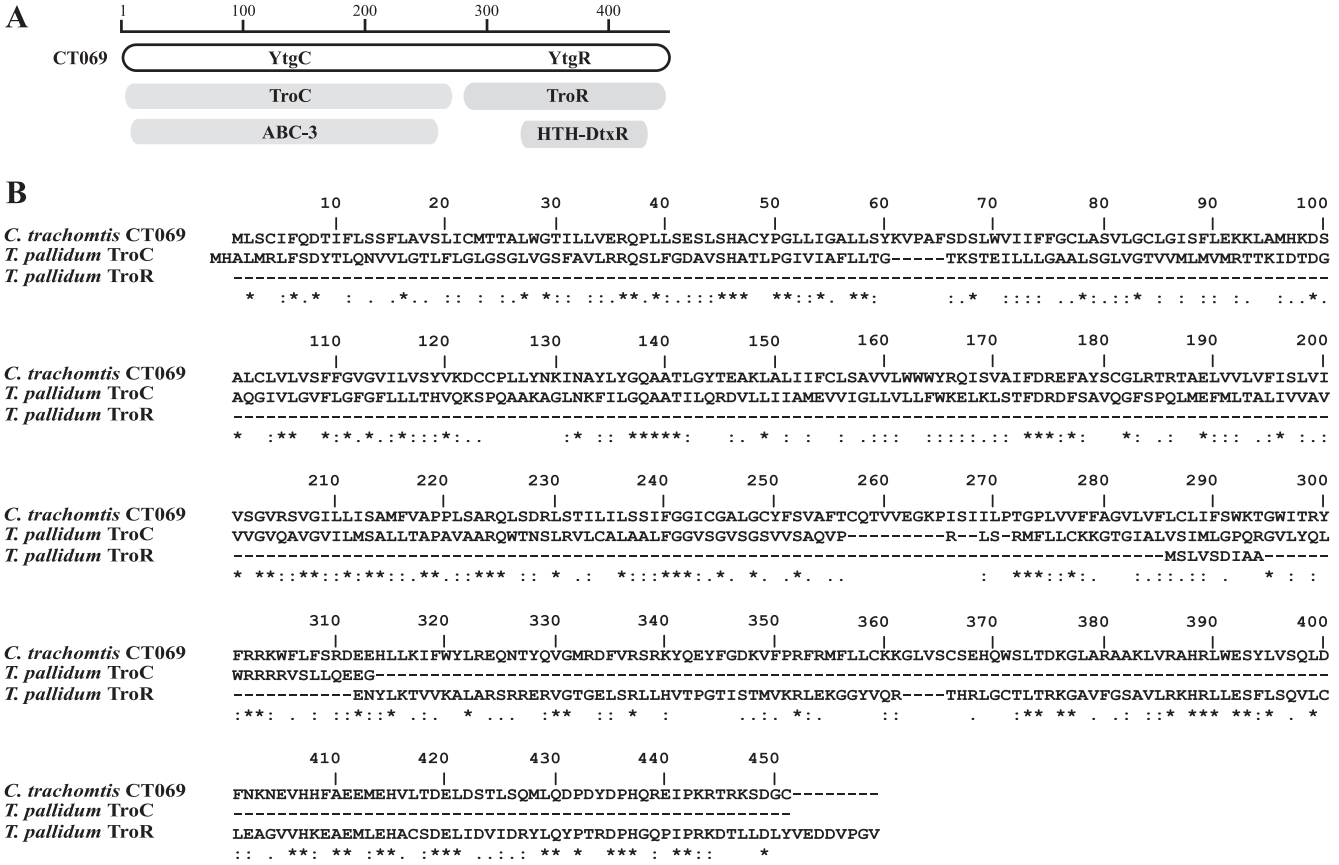

FIG. 2. Identification of CT069 as a candidate transcription factor. (A) BLAST analysis of YtgC showing homology at the N terminus to *T. pallidum* TroC and the transmembrane component of an ABC transporter (ABC-3), and at the C terminus to *T. pallidum* TroR and the helix-turn-helix (HTH) domain of *Corynebacterium diphtheriae* DtxR. (B) CLUSTAL W alignment between *C. trachomatis* CT069 and *T. pallidum* TroC and TroR. Identical, strongly similar, and weakly similar residues are marked by asterisks, colons, and periods, respectively.

