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Proteomic Identification of a Novel *Anaplasma phagocytophilum* DNA Binding Protein That Regulates a Putative Transcription Factor▽†

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***Anaplasma phagocytophilum*, the etiologic agent of human granulocytic anaplasmosis, is an obligatory intracellular bacterium. Little is known about the gene regulatory mechanisms for this bacterium. A gene encoding a putative transcription factor, *tr1*, upstream of three tandem genes encoding outer membrane proteins, including the major outer membrane protein P44, is driven by a strong promoter. In the present study, gel mobility shift assays revealed the presence of *A. phagocytophilum* proteins that interact with the promoter region of *tr1*. These proteins interacting with the *tr1* promoter region were purified by biotin-labeled DNA affinity chromatography from a large amount of host cell-free bacteria. Mass spectrometry identified the major protein as an *A. phagocytophilum* 12.5-kDa hypothetical protein, which was named ApxR. In a DNase I protection assay, recombinant ApxR (rApxR) bound cooperatively to four 24- or 25-bp sites within 235 bp upstream of *tr1*: regions III and IV proximal to *tr1* had higher affinity than regions I and II did. Deletion assays showed that regions III and IV were essential for rApxR binding, whereas regions I and II upstream of regions III and IV were not. The primary *cis*-acting region was region IV, since region IV alone was sufficient for rApxR to strongly transactivate the downstream gene in a *lacZ* reporter assay. Addition of regions I, II, and III did not enhance transactivation. These results show that ApxR is a novel transcriptional regulator that directly regulates *tr1*.**

Human granulocytic anaplasmosis (HGA) (formerly called human granulocytic ehrlichiosis) has recently been recognized as a zoonotic disease of public health importance and is currently one of the most common tick-borne zoonoses in the United States and Europe (4, 18). HGA is an acute febrile systemic illness accompanied by hematologic and liver enzyme abnormalities. It can cause severe and potentially fatal illness, especially in immunocompromised and elderly people (6). The etiologic agent of HGA, *Anaplasma phagocytophilum*, is a gram-negative, obligatory intracellular bacterium, which was initially determined to have a tropism for granulocytes (2, 9) and currently is also known to infect endothelial cells (11, 16).

A fundamental question in obligatory intracellular parasitism is how intracellular bacteria coordinate and finely tune their development and proliferation with the conditions of their host cells, as these bacteria are absolutely dependent on their host cells. In sequenced genomes of obligatory intracellular bacteria, fewer putative DNA binding proteins are detected than in free-living bacteria due to the relatively homeostatic host cell environment. Yet, very little experimental data are available on these genes or gene products. In the sequenced genome of *A. phagocytophilum* (7), there are eight putative DNA interacting proteins: two-component system response regulators CtrA and NtrX that contain a DNA binding motif (3), a putative transcriptional regulator encoded by *tr1* that has a winged helix-turn-helix motif (13), a basic histone-like protein HU (GenBank accession no. YP_505361.1) (5), a putative transcription factor of the Baf family (GenBank accession no. YP_505575.1), a transcriptional regulator of the MerB family (GenBank accession no. YP_505799.1), an integration host factor α (GenBank accession no. YP_505798.1), and an integration host factor β (GenBank accession no. YP_504885.1). Of these, *tr1*, which is upstream of tandem genes encoding outer membrane proteins, including the polymorphic major outer membrane protein P44 (13), has been the most studied. The transcriptional start site was previously found at 21 bp upstream of *tr1* with a typical $-35/-10$ σ^{70} promoter (13). The upstream region of *tr1* has strong promoter activity in *Escherichia coli* (1) and after transposition into the *A. phagocytophilum* genome (8). A gene in the *A. phagocytophilum* genome encodes a primary DNA-dependent RNA polymerase that is homologous to σ^{70} factor responsible for constitutive gene expression (7). This suggests that transcriptional activators or repressors play a role in *tr1* expression. The objectives of the present study were to investigate *A. phagocytophilum* proteins that may be involved in the transcriptional regulation of *tr1*. We performed electrophoretic mobility shift assays (EMSAs), using *A. phagocytophilum* lysate and a DNA probe within the potential regulatory region upstream of *tr1*. Using this approach, we isolated a novel *A. phagocytophilum* protein that we named ApxR that binds to a *cis* element involved in *tr1* regulation. A DNase I protection assay and a *lacZ* reporter assay were carried out to characterize the *cis* element and ApxR regulation. Our results indicate that ApxR is the first functionally characterized transcription factor of a member of the order *Rickettsiales*.

