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RESEARCH ARTICLE

Analysis of *Orientia tsutsugamushi* promoter activity

Jason R. Hunt and Jason A. Carlyon[*],[†]

Department of Microbiology and Immunology, Virginia Commonwealth University (VCU) Medical Center, School of Medicine, VCU, Richmond, VA 23298, USA

[*]**Corresponding author:** Department of Microbiology and Immunology, VCU Medical Center, School of Medicine, VCU, Richmond, VA 23298, USA. Tel: 804-628-3382; E-mail: jason.carlyon@vcuhealth.org

One sentence summary: This study identifies and functionally validates promoter elements in *Orientia tsutsugamushi*.

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[†]Jason A. Carlyon, https://orcid.org/0000-0003-2778-5066

ABSTRACT

Orientia tsutsugamushi is an obligate intracellular bacterium that causes scrub typhus, a potentially fatal rickettsiosis, and for which no genetic tools exist. Critical to addressing this technical gap is to identify promoters for driving expression of antibiotic resistance and fluorescence reporter genes in *O. tsutsugamushi*. Such promoters would need to be highly conserved among strains, expressed throughout infection, and exhibit strong activity. We examined the untranslated regions upstream of *O. tsutsugamushi* genes encoding outer membrane protein A (*ompA*), 22-kDa type-specific antigen (*tsa22*) and *tsa56*. The bacterium transcribed all three during infection of monocytic, endothelial and epithelial cells. Examination of the upstream noncoding regions revealed putative ribosome binding sites, one set of predicted −10 and −35 sequences for *ompA* and two sets of −10 and −35 sequences for *tsa22* and *tsa56*. Comparison of these regions among geographically diverse *O. tsutsugamushi* patient isolates revealed nucleotide identities ranging from 84.8 to 100.0%. Upon examination of the candidates for the ability to drive green fluorescence protein expression in *Escherichia coli*, varying activities were observed with one of the *tsa22* promoters being the strongest. Identification and validation of *O. tsutsugamushi* promoters is an initial key step toward genetically manipulating this important pathogen.

Keywords: *Orientia tsutsugamushi*; scrub typhus; *Rickettsia*; promoter; *Rickettsiales*

INTRODUCTION

Obligate intracellular bacterial pathogens have significantly impacted human health throughout history and continue to pose substantial public health threats. Their evolutionarily reliance on eukaryotic cells makes them excellent models for studying host–pathogen interactions and for uncovering novel aspects of host cell biology (McClure *et al.* 2017). Among all species in the six genera that infect humans (*Anaplasma*, *Chlamydia*, *Coxiella*, *Ehrlichia*, *Orientia* and *Rickettsia*), *Orientia tsutsugamushi* is the deadliest (Xu *et al.* 2017). Trombiculid mites are the primary reservoir for the bacterium and transmit it to vertebrate animals and humans. *Orientia tsutsugamushi* infection in humans results in scrub typhus, a disease long known to be endemic to the Asia-Pacific (reviewed in Xu *et al.* 2017; Luce-Fedrow *et al.* 2018). Notably, recent reports of non-travel related cases of scrub typhus in Chile, Peru and the United Arab Emirates indicate that the disease is endemic to South America and the Middle East (Izzard *et al.* 2010; Weitzel *et al.* 2016, 2019; Kocher *et al.* 2017; Abarca *et al.* 2020). Phylogenetic analyses of isolates recovered from patients in Chile and Dubai identified two novel species, *Candidatus Orientia chiloensis* and *Candidatus Orientia chuto*, respectively (Izzard *et al.* 2010; Abarca *et al.* 2020). The reservoirs and vectors of these two new species are unknown. *Orientia tsutsugamushi* invades dendritic cells, monocytes and macrophages at the mite bite site, after which it is presumed that the infected leukocytes disseminate via the lymphatics (Paris *et al.* 2012). The bacterium then egresses to infect endothelial cells of the skin and other major

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organs. Scrub typhus clinical manifestations include nonspecific flu-like symptoms, fever, rash, eschar at the mite bite site, headache, myalgia, cough, lymphadenopathy, nausea, vomiting and abdominal pain. Severe complications can occur and include multiorgan failure, acute respiratory distress syndrome, interstitial pneumonia, myocarditis, pericarditis, meningoencephalitis and disseminated vascular coagulation (Luce-Fedrow *et al.* 2018). In the absence of antibiotic therapy or when it is delayed, the scrub typhus median mortality rate of 6% can be as high as 70% (Xu *et al.* 2017). Pharmacologic treatment options are limited, immunity is short-lived and there is no preventative vaccine (Xu *et al.* 2017; Luce-Fedrow *et al.* 2018; Wongsantichon *et al.* 2020).