CT069 may be a fusion protein composed of two proteins that are both involved in the function of a metal ion transporter. Following the precedent of naming the chlamydial ortholog of TroC as YtgC (45), we have called our putative TroR ortholog, YtgR, Since CT069 is a putative fusion protein of YtgC and YtgR, we will also refer to it as YtgC-YtgR (Fig. 2A).

Evidence that CT069 encodes a fusion protein. To look for other evidence that CT069 is a fusion of YtgC and YtgR, we examined the relationship of *troC* and *troR* in the *T. pallidum* genome and compared it to the analogous situation for *ct069* in the *C. trachomatis* genome. *troC* and *troR* are coexpressed in the *T. pallidum tro* operon, together with *troA, troB*, and *troD*, and *gpm*, which encodes a glycolytic enzyme, phosphoglyceromutase (Fig. 3A) (19). TroA, TroB, TroC, and TroD are the structural components of the metal-dependent transporter, and TroR is a transcriptional regulator that regulates the expression of this operon (20, 34). Intriguingly, *ct069* is adjacent to *ytgA, ytgB*, and *ytgD*, which are the respective chlamydial orthologs of *T. pallidum troA, troB*, and *troD. ytgA* encodes a gene with sequence homology to periplasmic binding proteins of other ABC transporters, and *ytgB* encodes a predicted ATP-binding protein. *ytgD* encodes a putative transmembrane protein that, together with YtgC, is predicted to form the pore complex of the ABC transporter. Thus, all of the genes for a putative ABC transporter are together in one locus in the *C. trachomatis* genome. The relative arrangement of the *ytg* genes is slightly different from the *T. pallidum tro* operon, however, with *ytgR* swapping places with *ytgD* so that it is adjacent to *ytgC* (Fig. 3A). These findings provide additional support for a possible role of *ct069* as a single gene that encodes the orthologs of both TroC and TroR.

To determine whether these five *ytg* genes are also part of an operon in *C. trachomatis*, we examined their transcriptional organization. Using RT-PCR, we synthesized a cDNA fragment using a primer in *yaeM*, which is the gene immediately downstream of the *ytg* genes. *yaeM* encodes YaeM, a predicted 1-deoxy-D-xylulose 5-phosphate reductoisomerase that has a role in terpenoid biosynthesis. Since it is transcribed in the same direction as the *ytg* genes and there is a short intergenic region between *yaeM* and *ytgD*, we reasoned that it might be part of the same operon. Using gene-specific primers for each gene, we successfully amplified DNA for *ytgA, ytgB*, the *ytgC* and *ytgR* regions of *ct069, ytgD*, and *yaeM* from the same cDNA primed from within *yaeM* (Fig. 3A and B). These results indicate that *ytgA, ytgB, ytgC-ytgR, ytgD*, and *yaeM* are cotranscribed as a polycistronic message and are part of an operon in *C. trachomatis*. The *ytg* operon appears to be analogous to the *T. pallidum tro* operon except for a gene rearrangement of *ytgR* and *ytgD* that appears to have resulted in a gene fusion of *ytgC* and *ytgR* in *C. trachomatis.*