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MATERIALS AND METHODS

***A. phagocytophilum* and host cell-free bacteria.** Host cell-free *A. phagocytophilum* HZ strain was prepared by sonication of infected HL-60 cells as previously described (19). A bacterial suspension was passed sequentially through syringe-driven 5-μm nylon (Whatman, Clifton, NJ) and 0.8-μm glass-fiber membrane filters (Millipore, Billerica, MA) for EMSA analysis or through the 5-μm filter only for isolation of the DNA binding protein. The filtrate was centrifuged at 10,000 × *g* for 10 min, and the pellet enriched with *A. phagocytophilum* was washed with 2× phosphate-buffered saline (2× phosphate-buffered saline consists of 274 mM NaCl, 20 mM Na_2HPO_4, 5.4 mM KCl, and 3.6 mM KH_2PO_4 [pH 7.2]).

EMSA. The DNA fragments upstream of *tr1* shown in Fig. 1, and DNA fragments R3-1 and R3-2 with various deletions, were amplified with the primer pairs shown in Table S1 in the supplemental material. Approximately 5 pmol each of the purified PCR product was biotinylated with a biotin 3′-end DNA labeling kit (Pierce Biotechnology, Rockford, IL). The biotinylated probes were purified and denatured at 94°C for 3 min and then slowly cooled to room temperature to allow proper annealing. The bacterial pellet (approximately 100 μg) was lysed by sonication in 100 μl buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM dithiothreitol (DTT), 0.4% NP-40, and 1% protease inhibitor cocktail (Calbiochem, San Diego, CA) and centrifuged at 18,000 × *g* for 30 min at 4°C. The supernatant of bacterial lysate, bovine serum albumin (BSA), uninfected HL-60 cell lysate (5 μg of each protein, unless otherwise stated), purified recombinant ApxR (rApxR), or purified recombinant GroES (rGroES) was incubated with biotinylated DNA probes (0.1 pmol each) for 30 min at 4°C in 20 μl of binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 2.5% glycerol, 0.1% NP-40, 50 ng/μl of poly(dI-dC) or salmon sperm DNA]. As a control, the sample was incubated with an excess (15×) of unlabeled probe. A 6% nondenaturing polyacrylamide gel in 0.5× Tris-borate-EDTA (TBE) (0.44 M Tris base, 0.44 M boric acid, and 0.01 M EDTA [pH 8.0]) had been prerun for 1 h, and then samples were loaded onto the gel and electrophoresed at 100 V for 1.5 h at 4°C. The biotinylated probes were transferred to a nylon membrane (Amersham Biosciences, Piscataway, NJ) at 380 mA for 45 min. The transferred DNA was cross-linked to the membrane with UV light. The biotin-labeled DNA was detected with a LightShift chemiluminescence EMSA kit (Pierce).

Affinity purification of DNA binding proteins. The R3-1 probe was PCR amplified using the 5′ biotin-labeled forward primer of primer set 3 (see Table S1 in the supplemental material). The PCR product was precipitated with sodium acetate, pH 5.2 (final concentration of 0.3 M), and cold 100% ethanol. The supernatant of the lysate derived from 1 mg of bacteria purified as described above was incubated with 15 μg salmon sperm DNA at 4°C for 15 min, followed by incubation with 50 pmol biotinylated R3-1 probe for 30 min at 4°C in 600 μl of the binding buffer described above. The DNA-bound proteins were affinity purified with a μMACS streptavidin kit (Miltenyi Biotec, Berglsch Gladbach, Germany). The binding reaction mixture was applied to the column and washed sequentially with 400 μl binding buffer and 300 μl binding buffer supplemented with 1 M NaCl. DNA-bound proteins were solubilized in 10 μl sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel loading buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 5% β-mercaptoethanol) at 100°C for 5 min and subjected to 15% SDS-PAGE.

Mass spectrometry. After electrophoresis, the gel was fixed in 50% methanol and 10% acetic acid overnight and stained with GelCode Blue (Pierce). After the gel was destained in water, the bands were cut out and digested with trypsin in the gel (Promega, Madison, WI). Capillary liquid chromatography-nanospray tandem mass spectrometry (LC/MS/MS) was performed on a Thermo Finnigan linear quadrupole mass spectrometer (Thermo, San Jose, CA) equipped with a nanospray source operated in positive-ion mode. The LC system was an UltiMate Plus system (LC-Packings A; Dionex Co., Sunnyvale, CA). Sequence information from the MS/MS data was processed using the MASCOTT MA/MA search engine and Turbo SEQUEST algorithm in BioWorks 3.1 software.

Cloning and expression of rApxR and rGroES. *apxR* and *groES* were amplified using *A. phagocytophilum* genomic DNA as a template and directionally cloned into the NdeI and XhoI sites of the pET29a(+) vector (Novagen Inc., Madison, WI). The resulting plasmids (pApxR and pGroES) were amplified in *E. coli* Novablue cells (Novagen) and expressed in *E. coli* BL21(DE3) cells (Novagen) as previously described (21). The soluble fraction of *E. coli* lysate was Ni affinity purified with a His-Select cartridge (Sigma, St. Louis, MO). Purified rApxR and rGroES were dialyzed in EMSA binding buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, and 1 mM DTT.