Scrub typhus molecular pathogenesis is poorly understood. Conspicuously, mutational approaches for nonessential genes have been developed for species of all obligate intracellular genera except *Orientia* (reviewed in McClure *et al.* 2017). Hence, there is a major technical gap in the ability to study this global health threat. Gene disruption by plasmid-based allelic exchange or transposon-mediated insertion of antibiotic resistance and fluorescence reporter cassette would allow for selection of mutants and identification/recovery of host cells containing the mutants. These feats have been successfully performed for *Rickettsia*, *Anaplasma*, *Ehrlichia*, *Coxiella* and *Chlamydia* spp. (Baldridge *et al.* 2005; Felsheim *et al.* 2006; Liu *et al.* 2007; Binet and Maurelli 2009; Driskell *et al.* 2009; Baldridge *et al.* 2010; Felsheim *et al.* 2010; Clark *et al.* 2011; Beare 2012; Beare *et al.* 2012; Chen *et al.* 2012; Cheng *et al.* 2013; Beare and Heinzen 2014; Crosby *et al.* 2014, 2015, 2020; Kokes *et al.* 2015; Noriea, Clark and Hackstadt 2015; Oliva Chávez *et al.* 2015; Mueller, Wolf and Fields 2016; McKuen *et al.* 2017; Mueller, Wolf and Fields 2017; Wang *et al.* 2017; Keb, Hayman and Fields 2018; LaBrie *et al.* 2019; Wang *et al.* 2019; Wang *et al.* 2020; Arroyave *et al.* 2021; O'Conor *et al.* 2021). For optimal expression, in addition to codon-optimizing the antibiotic resistance and reporter genes for the high AT content of the *O. tsutsugamushi* chromosome (Nakayama *et al.* 2008), their expression would have to be driven from an *O. tsutsugamushi* promoter. In this study, we sought to identify suitable *O. tsutsugamushi* promoters of genes that are expressed throughout infection, are conserved among clinically relevant isolates that are geographically diverse and/or commonly used in laboratory studies, and exhibit strong activity. The findings presented herein are a first step toward developing genetic tools for this understudied pathogen.

METHODS

Cultivation of uninfected and *O. tsutsugamushi* infected cells

HeLa 229 human cervical epithelial cells (CCL-2; American Type Culture Collection [ATCC], Manassas, VA) and THP-1 cells (TIB-202; ATCC) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented 2 mM L-glutamine (ThermoFisher Scientific, Waltham, MA) and 10% fetal bovine serum (FBS; Gemini Bio-Products, Sacramento, CA, USA) at 35°C in a humidified incubator with 5% CO_2. RF/6A monkey choroidal endothelial cells (CRL-1780; ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; ThermoFisher Scientific) supplemented with 10% FBS, 2 mM L-glutamine, 19 MEM nonessential amino acids (Invitrogen) and 15 mM HEPES. *Orientia tsutsugamushi* str. Ikeda was maintained in HeLa cells as described for uninfected cells except that it had 1× Anti-Anti (ThermoFisher Scientific) and the FBS concentration was 1%, the latter of which slowed host cell growth to allow for a high percentage (>90%) of infection to be achieved by 3–4 days. To obtain *O. tsutsugamushi* for experimental use, infected HeLa cells were mechanically disrupted by adding glass beads and shaking the culture flasks followed by differential centrifugation at 250 × *g* for 5 min to remove intact cells and cellular debris. The resulting supernatant was centrifuged at 2739 × *g* for 10 min to recover *O. tsutsugamushi* for use in infection studies. In infection experiments, *O. tsutsugamushi* infected and uninfected control cells were maintained in the appropriate media containing 1% FBS.

Synchronous infection, RNA isolation and reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

HeLa, THP-1 and RF/6A cells were synchronously infected with *O. tsutsugamushi* at a multiplicity of infection (MOI) of 10 as verified by assessing duplicate coverslips using antiserum targeting the bacterium's TSA56 (56-kDa type-specific antigen) (Beyer *et al.* 2017) and immunofluorescence microscopy. The cells, which had been seeded onto coverslips and infected, were washed with PBS and then fixed and permeabilized with –20°C methanol at 2 h postinfection. Blocking in 5% (vol/vol) bovine serum albumin (BSA) in PBS was performed followed by successive incubations with rabbit anti-TSA56 (1:1000) and Alexa Fluor 488-conjugated goat anti-rabbit (Invitrogen, Carlsbad, CA, 1:1000) in 5% BSA. Blocking and antibody incubations were performed for 1 h at room temperature with three PBS washes between each step. Samples were incubated with 0.1 μg ml^{-1} 4′6-diamidino-2-phenylindole (DAPI; Invitrogen) in PBS for 1 min, washed three times with PBS and mounted with ProLong Gold Antifade mounting media (Invitrogen). Coverslips were viewed with an Olympus BX51 spinning disc confocal microscope (Olympus, Shinjuku City, Tokyo, Japan) for enumeration of the mean number of *O. tsutsugamushi* per cells. Cells were scored for immunosignal subcellular localization by counting 100 cells per coverslip. Triplicate infected cultures in 25-cm^2 flasks were washed at 2 h to remove unbound bacteria. Fresh media was added and the cells were placed in a humidified incubator with 5% CO_2 and set to 35°C. Total RNA was isolated at 24, 48 and 72 h using the RNeasy Mini Kit (Qiagen, Germantown, MD). One microgram RNA was treated with amplification grade DNase (Invitrogen). cDNA was generated using iScript Reverse Transcription Supermix according to the manufacturer's protocol (Bio-Rad, Hercules, CA). To verify successful removal of genomic DNA, parallel reactions performed in the absence of reverse transcriptase were used as template for PCR with human *GAPDH*-specific primers (Rodino *et al.* 2019) and MyTaq polymerase (Bioline, Taunton, MA). After an initial denaturing step at 95°C for 1 min, thermal cycling conditions were 35 cycles of 95°C for 15 s, 55°C for 15 s and 72°C for 10 s, followed by a final extension at 72°C for 30 s. Amplicons were analyzed with 2.0% agarose gels in 40 mM Tris-acetate-2 mM EDTA (pH 8). qPCR using cDNA from synchronously infected cells as template was performed with SsoFast EvaGreen supermix (Bio-Rad) and primers targeting *O. tsutsugamushi* 16S rDNA (*ott16S*) nucleotides 911–1096 (VieBrock *et al.* 2014), *ompA* nucleotides 57–260 (Evans *et al.* 2018a), *tsa22* nucleotides 339–509 (Table 1) and *tsa56* nucleotides 21–176 (Table 1). *Orientia tsutsugamushi* gene-specific primers were designed according to the annotated Ikeda str. genome (GenBank accession NC_010793.1) (Nakayama *et al.* 2008). Thermal cycling conditions used were 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 55°C for 5 s. Relative expression was determined using the $2^{-\Delta\Delta CT}$ method (Livak and

Table 1. Oligonucleotides utilized in this study.