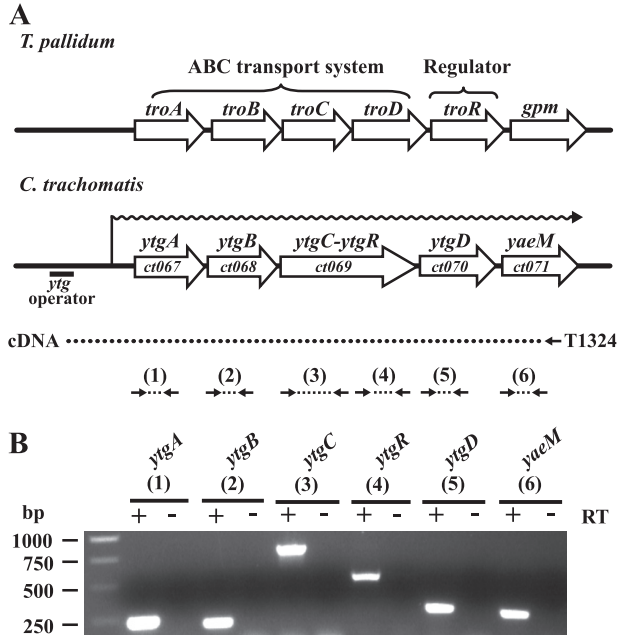

FIG. 3. Transcriptional organization of the *ytg* operon. (A) Schematic diagram of the *tro* operon in *Treponema pallidum* and the *ytg* operon in *C. trachomatis* (not drawn to scale). The polycistronic message is shown as a wavy line, and the location of the operator upstream of the operon is marked. The location of PCR primer pairs and the RT-PCR product for each gene in the operon are shown at the bottom of the panel and labeled 1 to 6. (B) RT-PCR analysis. cDNA was synthesized from chlamydial mRNA using a primer in *yaeM* (T1324) and then amplified using primers for *ytgA* (product 1, shown in panel A), *ytgB* (product 2), *ytgC* (product 3), *ytgR* (product 4), *ytgD* (product 5), and *yaeM* (product 6). A no-RT control was included for each reaction.

To determine whether this predicted YtgC-YtgR fusion protein is unique to *C. trachomatis*, we examined its orthologs in other *Chlamydia* spp. A similar YtgC-YtgR fusion is predicted for all eight *Chlamydia* spp. whose genomes have been sequenced (4, 17, 23, 30, 38, 39, 45, 50), as well as a *Chlamydia*-like endosymbiont of amoeba called *Protochlamydia amoebophila* (22). Thus, the apparent fusion of *C. trachomatis ytgC* and *ytgR* cannot be explained by an isolated sequencing error that artifactually placed the *ytgR* coding sequence in frame with *ytgC*. Moreover, the gene order of the operon in the other *Chlamydia* spp. is the same as in *C. trachomatis* with *ytgD* downstream of the *ytgC-ytgR* fusion (Fig. 3A). Thus, this comparative analysis indicates that the YtgC-YtgR fusion is a conserved feature of *Chlamydia*.

We attempted to detect this fusion protein in *C. trachomatis*-infected cells by performing Western blot analysis on cell lysates probed with polyclonal antibodies raised against the YtgR region of CT069. However, we were unable to detect either YtgR alone or the YtgC-YtgR fusion (data not shown). This inability to detect CT069 *in vivo* is similar to our experience with other chlamydial transcription factors such as HrcA (53) and TrpR (J. C. Akers and M. Tan, unpublished observation) and is likely due to the low abundance of these regulators.

Mapping the promoter of the *ytg* operon and identification of a candidate operator. By analogy to TroR and the *T. pallidum tro* operon, CT069 is predicted to be a transcription factor that regulates the *C. trachomatis ytg* operon. In order to test this prediction, we first identified the promoter of the *ytg* operon by mapping its transcription start site in *C. trachomatis* serovar D by 5′RACE (Fig. 4). By sequence inspection, we located a candidate σ^{66} promoter immediately upstream of the transcription start site with −35 and −10 elements (TTGAGG and TAAAAG spaced 17 nucleotides apart; Fig. 4) that are 4/6 and 5/6 respective matches to the preferred promoter elements recognized by *C. trachomatis* σ^{66} RNA polymerase (42, 46, 48). We verified that this promoter was transcribed by σ^{66} RNA polymerase *in vitro* (data not shown). Upstream of the −35 element, we identified a candidate CT069 binding site from −75 to −58 that consists of an 18-nucleotide region of dyad symmetry with 44% identity to the *T. pallidum tro* operator (Fig. 4). A similar candidate operator can be predicted, in the same location relative to the putative −35 and −10 promoter elements, for the *ytg* operon of other chlamydial species and strains (Fig. 4).