DNase I footprint analysis. A 314-bp DNA fragment upstream of the *tr1* translational start site, including 79 bp upstream of R3 and 235 bp of R3 (20 bp less than the R3 probe used for EMSA at the 3′ end) was amplified and cloned into a pCRII vector of a TA cloning kit (Invitrogen) to make the probe template. The probe was generated by PCR with primer set 5 (see Table S1 in the supplemental material), except that the 5′ part of the 5′-end primer was labeled with 6-carboxyfluorescein (FAM) at Applied Biosystems, Inc. (ABI) (Foster City, CA). The FAM-labeled probe at 300 ng was incubated with 5 or 1.25 μM rApxR or BSA as a control under the conditions described previously (22). Based on the results of optimization experiments using 10-fold dilutions (from 0.05 to 5 units) of DNase I (Worthington Biochemicals, Lakewood, NJ), 0.05 unit of DNase I was added to the reaction mixture and incubated for 5 min at 25°C. The reaction was terminated by heating at 75°C for 10 min. The digested DNA fragments were purified using a QIAquick PCR purification kit (QIAGEN) and eluted in 40 μl

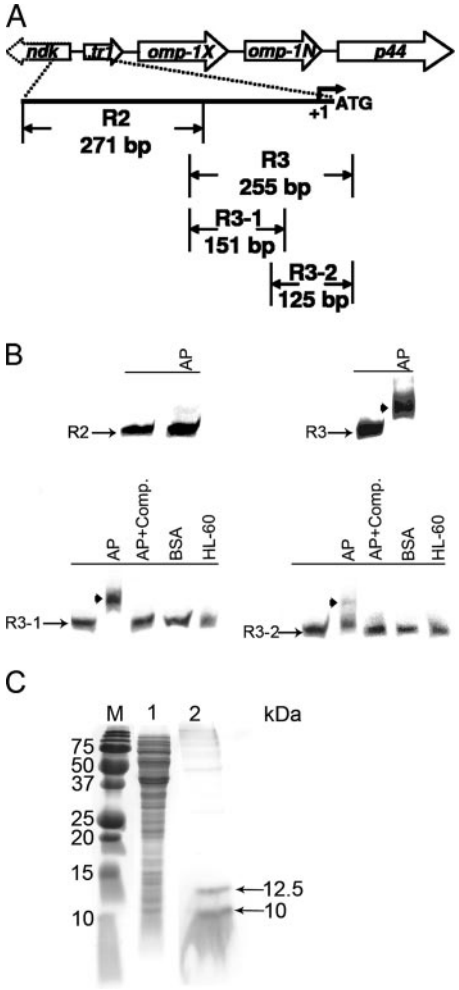

FIG. 1. Gel mobility shift assay of native *A. phagocytophilum* proteins bound to regulatory regions upstream of *tr1*. (A) Schematic representation of probe locations upstream of the *tr1* gene in the *p44* expression locus of *A. phagocytophilum*. The bent arrow indicates the transcriptional start site. (B) Native *A. phagocytophilum* proteins (AP) bound to the R3, R3-1, and R3-2 probes, but not R2. In each gel, the position of unshifted DNA is seen in the leftmost lane, which contained no protein. Shifted bands are indicated by black arrowheads. Comp., 15-fold excess amount of the unlabeled probe as a competitor. BSA and HL-60, BSA or uninfected HL-60 cell lysate was added instead of AP. The figure is representative of three independent experiments. (C) Isolated *A. phagocytophilum* proteins bound to the biotinylated DNA probe in streptavidin affinity chromatography subjected to 15% SDS-PAGE and Gel-Code blue staining. Lane 1, *A. phagocytophilum* lysate flowthrough; lane 2, bound proteins analyzed by proteomics (arrows). The positions of molecular mass standards in lane M (in kilodaltons) are shown to the left of the gel.

H_2O. Nondigested DNA was used for sequencing reactions with the 5′ FAM-labeled forward primer of primer set 5 (see Table S1 in the supplemental material) and a Thermo Sequenase dye primer manual cycle sequencing kit (USB, Inc., Cleveland, OH). The digested DNA and sequencing reaction products were analyzed with a 3730 DNA analyzer (ABI) (22). The sequences were then analyzed with GeneMapper software (ABI) to convert the DNase I digestion map into sequencing data to identify the exact sequences that were protected.