Designation[a]	Sequence (5′–3′)
tsa22-339F	TGTAGCTGCAGGCATACAAAC
tsa22-509R	GCGCTAGCAGCTTGAAGTTTAG
tsa56-21F	TGCTAGTGCAATGTCTGCGT
tsa56-176R	GCATCAGCTGAATCCAAGCG
P*ompA*-fl BamHI F[b]	**GACTGC**GGATCCATCTAGTTTAAAGGCGATTTTAAAAAAAATTG
P*ompA*-fl EcoRI R[b]	**GACTGC**GAATTCACTTGCTTATCCATCAAAATATTTAAAATG
P*tsa22*-fl BamHI F[b]	**GACTGC**GGATCCAGCCTGTTTTAATATTTAGCTAAC
P*tsa22*-fl EcoRI R[b]	**GACTGC**GAATTCAAAGATAATTCCTTATAATATTAGTAATACTTAAAAATATAC
P*tsa22*-down BamHI F[b]	**GACTGC**GGATCCCATGATATATGATAGAGGATCAATAC
P*tsa22*-up BamHI, EcoRI F[b]	**GACTGC**GGATCCAGCCTGTTTTAATATTTAGCTAACTAAAAGAAATATTTTGTAAGATAATACAGTATATGT ATAATGAATTCGCAGTC
P*tsa22*-up EcoRI, BamHI R[b]	**GACTGC**GAATCCATTATACATATACTGTATTATCTTACAAAATATTTCTTTTAGTTAGCTAAATATT AAAACAGGCTGGATCCGCAGTC
P*tsa56*-fl BamHI F[b]	**GACTGC**GGATCCTTTTTATGTGGGCTAATTTTAG
P*tsa56*-fl EcoRI R[b]	**GACTGC**GAATTCTCTAATCTCCTTAAAAGAATTAAATTTATTTTTTAG
P*tsa56*-down BamHI F[b]	**GACTGC**GGATCCAAATAAAAATAAATTTTACAATGGATAAAAC
P*tsa56*-up EcoRI R[b]	**GACTGC**GAATTCTTTATATATAACTTAAAGACAACATTCAATAG
pPROBEseqF	TAAACTGCCAGGAATTGGGGA
pPROBEseqR	CACCCTCTCCACTGACAGAAAA

[a]F and R refer to primers that bind to the sense and antisense strands, respectively. The terms down and up refer to each of the two putative promoter elements that are closest and furthest, respectively, from the start codon. P, putative promoter; fl, full length.
[b]Boldface text indicates extra nucleotides upstream of restriction sites. Restriction sites are underlined.

Schmittgen 2001) as part of the CFX Maestro for Mac 1.0 software package (Bio-Rad).

In silico analyses

Noncoding regions upstream of the *ompA*, *tsa22* and *tsa56* initiation codons were assessed for the presence of sequences exhibiting similarity to the consensus *Escherichia coli* −10 and −35 RNA polymerase binding sites using BPROM (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb) (Solovyev and Salamov 2011). The upstream regions were defined as the nucleotides beginning at the −1 position relative to the initiation codon and extending upstream until the next open reading frame of each annotated chromosome. CLUSTAL Ω (Madeira *et al.* 2019) was used to generate alignments of and calculate % nucleotide identities among the *ompA*, *tsa22* and *tsa56* upstream regions of *O. tsutsugamushi* strains Boryong (NC_009488.1), Gilliam (NZ_LS398551.1), Karp (NZ_LS398548.1), Kato (NZ_LS398550.1), TA686 (NZ_LS398549.1), UT76 (NZ_LS398552.1), UT176 (NZ_LS398547.1) and Wuj/2014 (NZ_CP044031.1) relative to Ikeda.

Promoter constructs

pPROBE-NT was a gift from Dr Steven Lindow (Addgene plasmid # 37818; http://n2t.net/addgene:37818; RRID:Addgene 37818). The 5′ untranslated regions (UTRs) upstream of the *O. tsutsugamushi* str. Ikeda *ompA*, *tsa22* and *tsa56* start codons were PCR amplified using primers listed in Table 1, Platinum Taq DNA Polymerase High Fidelity (ThermoFisher, Waltham, MA), and DNA that had been isolated from *O. tsutsugamushi* str. Ikeda infected HeLa cells using the DNeasy Blood and Tissue kit (Qiagen, Valenica, CA) according to the manufacturer's protocol. The primers utilized contained BamHI or EcoRI sites for the purpose of cloning into pPROBE-NT. After an initial denaturing step at 94°C for 2 min, thermal cycling conditions were 30 cycles of 94°C for 15 s, 53°C for 30 s and 68°C for 30 s, followed by a final extension at 68°C for 5 min. The resulting amplicons were purified using the NucleoSpin Gel and PCR Clean-up Kit (Machery-Nagel, Bethlehem, PA), digested with BamHI and EcoRI (New England Biolabs, Ipswich, MA), and subsequently cloned using T4 DNA ligase (New England Biolabs) into pPROBE-NT that had been digested with BamHI and EcoRI. The recombinant plasmids were transformed into chemically competent Stellar *E. coli* HST08 cells (Takara, Mountain View, CA) followed by the addition of SOC (super optimal broth with catabolite repression) medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) (Takara) and incubation at 37°C with agitation at 250 RPM for 1 h. Aliquots of each culture were plated onto Luria–Bertani (LB) agar plates containing 50 μg ml^{-1} kanamycin and incubated at 37°C overnight. Colony PCR using vector-specific primers pPROBEseqF and pPROBEseqR (Table 1) was performed to identify colonies that harbored plasmids containing inserts of the expected size. Plasmids were isolated from PCR-positive colonies using the QIAprep Spin Miniprep Kit (Qiagen). Recombinant plasmid insert integrity was confirmed by sequence analysis (Genewiz, South Plainfield, NJ).