CT069 binds upstream of the *ytg* operon *in vitro*. Since there is no experimental genetic system in *Chlamydia*, we examined whether CT069 is a transcription factor by performing studies with a recombinant form of the protein. We were unable to express full-length CT069 because it was toxic for *E. coli* (data not shown), and so we expressed the region with sequence similarity to the TroR repressor. We used CLUSTAL W to identify this YtgR region from aa 281 to the C terminus of CT069 (Fig. 2B). We cloned the coding sequence into the protein expression vector pBAD-ITO and overexpressed the YtgR polypeptide with C-terminal histidine and myc tags in *E. coli* strain Top10. Recombinant YtgR was purified under denaturing conditions by cobalt affinity chromatography. The pu-

FIG. 4. Alignment of the predicted promoter region of the *ytg* operon from five chlamydial genomes. The transcription start site of the *ytg* operon in *C. trachomatis* serovar D was mapped by 5′RACE and is marked by an asterisk. The −35 and −10 elements of the predicted σ^{66} promoter are highlighted in gray. A putative operator upstream of the −35 promoter element is boxed and aligned with the *T. pallidum tro* operator. This predicted operator forms an inverted repeat as shown by a pair of arrows.

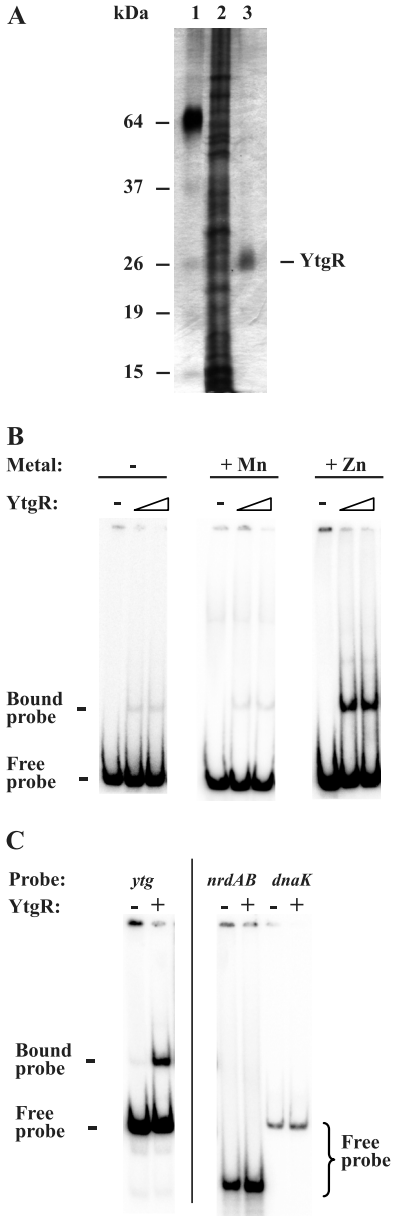

FIG. 5. EMSA analysis of CT069 binding. (A) Silver stain of SDS-PAGE gel showing purification of YtgR region of CT069. Lane 1, molecular weight markers; lane 2, lysate of *E. coli* overexpressing recombinant YtgR; lane 3, YtgR after purification by cobalt chromatography. (B) The YtgR region of CT069 was incubated at a concentration of 200 or 600 nM with a DNA probe containing the *C. trachomatis ytg* promoter region in the absence of exogenous metal ion (−) or in the presence of 100 μM $Mn2^{+}$ or Zn^{2+} (labeled as +Mn or +Zn, respectively). (C) To verify that binding is sequence specific, 400 nM YtgR was incubated with a DNA probe containing either the *ytg, nrdAB*, or *dnaK* promoter region in the presence of 100 μM Zn^{2+}.

rified YtgR protein was >95% pure, as assayed by silver staining (Fig. 5A) and confirmed by Western blot analysis with anti-myc antibodies (data not shown).

We used an EMSA to determine whether the YtgR region of CT069 binds to the candidate operator that we predicted upstream of the *ytg* operon. In the absence of added metal ions, <5% of a labeled DNA probe containing the predicted operator was bound by YtgR (Fig. 5B). Since Zn^{2+} and Mn^{2+} have been proposed as corepressors for *T. pallidum* TroR (20, 34), we tested the effect of adding these divalent metal cations to the EMSA reaction and gel running buffer. When we added 100 μM Zn^{2+}, 25% of the probe was bound by YtgR. In contrast, Mn^{2+} up to a concentration of 100 μM did not facilitate YtgR binding (Fig. 5B). YtgR in the presence of Zn^{2+} did not bind to control probes containing either the *nrdAB* promoter and its NrdR-box operator (6a) or the *dnaK* promoter and its CIRCE operator (52) (Fig. 5C). These results demonstrate that the YtgR region of CT069 binds in a Zn^{2+}-dependent and sequence-specific manner to a DNA probe containing the predicted operator of the *ytg* operon.