***tr1* promoter-*lacZ* reporter fusion constructs.** The *lacZ* gene was amplified from plasmid pQF50, which contains full-length *lacZ* with *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Three different *tr1* promoter regions, the entire R3 fragment (235 bp), the 3′ R3 fragment containing regions III and IV (116 bp), and the 3′ R3 fragment containing region IV (90 bp), were PCR amplified using *A. phagocytophilum* genomic DNA as the template. The transcriptional fusion was constructed by placing each *tr1* promoter region upstream of the promoterless *lacZ* gene in pACYC184 (New England Biolabs, Beverly, MA). *E. coli* BL21(DE3) pretransformed with pApxR or pET29a(+) without an insert was cotransformed with pACYC184 containing each one of the *tr1* promoter-*lacZ* fusion inserts. After overnight culture, transformants were subcultured in LB medium supplemented with 50 μg/ml kanamycin and 25 μg/ml chloramphenicol at 37°C for 2.5 h, followed by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) induction for 1 h. β-Galactosidase activity was measured as described previously (15). Plasmids were extracted from each sample by using a GenScript Quickclean 5 M Miniprep kit (GenScript, Piscataway) and run on a 1% agarose gel to confirm the copy number of each plasmid per bacterium. The *E. coli* suspension was dissolved in 1× SDS-PAGE gel loading buffer at 95°C for 10 min. Approximately 20 μg of total protein was loaded in each well on a 15% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated with peroxidase-conjugated antipolyhistidine antibody (Sigma) at a 1:500 dilution, and reacting bands were visualized by enhanced chemiluminescence (ECL kit; GE Healthcare, Piscataway, NJ).

Statistical analysis. Statistical analyses were performed by using analysis of variance and Tukey's honestly significant difference test, and a P of <0.05 was considered significant.

RESULTS

Gel mobility shift assays with *A. phagocytophilum* native protein binding. Previously, the transcriptional start site was found 21 bp upstream of the *tr1* translational start site (ATG) (13), and the 40-bp upstream region was shown to have strong promoter activity in *E. coli* (1). To investigate whether there is an *A. phagocytophilum* protein that binds to a *cis* element to regulate *tr1*, the ~500-bp region immediately upstream of *tr1* was analyzed by an EMSA. To construct DNA probes within the ~500-bp region, overlapping DNA fragments R2 (271 bp) and R3 (255 bp) were PCR amplified from *A. phagocytophilum* genomic DNA and biotinylated (Fig. 1A). Lysate from host cell-free *A. phagocytophilum* was incubated with the probes, and the mixture was analyzed by an EMSA. The results showed a shift of DNA fragment R3 but not DNA fragment R2 (Fig. 1B). To narrow down the target DNA region, fragment R3 was subdivided into overlapping two fragments, R3-1 (151 bp) and R3-2 (125 bp) (Fig. 1A), and biotinylated. EMSA results revealed the binding of both R3-1 and R3-2 probes to an *A. phagocytophilum* protein (Fig. 1B). The binding of probe R3-2 to the *A. phagocytophilum* protein was weaker than the binding of R3 and R3-1 probes (Fig. 1B). The addition of unlabeled R3-1 and R3-2 DNA competitively reduced *A. phagocytophilum* protein binding to the labeled probe, indicating that the binding was specific to each probe (Fig. 1B). The negative controls, BSA and the uninfected HL-60 cell lysate, did not bind to these DNA fragments (Fig. 1B).

Affinity capture and identification of the DNA binding protein by MS. To identify the *A. phagocytophilum* protein bound to the R3-1 DNA fragment, the protein bound to biotinylated DNA was affinity purified with streptavidin affinity chromatography. SDS-PAGE of affinity-purified proteins revealed two major bands (12.5 and 10 kDa) and several weak bands of larger molecular sizes (Fig. 1C). MS/MS of the 12.5-kDa band revealed two peptide sequences (VIGVDQPK and VSQII NGK) that matched a 111-amino-acid 12.5-kDa hypothetical protein (GenBank accession no. YP_505110.1). We named this protein *A. phagocytophilum* expression regulator, ApxR. Another major band contained a peptide sequence (VVSVGPG ASNSDGK) that matched a 10-kDa chaperonin, GroES (GenBank accession no. YP_504858.1). Proteins of larger sizes