Promoter activity assays

To qualitatively assess for the abilities of selected putative promoter *O. tsutsugamushi* sequences to drive green fluorescence protein (GFP) expression, *E. coli* HST08 (Takara Bio, Mountain View, CA) transformed with pPROBE-NT constructs were streaked onto LB agar containing 50 μg ml^{-1} kanamycin. After overnight incubation at 37°C, the plates were imaged under blue light illumination using the ChemiDoc Touch Imaging System (Bio-Rad, Hercules, CA). To quantitatively measure GFP expression, single colonies of *E. coli* HST08 transformants containing the recombinant pPROBE-NT plasmids or empty vector were inoculated into LB broth containing 50 μg ml^{-1} kanamycin and grown aerobically under constant agitation at 37°C to an optical density of 0.4 at 600 nm. Five lakh cells were pelleted, washed

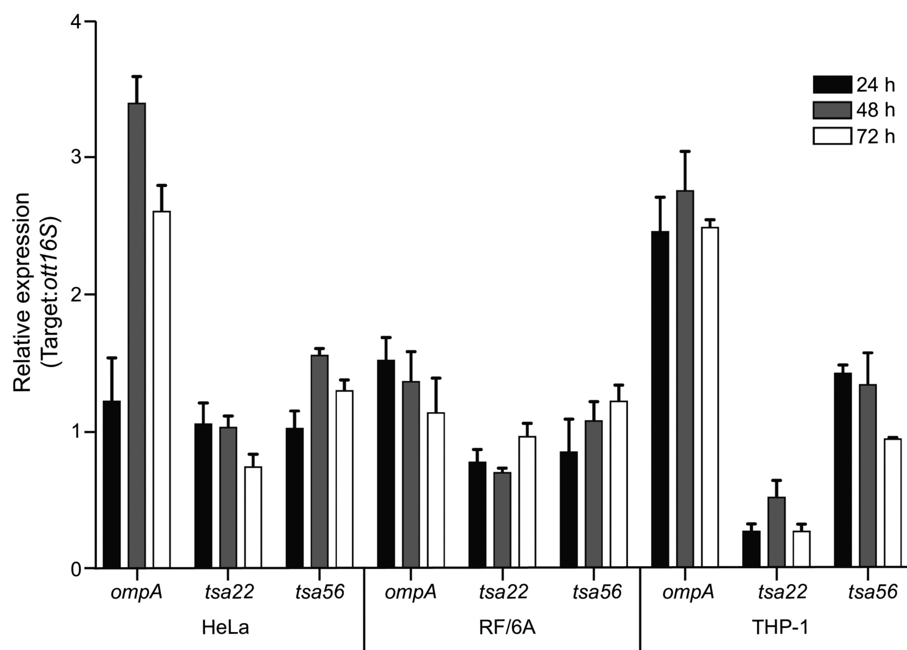

Figure 1. *Orientia tsutsugamushi* transcriptionally expresses *ompA*, *tsa22* and *tsa56* during infection of mammalian host cells. HeLa, RF/6A or THP-1 cells were synchronously infected with *O. tsutsugamushi* (Ot) followed by collection of total RNA at 24, 48 or 72 h. RT-qPCR was performed using gene-specific primers. Relative *ompA*-, *tsa22*- and *tsa56*-to-Ot 16S rRNA gene (*ott16S*) expression was determined using the $2^{-\Delta\Delta CT}$ method. Data are mean values ± SD from three experiments performed in triplicate.

three times with phosphate buffered saline (PBS; 1.05 mM KH_2PO_4, 155 mM NaCl, 2.96 mM Na_2HPO_4, pH 7.4) and lysed with radioimmunoprecipitation assay buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA [pH 8]) containing Halt Protease and Phosphatase Inhibitor Cocktail (ThermoFisher Scientific, Waltham, MA). Lysates were resolved by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) in 4–15% TGX polyacrylamide gels (Bio-Rad), transferred onto nitrocellulose membrane and screened by western blot analysis as described (VieBrock *et al.* 2014). Antibodies used were rabbit anti-GFP (Invitrogen, Carlsbad, CA; catalog #A6455) and horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling, Danvers, MA; #7074S) at concentrations of 1:1000 and 1:10 000, respectively. Blots were imaged using the ChemiDoc Touch Imaging System. Anti-GFP immunosignal intensities were quantified using the Image Lab 6.0 software package (Bio-Rad). Duplicate gels that were stained with Coomassie Brilliant Blue R250 (Bio-Rad) were also imaged for total protein. The GFP immunosignal was normalized to total protein signal per sample.