CT069 represses transcription of the *ytg* promoter in a heterologous reporter assay. We used an *E. coli* reporter assay to determine whether CT069 is able to function as a transcriptional repressor and repress the promoter of the *ytg* operon. We expressed the YtgR region of CT069 in *E. coli* and measured the effects on a luciferase reporter under the control of the *C. trachomatis ytg* promoter. When we expressed YtgR in wild-type *E. coli* K-12, we did not measure any effect on reporter activity (data not shown). In light of our finding that Zn^{2+} was necessary for binding of YtgR to its operator *in vitro*, we hypothesized that the failure to detect YtgR-mediated repression was due to insufficient intracellular levels of Zn^{2+} in *E. coli* K-12 under normal growth conditions in LB medium.

To test YtgR in the presence of higher Zn^{2+} concentrations, we expressed YtgR in an *E. coli* strain that is defective for Zn^{2+} efflux (*zntA zitB*), which causes elevated levels of intracellular Zn^{2+} (kindly provided by Christopher Rensing) (14). In this Zn^{2+} efflux mutant, there was a 63% decrease in reporter activity when we tested the *ytg* promoter region from −82 to +5, which includes the predicted *ytg* operator located between −75 and −58 (Fig. 6). This effect was promoter specific since there was no repression when we tested the *E. coli nrdH* promoter in control experiments. To examine whether our predicted operator is necessary for this CT069-dependent repression, we tested 5′-deletion constructs of the *ytg* promoter region. There was no repression when we used a promoter region from −60 to +5, which removes most of the predicted operator, or from −46 to +5, which lacks any operator sequence. These *in vivo* findings provide functional evidence that the YtgR region of CT069 is a transcriptional repressor and support the role of Zn^{2+} as a corepressor. Furthermore, they provide experimental support for the location of our proposed *ytg* operator, which is necessary for repression.

DISCUSSION

Only a small number of transcription factors have been identified in the *Chlamydia* genome. A limited repertoire of transcriptional regulators is to be expected for two reasons that are both related to the obligate intracellular lifestyle of this bacterium. First, *Chlamydia* has undergone reductive evolution because it obtains many essential nutrients from its host cell. Members of this genus of human and animal pathogens have a small genome of about one megabase encoding approximately 1,000 genes, compared to *E. coli*, which has a genome of 4.6 megabases and about 4,300 genes. Second, chlamydiae encounter a controlled environment within a cytoplasmic inclu-