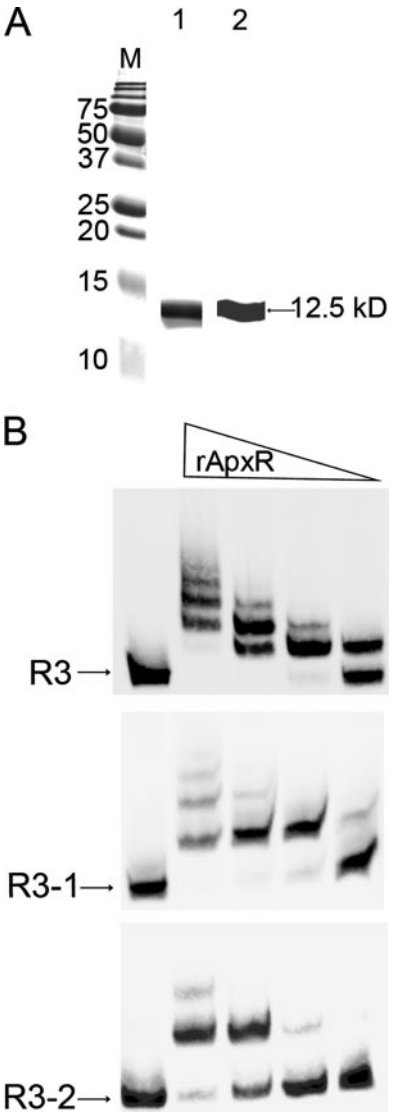

FIG. 2. Binding of rApxR to the region upstream of *tr1*. (A) Three micrograms of affinity-purified rApxR was subjected to SDS-PAGE. Lane 1, Coomassie brilliant blue staining; lane 2, Western blot analysis with an anti-His tag antibody. The positions of molecular mass standards in lane M (in kilodaltons) are shown to the left of the gel. The position of rApxR (12.5 kDa) is indicated to the right of the gel. (B) Dose response of rApxR (5, 0.5, 0.05, and 0.005 μg) binding to probes R3, R3-1, and R3-2 (0.1 pmol each) in an EMSA. In each panel, the position of unshifted DNA is seen in the leftmost lane, which contained no protein.