Statistical analyses

Statistical analyses were performed using the Prism 7.0 software package (GraphPad, San Diego, CA). Two-way analysis of variance with Tukey's *post-hoc* test was used to test for significant differences among the groups. *P*-values < 0.05 were considered statistically significant.

RESULTS

Orientia tsutsugamushi expresses *ompA*, *tsa22* and *tsa56* throughout infection of mammalian host cells

An *O. tsutsugamushi* promoter being considered for expressing antibiotic resistance and fluorescence reporter genes should first be confirmed to drive expression of its endogenous gene throughout infection. Genes encoding OmpA (outer membrane protein A), TSA22 (22-kDa type-specific antigen) and TSA56 were selected as candidates for promoter activity analysis because *O. tsutsugamushi* expresses all three proteins during infection of tissue culture cells, laboratory mice and/or scrub typhus patients (Lee *et al.* 2008; Chao *et al.* 2009; Cho *et al.* 2010; Lin *et al.* 2012; Beyer *et al.* 2017; Ha *et al.* 2017). The *tsa22* gene was also chosen because its 5′ untranslated region was shown to be highly conserved among seven strains and prior to this study was the only putative *O. tsutsugamushi* promoter sequence that had been assessed for conservation (Ge *et al.* 2005). Another reason for our inclusion of *ompA* was its demonstrated 93.6–100.0% nucleotide identity among 51 isolates (Evans *et al.* 2018a). *Orientia tsutsugamushi* str. Ikeda, which was isolated from a patient in Japan, causes severe disease and has a fully annotated genome (Tamura *et al.* 1984; Nakayama *et al.* 2008), was used for these studies. Total RNA that had been isolated from synchronously infected HeLa (epithelial), RF/6A (endothelial) and THP-1 (monocytic) cells at 24, 48 and 72 h was subjected to RT-qPCR analyses using gene-specific primers. All three were expressed at all time points examined in each cell line (Fig. 1), indicating that their promoters are active throughout *O. tsutsugamushi* infection of mammalian host cells.

Predicted *ompA*, *tsa22* and *tsa56* promoters are conserved among *O. tsutsugamushi* isolates

The noncoding regions upstream of *O. tsutsugamushi* str. Ikeda *ompA*, *tsa22* and *tsa56* were examined to identify sequences similar to *E. coli* −10 and −35 RNA polymerase binding sites and potential ribosome binding site (RBS) sequences. The bacterial σ70 promoter prediction program, BPROM (Solovyev and Salamov 2011), identified one set of −10 and −35 sequences beginning at 53 nucleotides upstream of the *ompA* start codon (Fig. 2A). BPROM identified two pairs of promoter elements

(A) ***ompA***

ATCTAGTTTAAAGGCGATTTTAAAAAAAATTGCCTTTAAATATCAATTGTTAATTTAAAAGTTAAATATT
AAGTTTAGAAGTAATTAGCTTAGTATTTAGCAACATTAATTACGTATAATGAAGATT**TTTATT**AATTTTT
TTTTGGTTAGAT**ATGTAAAAT**AGTTGCAATACTGCAATTACTACATTTTAAATATTTTGATGGATAAGCA
AGT**ATG**ATTAAAAAGTCAATTATTAGTATATGTGTATTAGTGCTACTTTCTGGTTACAAGTGGAAATGTT

(B) ***tsa22***

AGCCTGTTTTAATATTTAGCTAACTAAAAGAAATATT**TTGTAA**GATAATACAGTAT**ATGTATAAT**CATGA
TATATGATAGAGGATCAATACAACTGAAACAATATTAGTTA**TTGATA**ACTGCATAATATTT**AAGTATATT**
TTTAAGTATTACTAATATTATAAGGAATTATCTTT**ATG**AGTAAAGAAGCAACAGAACTCAAAGAAAGTAT

(C) ***tsa56***

TTTTTATGTGGGCTAATTTTAGATAATACAATGTTAGTATAATTATGTGGTTAATTAATGTATCTTGATT
TAAGATTTTATATAAAATATAATTAAGACAGGGCTTTATTATTAGCTTGAAAAACTGTTGCTATTTTAGC
TAAAAATAAACTTTTATGCAAGAAAAATTATTAATAATTGAAGGTAGTTGTTGCGTAAAAGTGGTGTTA
TGCTATCTAAGGTTAACTGTAACAAGGTGCTAATAGATAATTAATGTATTTTCGAACGTGTCTTTAAACA
TATATATAAGAGCAGTGTTCTA**TTGAAT**GTTGTCTTTAAGTT**ATATATAAA**AAATAAAAATAAATTTTAC
AATGGATAAAACGCTTTGAAGCGATATTTTTAACACAGTGTTTTATAGATTGTTTAAATTATTTTACAAG
TACTATTAAATATTAGTATACTAAATAATAGTTTTTTGATATAAAACTAAG**TTAGTG**TGGCTAAATAATT
A**GTTTAGAAT**GGTTACCACTAAAAAATAAATTTAATTCTTTTAAGGAGATTAGA**ATG**AAAAAAATTATGT

Figure 2. The UTR of *ompA*, *tsa22* and *tsa56* each contains a predicted RBS and −10 and −35 sequences. Upstream sequences of *ompA* **(A)**, *tsa22* **(B)** and *tsa56* **(C)** were evaluated for the presence of RBS and −10 and −35 sequences. Initiation codons are indicated by white boldface text and purple highlighting. Putative RBS sequences are underlined. (A) The *ompA* UTR −10 and −35 sequences are denoted by boldface text. The two sets of −10 and −35 sequences in the UTRs of *tsa22* (B) and *tsa56* (C), which are designated 'down' and 'up' based on proximity to the start codon, are indicated by blue and red boldface text, respectively. Nucleotides cloned into pPROBE-NT for assessing promoter activity of *tsa22*-down and *tsa56*-down are highlighted gray, while those for evaluating promoter activity of *tsa22*-up and *tsa56*-up are highlighted yellow.