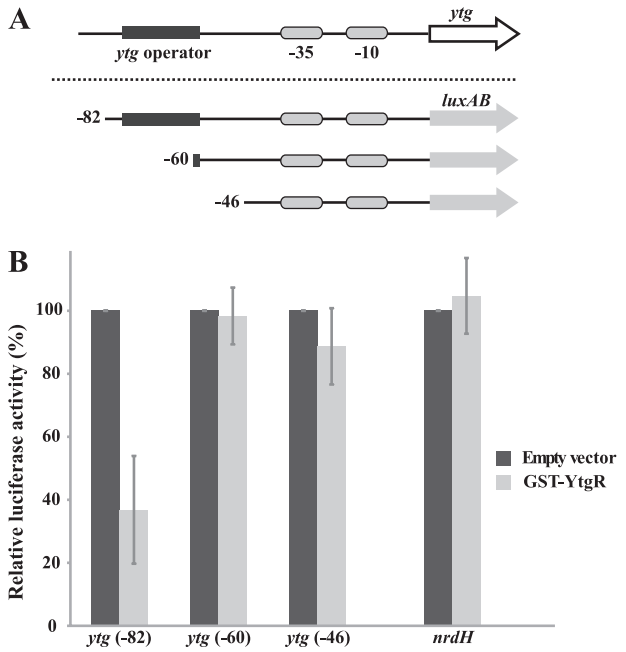

FIG. 6. The YtgR region of CT069 represses transcription of the *ytg* promoter in an *E. coli* reporter assay. (A) Schematic diagram of the *C. trachomatis ytg* promoter region (above) and three luciferase reporter constructs containing different lengths of the *ytg* promoter region cloned upstream of the *luxAB* reporter gene. Predicted −35 and −10 promoter elements and the *ytg* operator are marked. (B) Each *ytg* reporter plasmid was cotransformed with a plasmid expressing GST-YtgR or an empty expression vector into an *E. coli* strain that is defective for Zn^{2+} efflux (*zntA zitB*), which causes elevated levels of intracellular Zn^{2+}. The *E. coli nrdH* reporter was used as a negative control. For each reporter, the luciferase activity obtained in the presence of GST-YtgR was normalized to the levels obtained with the empty vector, which was defined as 100%. The values shown are the mean of three independent experiments, and standard deviations are marked by error bars.

sion that avoids phagolysosomal fusion. The temperature, pH, and osmolarity inside the inclusion are stable (9, 10), and the concentrations of Na^+, K^+, and Ca^{2+} are similar to the cytoplasm (16, 43). Nevertheless, the eight transcription factors that have been predicted in the *Chlamydia* genome represent a disproportionally small number compared to other obligate intracellular bacteria (26, 54), which prompted us to search for additional transcriptional regulators.

In the present study, we used a combined bioinformatics and functional approach to identify novel transcriptional regulators with the general structure and characteristic domains of a bacterial transcription factor. Our successful identification of CT069 demonstrates that this general approach can be used to discover transcription factors that were missed in a standard genome annotation. The NCBI database provides clues that CT069 is both a membrane transporter and a transcription factor since it provides BLAST analyses showing conserved domains within CT069 for these separate functions (3). However, this dual nature is not apparent from the description of CT069 as an "integral membrane protein," reflecting the limitations in both annotation and accessibility for the large amounts of information in a genome database. Our approach uncovered the function of CT069 as a transcription factor that was overlooked because current genome annotation is geared toward finding the "best hit" for each gene. Our bioinformatics approach is reliant, however, on the effectiveness of the prediction algorithms, but it has the potential to improve as computational methods for predicting protein structure and function become more accurate. In addition to CT069, we identified CT009 (YfgA) as a candidate chlamydial transcription factor that contains a predicted HTH binding domain. However, functional studies to evaluate whether CT009 is a transcription factor will be more challenging because its target genes are not known.

By analogy to *T. pallidum troABCDR*, the *ytg* operon in *Chlamydia* spp. appears to encode all of the components for an ABC transporter for importing divalent metal cations. The only component that has been studied in *Chlamydia* is YtgA, which is a homolog of the periplasmic metal-binding protein TroA. Raulston and coworkers (28) showed that *C. trachomatis* YtgA bound iron *in vitro*, and its expression was induced during iron-restrictive growth. The *T. pallidum* Tro transporter has been shown to import Zn^{2+}, Mn^{2+}, and possibly Fe^{2+} (20), but the metal ions that are transported by the chlamydial Ytg transporter have not yet been determined.

Our study demonstrates that Zn^{2+} is involved in the regulation of the *Chlamydia ytg* operon. We showed that Zn^{2+} was necessary for significant binding of the YtgR region of CT069 to the *ytg* operator *in vitro* and that *in vivo* repression could only be demonstrated in an *E. coli* Zn^{2+} efflux mutant that has been engineered to have higher intracellular Zn^{2+} levels (14, 15). These findings support the role of Zn^{2+} as a corepressor for CT069 and are consistent with observations in *T. pallidum* that Zn^{2+} was required as a cofactor for TroR (20). A separate report on *T. pallidum* TroR showed that it was regulated by Mn^{2+} instead (34), but Mn^{2+} had no effect on YtgR-operator binding in our studies. CT069 thus appears to be similar to other prokaryotic metal-dependent repressors, such as DtxR and Fur, that utilize specific divalent metal ions as cofactors (7, 18, 49). Although our *in vitro* and *in vivo* studies implicate Zn^{2+} as the cofactor for CT069 in regulating expression of the *ytg* operon, these findings have not been verified in *Chlamydia*.