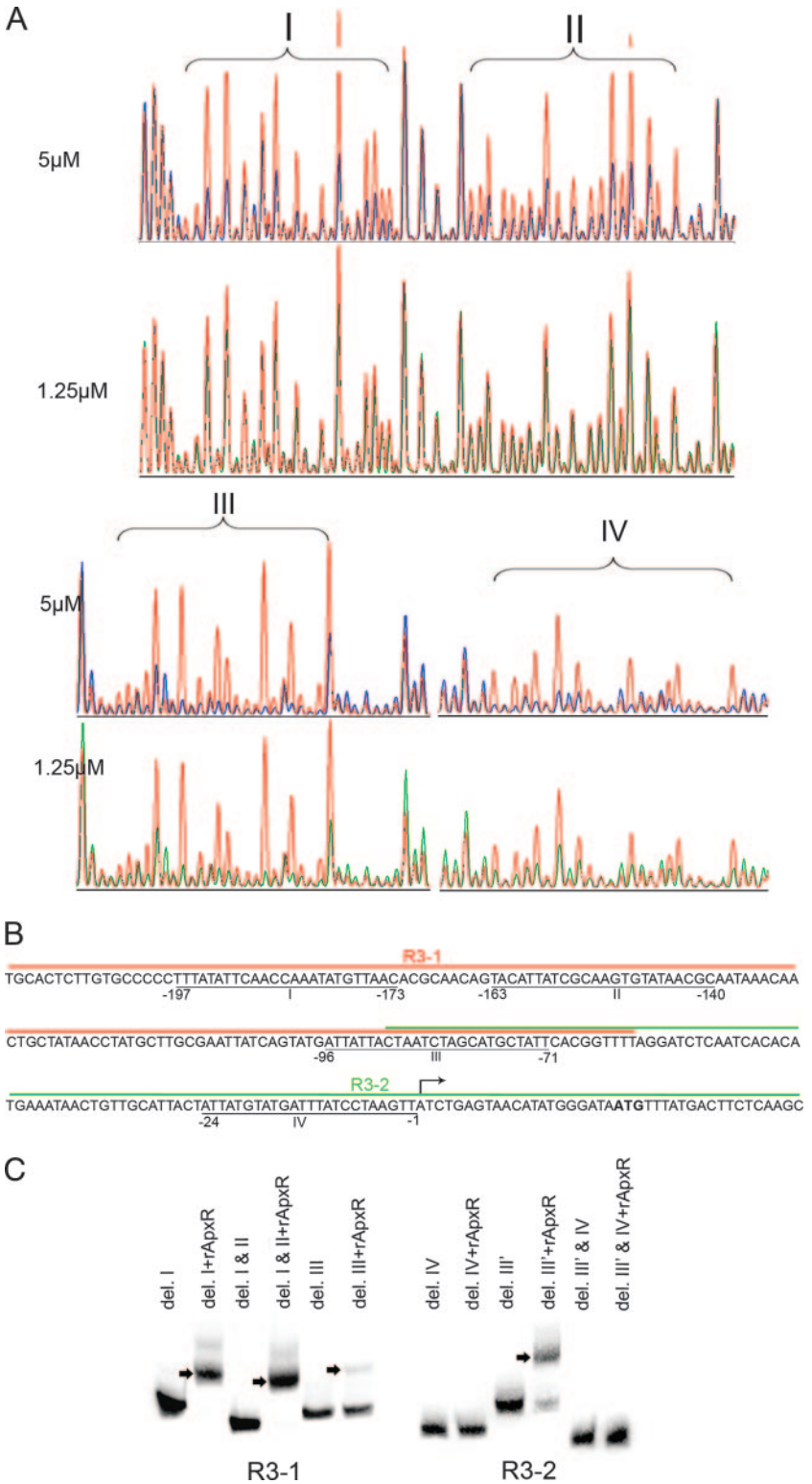

FIG. 3. Identification of the sequence of the ApxR-protected regions of the *tr1* promoter by DNase I protection footprinting. (A) Electropherograms superimposed to show the four regions (regions I to IV) protected by different concentrations of rApxR (5 μM [blue] and 1.25 μM [green]) or BSA (red) within region R3 after digestion with DNase I. (B) DNA sequences of four protected regions (underlined) within region R3. The sequences for the R3-1 and R3-2 probes are overlined in red and green, respectively. (C) EMSA showing that regions III and IV are required for rApxR binding to R3-1 and R3-2 probes, respectively. Probes used were R3-1 probes with deletions in region I (del. I), regions I and II (del. I & II), or region III (del. III), and R3-2 probes with deletions in region IV (del. IV), 14 bp at the 5′ end of region III (del. III'), or both (del. III' & IV). Shifted bands are indicated by black arrows.

could not be identified by MS due to insufficient amounts of protein.

rApxR and EMSA analysis. To further characterize ApxR binding to the regulatory DNA region, *apxR* was cloned into the pET29a(+) vector (pApxR) and expressed in *E. coli*. This expression yielded a 119-amino-acid rApxR (12.5 kDa), including an 8-amino-acid six-His tag in the C terminus derived from the pET29a(+) vector. The Ni-affinity-purified rApxR was detected as a single band of approximately 12.5 kDa by Coomassie brilliant blue staining on SDS-polyacrylamide gels and by Western blot analysis with an anti-His tag antibody (Fig. 2A).

Upon incubation with increasing concentrations of the rApxR protein, EMSA analysis showed the shift of R3, R3-1, and R3-2 probes in a dose-dependent manner (Fig. 2B). The binding of probe R3-2 to the rApxR protein was weaker than the binding of R3 and R3-1 probes (Fig. 2B). By EMSA, rGroES was also tested. However, rGroES did not bind to the R3 DNA fragment. Addition of rGroES did not influence the binding of rApxR to R3 (data not shown).

DNase I footprint analysis. The EMSA results demonstrated that rApxR directly interacts with two DNA fragments, R3-1 and R3-2, in a dose-dependent manner. Therefore, we performed DNase I protection assays to determine the DNA sequences to which rApxR would bind. The protected regions of the sense strand were determined by comparing sequences of DNA samples protected by 5 or 1.25 μM rApxR to the sequence of unprotected DNA samples treated with BSA (Fig. 3A). There were four 24- or 25-bp regions protected in tandem by rApxR from bases −197 to −173 (region I), −163 to −140 (region II), −96 to −71 (region III), and −24 to −1 (region IV) (Fig. 3A and B). These four protected regions were all located within R3 but not within the 79 bp upstream of R3. Regions I, II, and III were located within DNA fragment R3-1, whereas 14 bp of region III and all of region IV were located within DNA fragment R3-2. This result explains why the binding of native ApxR as well as rApxR to DNA fragment R3-2 was less or weaker than binding to DNA fragment R3-1 in EMSA (Fig. 1B and 2B).

Among these four regions, rApxR binding affinities were different. At the higher concentration of rApxR (5 μM), all four regions were protected (Fig. 3A). When the rApxR concentration decreased to 1.25 μM, regions I and II were not protected, whereas the protection of region III and IV was still evident. After combining EMSA data (Fig. 1B and 2B) and DNA footprint analysis data, region III appears to have a higher affinity to ApxR than region IV does.

Deletion analysis of DNA regions required for ApxR binding by EMSA. DNA footprint analysis showed four rApxR-protected regions. Consequently, we determined which regions are required for rApxR binding to R3-1 and R3-2 fragments. Deletion of region I or both regions I and II from fragment R3-1 had little effect on the binding of rApxR in an EMSA analysis (Fig. 3C). However, rApxR failed to bind when region III in fragment R3-1 or region IV in fragment R3-2 were deleted (Fig. 3C). This indicates regions III and IV were essential for rApxR binding, whereas regions I and II and the 14 bp of region III in fragment R3-2 are not essential for rApxR binding to fragment R3-1 or R3-2.