	−35	−10
E. coli	**TTGACA**	**TATAAT**
A. phagocytophilum tr1	TG**TTGCAT**TACTATTATGTATGATT--	**TATCCT**
E. chaffeensis p28-14	AC**TTGCTT**TTCTTTATTTCTTTCAT--	**TATTCT**
E. chaffeensis p28-19	AC**TTGCTT**TTATATGACACTTCTAC--	**TATTGT**
R. rickettsii rOmpA	TA**ATAGACA**TATTAAAAAAGTTGCGT-	**TATAAC**
R. rickettsii rOmpB	TG**CTTGTAA**TTATATACAATATATAG-	**TATGCT**
O. tsutsugamushi ompA	TT**TTTATT**AATTTTTTTTTGGTTAGAT	**ATGTAAAAT**
O. tsutsugamushi tsa22-down	TA**TTGATA**ACTGCATAATATTT-----	**AAGTATATT**
O. tsutsugamushi tsa22-up	TT**TTGTAA**GATAATACAGTAT------	**ATGTATAAT**
O. tsutsugamushi tsa56-down	AG**TTAGTG**TGGCTAAATAATTA-----	**GTTTAGAAT**
O. tsutsugamushi tsa56-up	TA**TTGAAT**GTTGTCTTTAAGTT-----	**ATATATAAA**

Figure 3. Comparison of −10 and −35 regions of O. *tsutsugamushi ompA*, *tsa22* and *tsa56* with those of other *Rickettsiales* species and *E. coli*. The −10 and −35 sequences are indicated by boldface text. Dashes were introduced to enable alignment with the portion of the O. *tsutsugamushi ompA* UTR sequence depicted.

beginning at 36 and 111 nucleotides upstream of the *tsa22* translational start site and 45 and 214 bases upstream of the *tsa56* initiation codon (Fig. 2B and C). Putative RBS sequences (Steitz and Jakes 1975) were detected seven to nine nucleotides upstream of the *ompA*, *tsa22* and *tsa56* start codons (Fig. 2). To differentiate between the respective *tsa22* and *tsa56* promoter candidates (P*tsa22* and P*tsa56*), the elements closest to the start codon were assigned the designation 'down' (P*tsa22*-down and P*tsa56*-down), and those further upstream were given the designation 'up' (P*tsa22*-up and P*tsa56*-up) (Table 2). Among the five promoter candidates, the −10 regions differed by 1 to 5 nucleotides and the −35 regions differed by 2 to 4 nucleotides relative to each other (Fig. 3). The distance between the −10 and −35 sequences was identical for the P*tsa22*-down, P*tsa56*-down and P*tsa56*-up, but was one nucleotide shorter for P*tsa22*-up and five nucleotides longer for P*ompA*. The five O. *tsutsugamushi* −10 and −35 sequences were similar to those that had been identified in other *Rickettsiales* species and validated for activity in *E. coli* (Policastro and Hackstadt 1994; Barbet *et al.* 2005; Peddireddi, Cheng and Ganta 2009), although the similarity was more discernible with the *Rickettsia rickettsii* sequences than with those for *Anaplasma phagocytophilum* or *Ehrlichia chaffeensis*. The predicted promoter regions for O. *tsutsugamushi* str. Ikeda were compared with corresponding sequences for the Boryong (South Korea), Gilliam (Burma), Kato (Japan) and Karp (New

Table 2. Putative O. *tsutsugamushi* Ikeda str. promoter regions assessed in this study.

Promoter designation	Nucleotide range[a]	Length	Genome coordinates
P*ompA*	−1 to −213	213	1338189 to 1338401
P*tsa22*-down	−1 to −110	110	1578808 to 1578917
P*tsa22*-up	−111 to −175	65	1578743 to 1578807
P*tsa56*-down	−1 to −213	213	988304 to 988516
P*tsa56*-up	−214 to −544	331	987973 to 988303

[a]Nucleotide range is relative to the initiation codon of each gene.

Table 3. Nucleotide identities of putative promoters among *O. tsutsugamushi* strains.