Although *Chlamydia* appears to have few transcription factors, our study demonstrates that it encodes two regulators that are responsive to metal ions. In addition to CT069, *Chlamydia* has been shown to have another metal-dependent transcriptional regulator DcrA (36, 55), which is a distant homolog of Fur. The importance of metal ion homeostasis has been shown by cell culture studies demonstrating that iron limitation causes chlamydiae to enter an altered growth state called persistence in which the production of infectious progeny is blocked (2, 37). By identifying CT069 as a Zn^{2+}-dependent transcription factor that controls the expression of a metal ion transporter, our study provides additional mechanisms for the regulation of metal ion levels in *Chlamydia*.

This is the first report of a predicted YtgC-YtgR fusion protein composed of a transmembrane domain and a transcription factor domain, but this arrangement is not unique to *C. trachomatis*. We have noted the presence of a *ytgC-ytgR* gene fusion in the genomes of all sequenced *Chlamydia* spp. (4, 17, 23, 30, 38, 39, 45, 50). We also predict that several other bacteria, including *Bacillus subtilis*, encode a similar YtgC-YtgR fusion protein since they also contain *ytgC-ytgR* gene

fusions in their genomes. In each case, *ytgR* is only fused to *ytgC*, and the gene order is the same as the *C. trachomatis ytg* operon (Fig. 3A). These conserved features at the level of the genes suggest that the function of the YtgC-YtgR fusion protein is also conserved and raise the possibility that the gene fusion has a common evolutionary origin. The *C. trachomatis* genome contains other examples of fusion proteins made up of functionally related proteins. For example, MurC-Ddl is a fusion of two enzymes involved in peptidoglycan synthesis (27), and LcrE is a fusion of two type III secretion proteins (12).

There is precedent for a membrane-associated transcription factor that can respond to changes in the external environment by altering gene expression. For example, *Vibrio cholerae* ToxR is an integral membrane protein that responds to environmental stimuli by activating the cholera toxin genes (8, 29). Similarly, *E. coli* CadC is an integral membrane protein that regulates *cadBA* expression in response to external pH (51). The YtgC-YtgR fusion may allow *Chlamydia* to sense the influx of metal ions and respond by regulating the expression of the putative metal ion transporter that is encoded by the *ytg* operon.

CT069 has features in common with three chlamydial transcription factors that regulate the biosynthesis or import of an essential nutrient. TrpR represses the operon encoding tryptophan biosynthesis genes when levels of the corepressor tryptophan are high (1). ArgR requires arginine as a cofactor to repress the *glnPQ* operon, which encodes a predicted arginine transporter (41). The recently described repressor NrdR regulates an operon encoding nucleotide reductase, which converts ribonucleotides into deoxyribonucleotides, and deoxynucleoside triphosphates have been proposed as its corepressor (6a). These aporepressors each appear to control a single operon that encodes a biosynthetic enzyme or transporter, and the operon is repressed when levels of the end product or imported molecule are sufficiently high. In addition, with the exception of NrdR, the gene encoding each transcription factor is also located within or adjacent to its respective target operon, thereby making up a regulatory unit. In fact, TrpR and ArgR are only present in some *Chlamydia* spp. and strains (1, 41), suggesting that some of these regulatory units have been acquired or lost during the course of chlamydial evolution. By analogy to *T. pallidum* TroR, CT069 may control the expression of its own operon as its sole regulatory target, which it represses in coordination with a metal ion corepressor as part of a negative-feedback loop.

We demonstrate here that the original genome annotation has not identified the full complement of transcription factors in *Chlamydia*. The total number of transcription factors, however, is likely to be small because of this pathogen's obligate intracellular lifestyle and reductive genome. Any additional transcription factors are likely to be highly diverged members of known families of bacterial transcription factors, novel chlamydial transcription factors without orthologs in other bacteria, or unusual transcription factors such as CT069. The small number of transcription factors in *Chlamydia* underscores the importance of the remaining ones as critical regulators of gene expression and homeostatic control.

ACKNOWLEDGMENTS

We thank Christopher Rosario, Elizabeth Di Russo Case, Allan Chen, Kirsten Johnson, and Jennifer Lee for critical reading of the manuscript and helpful advice.

This study was supported by a grant from the National Institutes of Health (NIH) (AI 44198). M.T. was also supported by an NIH Independent Scientist Award (AI 057563). J.C.A. was supported by a predoctoral training grant from the NIH (National Research Service Award LM007443 from the National Library of Medicine).

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