Regulation of *tr1* promoter-*lacZ* fusion with ApxR. Since ApxR bound four regions within the R3 fragment, we examined whether these regions are involved in *tr1* transactivation by ApxR. The *lacZ* reporter fusions were constructed by placing the entire R3 fragment (R3-*lacZ*), the 3′ R3 fragment containing regions III and IV (III&IV-*lacZ*), and the 3′ R3 fragment containing only region IV (IV-*lacZ*) upstream of the translational start site of a promoterless *lacZ* gene in pACYC184. Each one of the *lacZ* reporter fusions was transformed into pApxR-transformed or pET29a(+)-transformed *E. coli*. In pApxR-transformed *E. coli* that was cotransformed with any one of three *lacZ* reporter constructs, the β-galactosidase activity was significantly greater when rApxR expression was induced with IPTG than without IPTG induction (Fig. 4). In each of the three *lacZ* reporter constructs, β-galactosidase activity with IPTG induction was significantly greater in pApxR-transformed *E. coli* than in pET29a(+)-transformed *E. coli* (Fig. 4). The primary *cis*-acting region was region IV, since rApxR strongly transactivated the *lacZ* reporter gene downstream of region IV in the region IV-*lacZ* construct. R3 that contains all four regions and region III&IV-*lacZ* did not differ significantly from the region IV-*lacZ* construct in the *lacZ* reporter assay (Fig. 4). Thus, regions I, II, and III did not enhance the transactivation of region IV by ApxR. The relative

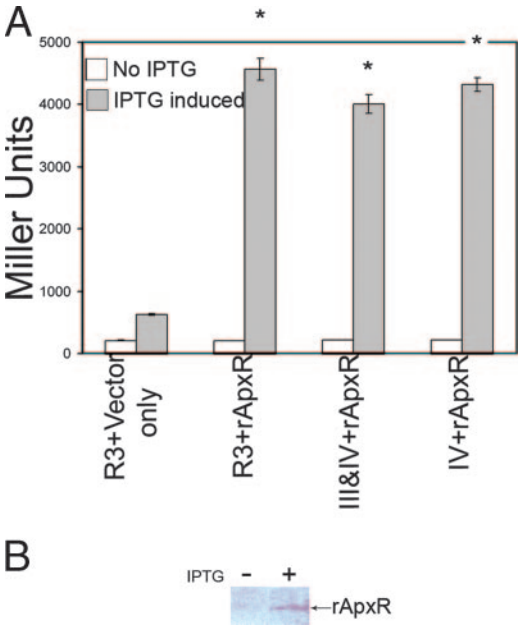

FIG. 4. ApxR activates the transcription of *tr1* promoter-*lacZ* fusion. (A) β-Galactosidase assays were used to measure the transcriptional activity of the entire R3 fragment, regions III and IV, or region IV-*lacZ* fusion in *E. coli* strains containing pApxR or the pET29a(+) vector alone. The activation of three *tr1* promoter-*lacZ* fusion constructs was compared with or without rApxR induction and between *E. coli* strains containing pET29a(+) vector and pApxR. Data are presented as means ± standard deviations (error bars) of triplicate samples. Values that were significantly different ($P < 0.01$) from the value for the *E. coli* strains with pET29a(+) alone or the value for the *E. coli* strains without IPTG induction by Tukey's honestly significant difference test are indicated by an asterisk. A representative result from four independent experiments is shown. (B) Western blot analysis of the expression of rApxR protein (arrow) upon IPTG induction (+) in the *E. coli* strain cotransformed with pApxR and pACYC184 encoding the entire R3 fragment. The expression of rApxR was not detected in the absence of IPTG (−).

TABLE 1. *Anaplasma phagocytophilium* ApxR orthologs found in sequenced genomes of the order of *Rickettsiales*[a]

Bacterium	GenPept accession no.(s)	Annotation	E value
Anaplasma marginale St. Maries	YP_153746.1	Hypothetical protein	1e−25
Ehrlichia canis Jake	YP_302926.1	Hypothetical protein	4e−14
Ehrlichia chaffeensis Arkansas	YP_507593.1	Hypothetical protein	6e−13
Ehrlichia chaffeensis Sapulpa	ZP_00545133.1	Hypothetical protein	6e−13
Ehrlichia ruminantium Gardel	YP_196226.1	Hypothetical protein	1e−12
Ehrlichia ruminantium Welgevonden	YP_180165.1, YP_197182.1	Hypothetical protein	5e−12

[a] An E value of ≤ 5e−12 based on amino acid sequences was used as a cutoff.

copy numbers of pApxR and pACYC184 per bacterium were similar among three *lacZ* reporter constructs with or without IPTG induction and in each *lacZ* reporter construct with or without IPTG induction. Expression of the rApxR protein upon IPTG induction was confirmed by Western blot analysis (Fig. 4). Taking all the data together, ApxR can function as a transactivator for *tr1*.