Strain	Genome coordinates	Length	Percent identity[a]
PompA			
Boryong	1771158 to 177364	207	84.79
Gilliam	442789 to 442967	179	91.67
Karp	261035 to 261245	211	97.66
Kato	315245 to 315458	214	99.53
TA686	1656491 to 1656693	203	88.26
UT76	469266 to 469480	215	94.50
UT176	141889 to 142103	216	94.50
Wuj/2014	1335472 to 1335684	213	95.37
Ptsa22-down			
Boryong	24947 to 25056	110	99.09
Gilliam	22852 to 22961	110	100.00
Karp	19481 to 19590	110	96.36
Kato	72271 to 72380	110	100.00
TA686	2061082 to 2061191	110	99.09
UT76	21335 to 21445	111	99.10
UT176	347754 to 347863	110	100.00
Wuj/2014	965889 to 965999	111	99.10
Ptsa22-up			
Boryong	24883 to 24946	64	93.85
Gilliam	22787 to 22851	65	96.92
Karp	19417 to 19480	64	90.77
Kato	72381 to 72445	65	100.00
TA686	2062017 to 2061081	65	98.46
UT76	21446 to 21510	65	100.00
UT176	348864 to 347928	65	100.00
Wuj/2014	965824 to 965888	65	100.00
Ptsa56-down			
Boryong	581099 to 581311	213	100.00
Gilliam	2036332 to 2036544	213	99.06
Karp	1873572 to 1873785	214	97.66
Kato	860782 to 860994	213	100.00
TA686	2031496 to 2031708	213	99.06
UT76	1676193 to 1676405	213	100.00
UT176	840850 to 841062	213	97.65
Wuj/2014	463969 to 464181	213	100.00
Ptsa56-up			
Boryong	581312 to 581645	334	98.20
Gilliam	2036002 to 2036331	330	97.89
Karp	1873238 to 1873571	334	92.84
Kato	860995 to 861325	331	100.00
TA686	2031707 to 2032041	335	91.45
UT76	1675864 to 1676192	329	98.19
UT176	841063 to 841394	332	97.60
Wuj/2014	463640 to 463968	329	98.19

[a]Identity relative to *O. tsutsugamushi* str. Ikeda.

Guinea) strains along with Karp isolates UT76, UT176 (both from northeastern Thailand) and the Wuj/2014 isolate (China), each of which were recovered from scrub typhus patients (Bengtson 1945; Rights and Smadel 1948; Shishido *et al.* 1958; Chang *et al.* 1990; Blacksell *et al.* 2008; James *et al.* 2016; Yao *et al.* 2020). Nucleotide identities ranging from 84.79 to 100.00% were observed (Table 3).

Evaluation of promoter activities of the sequences upstream of the *ompA*, *tsa22* and *tsa56* coding regions

Functional activity of the candidate promoter regions was evaluated using an approach similar to those used to validate *R. rickettsii*, *E. chaffeensis* and *Anaplasma* spp. promoters (Policastro and Hackstadt 1994; Barbet *et al.* 2005; Peddireddi, Cheng and Ganta 2009). *PompA*, *Ptsa22*-down, *Ptsa22*-up, *Ptsa56*-down and *Ptsa56*-up were examined. The entire noncoding sequences upstream of *tsa22* and *tsa56* containing both the 'down' and 'up' promoter elements, referred to as *Ptsa22*-fl (full length) and *Ptsa56*-fl, respectively, were also tested. Each sequence was inserted upstream of the green fluorescent protein (*gfp*) open reading frame in pPROBE-NT followed by examination of GFP expression by recombinant *E. coli* transformed with each construct. Qualitative assessment of LB agar plates streaked with bacteria under blue light illumination revealed that all of the promoter regions drove relatively strong GFP expression with the exception of *Ptsa56*-fl, which exhibited GFP signal that was comparable to the background level observed for *E. coli* transformed with empty vector (Fig. 4A). To quantitatively measure promoter activity, GFP antibody was used to probe Western-blotted whole cell lysates. GFP signal was normalized to total protein signal of duplicate Coomassie brilliant blue-stained samples. Consistent with the qualitative assessment, the *Ptsa56*-fl-driven GFP level was barely above the background of empty pPROBE-NT (Fig. 4B). The six other promoters exhibited activities that were significantly greater than the vector control, thereby confirming their functional activity. Of these six, *PompA* exhibited the weakest activity. Notably, *Ptsa22*-up displayed the most robust promoter activity, as the GFP level for this transformant significantly exceeded those of the other five including *Ptsa22*-fl. These results functionally validate promoter activities of noncoding regions upstream of three *O. tsutsugamushi* genes and signify *Ptsa22*-up as the strongest.

DISCUSSION

Orientia tsutsugamushi and other *Orientia* species are the etiologic agents of scrub typhus, an infection that can be debilitating, has a high fatality rate and is an emerging global health threat (Xu *et al.* 2017; Luce-Fedrow *et al.* 2018). Their obligate intracellular lifestyle makes them challenging to study, as deleting or disrupting essential genes would prevent recovery of the mutants. Clever approaches have been applied to studying *O. tsutsugamushi* that circumvent its genetic intractability including using fluorescent probes to label and track live bacteria during infection of tissue culture cells, validating the ability of adhesin candidates to mediate binding to/infection of host cells when heterologously expressed on the *E. coli* surface, and ectopically expressing oriential effectors and mutated versions thereof in host cells to determine if they phenocopy aspects of infection, identify eukaryotic interaction partners and discern their functional roles (Ha *et al.* 2011; VieBrock *et al.* 2014; Atwal *et al.* 2016; Beyer *et al.* 2017; Evans *et al.* 2018b; Nguyen *et al.* 2021). Still, the development of genetic tools for *Anaplasma*, *Chlamydia*, *Coxiella*, *Ehrlichia* and *Rickettsia* spp. has arguably enabled greater strides in understanding the molecular pathogenesis of these organisms. For instance, random transposon insertional mutagenesis and allelic exchange, some cases of which were followed up by genetic complementation, facilitated discovery of new virulence factors and immunopathological molecules, genes required for optimal fitness or survival in host cells and mammals, and novel vaccine targets (Binet and Maurelli 2009; Driskell *et al.* 2009; Chen *et al.* 2012; Cheng *et al.* 2013; Crosby *et al.* 2015, 2020; Oliva Chávez *et al.* 2015; Mueller, Wolf and Fields 2016; McClure *et al.* 2017; McKuen *et al.* 2017; LaBrie *et al.* 2019; Wang *et al.* 2020; Arroyave *et al.* 2021; O'Conor *et al.* 2021). These approaches involve

Figure 4. Promoter activity evaluation of *ompA*, *tsa22* and *tsa56* upstream noncoding regions. Putative promoter regions of *tsa56* (P*tsa56*-fl [full length], P*tsa56*-down [dn], P*tsa56*-up, *ompA* [P*ompA*] and *tsa22* [P*tsa22*-fl, P*tsa22*-down and P*tsa22*-up]) were cloned upstream of promoter-less *gfp* in pPROBE-NT. The recombinant plasmids or empty pPROBE-NT (control) were transformed into *E. coli*. **(A)** LB agar plate streaked with *E. coli* transformed with the indicated constructs imaged under blue light illumination to detect GFP fluorescence. **(B)** Western-blotted whole cell lysates of *E. coli* transformed with the indicated vector were screened with GFP antibody. **(C)** Mean ± GFP signal intensity of triplicate samples each normalized to total protein signal of duplicate Coomassie brilliant blue stained gels. Data in panels (B) and (C) are representative of three experiments with similar results. Statistically significant (*$P < 0.05$; ***$P < 0.003$; ****$P < 0.0001$) values are indicated. Black asterisks denote GFP signal values that are statistically significant versus control, whereas green asterisks indicate statistical significance of P*tsa22*-up GFP signal compared with those produced from other *O. tsutsugamushi* promoter sequences.

chromosomal insertion of a cassette that expresses an antibiotic resistance gene that permits mutant selection and often a fluorescence reporter that enables visualization of mutants and recovery of host cells harboring them. The essential first step in constructing such cassettes is to identify promoters that will drive selection and reporter gene expression in the transformed bacterium. The driving rationale for this study was to identify promoters in *O. tsutsugamushi* that can ultimately be used in constructs for genetically manipulating this important pathogen.

Orientia tsutsugamushi infects leukocytes and endothelial cells during mammalian infection and a variety of cell lines *in vitro*, including HeLa cells, which are commonly used to study host interactions with the pathogen (VieBrock *et al.* 2014; Giengkam *et al.* 2015; Beyer *et al.* 2017; Evans *et al.* 2018a; Rodino *et al.* 2019). Any promoter used for selection and reporter marker expression would need to be active throughout infection of host cells. All three candidates assessed—*ompA*, *tsa22* and *tsa56*—met this criterion as they were expressed at throughout infection of HeLa, THP-1 and RF/6A cells. Examination of the UTRs upstream of each revealed that they are highly conserved among geographically diverse *O. tsutsugamushi* isolates. This is important because it suggests that cassettes/constructs harboring these promoters could be used to drive gene of interest expression in multiple *O. tsutsugamushi* strains. Examination of the *ompA*, *tsa22* and *tsa56* noncoding regions revealed the presence of a putative RBS in each and a total of five potential sets of −10 and −35 sequences that were similar to the consensus *E. coli* sequences and to several that had been previously confirmed in other *Rickettsiales* members (Policastro & Hackstadt 1994; Barbet *et al.* 2005; Peddireddi, Cheng and Ganta 2009).

In the absence of being able to directly assess functionality of the candidate promoter regions in *O. tsutsugamushi*, each was examined for the ability to drive promoter-less GFP expression in *E. coli*. Notably, this and similar approaches using *E. coli* as a heterologous host validated promoters in *Anaplasma*, *Ehrlichia* and *Rickettsia* that were able to be subsequently used to drive selection marker and fluorescence reporter gene expression when transpositionally inserted into the chromosomes of these bacteria (Policastro and Hackstadt 1994; Baldridge *et al.* 2005; Barbet *et al.* 2005; Felsheim *et al.* 2006; Peddireddi, Cheng and Ganta 2009; Baldridge *et al.* 2010; Felsheim *et al.* 2010; Clark *et al.* 2011; Chen *et al.* 2012; Cheng *et al.* 2013; Crosby *et al.* 2015, 2020; Oliva Chávez *et al.* 2015; Wang *et al.* 2017, 2020; O'Conor *et al.* 2021). This precedent highlights that promoters identified herein should be functional if inserted into the *O. tsutsugamushi* chromosome. P*tsa22*-up drove GFP expression significantly greater than all other candidates, denoting it as the top choice for inclusion in constructs for future genetic manipulation studies. P*tsa22*-down, P*tsa22*-fl, P*tsa56*-up and P*tsa56*-down also exhibited strong promoter activities and therefore also remain in contention for inclusion in future studies. As P*tsa56*-fl exhibited no promoter activity, it has been eliminated from further consideration. Likewise, P*ompA* is no longer being considered because, in addition to exhibiting relative weak promoter activity, its nucleotide identity among the isolate sequences examined ranged from 84.79 to 95.47% relative to Ikeda, whereas the identities of the P*tsa22* and P*tsa56* promoters ranged from 90.77 to 100%.

In closing, this study identifies a total of five *O. tsutsugamushi* promoter elements that exhibit strong activity when transformed into *E. coli*, drive relatively consistent gene expression throughout infection of three different mammalian host cell types and exhibit high degrees of nucleotide identity among diverse strains. By satisfying each of these criteria, they represent excellent candidates for driving selection marker and reporter expression when inserted into the *O. tsutsugamushi* chromosome and are a foundation on which genetic manipulation approaches for this understudied pathogen can be built.

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