DISCUSSION

Herein, we have isolated an *A. phagocytophilum* hypothetical protein by biotin-labeled DNA affinity chromatography and provided several lines of evidence that this protein (ApxR) is a novel transcriptional regulator. In support of this conclusion, the amino acid sequence from positions 8 to 57 of ApxR matches COG5606 that includes the XRE protein, which contains a helix-turn-helix motif based on the analysis of the NCBI conserved domain database. ApxR bound up to four tandem regions of a similar length of approximately 25 bp each within the 230-bp promoter region of *tr1*. The *tr1* promoter regions from positions −96 to −71 (region III) and −24 to −1 (region IV) were essential for rApxR binding, since binding was lost if these regions were deleted. These two regions bound at higher affinity to rApxR than regions I and II did. The *tr1* promoter-*lacZ* fusion protein reporter assay showed that strongly positive regulation of the region IV by ApxR and the remaining three upstream regions did not enhance transactivation. The primary *cis*-active region IV resides within the 40-bp sequence upstream of *tr1*, which is active in *E. coli* (1) and after transposition into the *A. phagocytophilum* genome (8). This region overlaps the −10 consensus sequence of the −10/−35 promoter. This is consistent with the observations of the σ^{70}-type promoter preceding *tr1* (13) and that the σ^{70}-type promoter is activated by direct interaction between RNA polymerase and a neighboring activator (10). In the *A. phagocytophilum* genome, there is only a single alternative σ factor, σ^{32}. The paucity of alternative sigma factors suggests an important role of transcription factors that regulate the σ^{70}-type promoter in *A. phagocytophilum*. Based on the frequency of *A. phagocytophilum* genes preceded by the σ^{70}-type promoter, the transactivating factor ApxR may regulate more genes. In fact, *apxR* (monocistronic gene) itself is preceded by the σ^{70}-type promoter and rApxR bound to the promoter region in an EMSA (Xueqi Wang and Yasuko Rikihisa, unpublished data). Thus, it is possible that *apxR* itself is autoregulated. Diversity of base sequences found among four ApxR binding regions is characteristic of a global regulator (5). In *Chlamydia trachomatis*, a constitutively activated mutant form of a response regulator, ChxR serves as a global transcriptional regulator to regulate its intracellular developmental cycle (12). Considering the limited numbers of putative transcription factors in the *A. phagocytophilum* genome, ApxR may also serve as a global regulator in *A. phagocytophilum* to regulate its intracellular developmental cycle.

The role of GroES coaffinity purified with ApxR in *tr1* transcription was not further investigated in the present study, since it did not bind to the *tr1* promoter region in the EMSA. GroES is a prominent heat shock chaperon in the cytoplasm of *A. phagocytophilum*, and as such, it may indirectly modulate ApxR function by stabilizing or sequestrating ApxR.

Functions of Tr1 (estimated molecular mass of 21 kDa) are currently under investigation. Tr1 (a hypothetical transcriptional regulator also known as UN1 [17]) has a helix-turn-helix DNA binding motif and is associated with a multigene family encoding major outer membrane proteins of *Ehrlichia* and *Anaplasma* species. Initially, *tr1* was found upstream of the 22 tandem *omp-1* (*p28*) and *p30* loci of *Ehrlichia chaffeensis* and *Ehrlichia canis*, respectively, and these *omp-1* genes and *p30* genes were shown to be polycistronically transcribed from the *tr1* promoter (17). Homologs of *tr1* were subsequently detected upstream of the *p44/msp2* expression locus of *A. phagocytophilum* (13), the *Anaplasma marginale* major surface protein 2 (*msp2*) operon-associated protein (*opag* genes) expression locus (14), and the *Ehrlichia ruminantium map1* locus (20). *omp-1*, *msp2*, *p44*, *p30*, and *map-1* belong to the outer membrane protein 1/Msp2/P44 superfamily Pfam PF0167 (7). *A. marginale*, a species closely related to *A. phagocytophilum*, contains a sequence that is identical to −24 to −1 (region IV) upstream of a gene encoding a hypothetical protein that is a homolog of the *tr1* protein of *A. phagocytophilum* (1). A search of the GenBank database identified *apxR* homologs in the genomes of *A. marginale* St. Maries, *E. canis* Jake, *E. chaffeensis* Arkansas and Sapulpa, and *E. ruminantium* Welgevonden and Gardel (E value of ≤5e−12; Table 1). Therefore, there has been evolutionary pressure to conserve *apxR* and *tr1* among *Ehrlichia* and *Anaplasma* species, and the means by which the *tr1* of *A. phagocytophilum* is directly regulated by ApxR may be also used to regulate *tr1* homologs among other members of *Ehrlichia* and *Anaplasma* species.

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