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Transcriptional profiling of *Rickettsia prowazekii* coding and non-coding transcripts during *in vitro* host-pathogen and vector-pathogen interactions

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ABSTRACT

Natural pathogen transmission of *Rickettsia prowazekii*, the etiologic agent of epidemic typhus, to humans is associated with arthropods, including human body lice, ticks, and ectoparasites of eastern flying squirrel. Recently, we have documented the presence of small RNAs in *Rickettsia* species and expression of *R. prowazekii* sRNAs during infection of cultured human microvascular endothelial cells (HMECs), which represent the primary target cells during human infections. Bacterial noncoding transcripts are now well established as critical post-transcriptional regulators of virulence and adaptation mechanisms in varying host environments. Despite their importance, little is known about the expression profile and regulatory activities of *R. prowazekii* sRNAs (*Rp*_sRs) in different host cells encountered as part of the natural life-cycle. To investigate the sRNA expression profile of *R. prowazekii* during infection of arthropod host cells, we employed an approach combining *in vitro* infection, bioinformatics, RNA sequencing, and PCR-based quantitation. Global analysis of *R. prowazekii* transcriptome by strand-specific RNA sequencing enabled us to identify 67 *cis*-acting (antisense) and 26 *trans*-acting (intergenic) *Rp*_sRs expressed during the infection of *Amblyomma americanum* (AAE2) cells. Comparative evaluation of expression during *R. prowazekii* infection of HMECs and AAE2 cells by quantitative RT-PCR demonstrated significantly higher expression of four selected *Rp*_sRs in tick AAE2 cells. Examination of the coding transcriptome revealed differential up-regulation of > 150 rickettsial genes in either HMECs or AAE2 cells and yielded evidence for host cell-dependent utilization of alternative transcription start sites by 18 rickettsial genes. Our results thus suggest noticeable differences in the expression of both *Rp*_sRs as well as the coding transcriptome and the exploitation of multiple transcription initiation sites for select genes during the infection of human endothelium and tick vector cells as the host and yield new insights into rickettsial virulence and transmission mechanisms.

1. Introduction

Pathogenic bacteria in the genus *Rickettsia* belong to two major groups, namely spotted fever and typhus, which continue to pose significant health threats to humans across the globe. *Rickettsia prowazekii*, the causative agent of epidemic typhus, is an obligate intracellular, Gram-negative bacterium transmitted primarily by the body lice (*Pediculus humanus corporis*). Consequently, outbreaks of epidemic typhus tend to occur with conditions of crowding in close quarters and compromised hygiene during the times of war, famine, or natural disasters and the disease is also known as camp/famine/jail fever. During human infections, vascular endothelial cells lining the small and medium-sized blood vessels are the primary targets of infection and salient features of disease pathogenesis include vascular inflammation/dysfunction and perturbation of the vasculature's barrier function manifesting as altered permeability and fluid leakage from the intravascular compartment to the interstitium (Bechah et al., 2008b; Walker and Ismail, 2008). Considered to be one of the most severe forms of human rickettsioses, epidemic typhus due to *R. prowazekii* is associated with high mortality rates, in particular during the absence of

Abbreviations: AAE2, *Amblyomma americanum* cells; CDS, coding sequence; *gltA*, citrate synthase gene; HMECs, human microvascular endothelial cells; MEV, mean expression value; *Rp*_sR, *Rickettsia prowazekii* small ribonucleic acid; RPKM, reads per kilobase per million; TEX, terminator 5′-phosphate-dependent exonuclease; TPM, transcripts per kilobase million; TSS, transcription start site

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appropriate sanitation and timely intervention with antibiotics-based therapies (Bechah et al., 2008a; Raoult et al., 2004; Uchiyama, 2012). Also, the recrudescent form of epidemic typhus or Brill-Zinsser disease can manifest in patients years after the primary infection and clinical recovery (Bechah et al., 2008a; Uchiyama, 2012), and such a reoccurrence can lead to new cases or outbreaks of epidemic typhus. Although human body lice are the established principal vectors, ectoparasites (fleas and lice) of the flying squirrel maintain *R. prowazekii* in the sylvatic cycle. The presence of *R. prowazekii* in *Amblyomma* ticks from Mexico and *Ixodes* ticks in the Netherlands has also been demonstrated recently, suggesting the possibility of tick transmission in natural infections (Bozeman et al., 1975; Medina-Sanchez et al., 2005; Philip et al., 1966).

Once thought to be junk DNA, bacteria encode small regulatory RNAs (sRNAs) that act as critical post-transcriptional regulators of gene expression. These sRNAs typically range from 50 to 500 nucleotides and regulate a variety of processes such as environmental sensing, metabolism, stress responses, and virulence in pathogenic bacteria. The major families of sRNAs include true antisense RNAs originating from the 'opposite' complementary strand to the mRNA (*cis*-acting), sRNAs that act by limited complementarity base pairing with their targets (*trans*-acting), and sRNAs that exhibit binding interactions with proteins affecting their activity. *Trans*-acting sRNAs are encoded within the intergenic regions and act on target RNAs located elsewhere in the genome. In essence, such sRNAs are akin to eukaryotic microRNAs in their ability to modulate the activity and stability of multiple mRNAs (Gottesman and Storz, 2011; Liu and Camilli, 2010). Unlike *cis*-acting sRNAs, *trans*-acting sRNAs display only partial nucleotide complementarity and generally require an RNA chaperone to facilitate interactions with their targets (Waters and Storz, 2009).

Using a bioinformatics-based approach, we recently predicted the presence of over 1700 *trans*-acting sRNAs in the genomes of 16 different strains encompassing 13 rickettsial species (Schroeder et al., 2015). Using infection of cultured human microvascular endothelial cells (HMECs) with *R. prowazekii*, we further identified expression of 35 novel *trans*-acting and 23 *cis*-acting sRNAs through Next Generation Sequencing and confirmed expression of four novel *R. prowazekii* sRNAs (named *Rp*_sRs) along with the highly conserved and known bacterial sRNAs, namely α-tmRNA, RNaseP_bact_a, *ffs*, and 6S RNA (Schroeder et al., 2016). The present study was undertaken to conduct a compare and contrast analysis of the expression of *R. prowazekii* transcriptome during infection of human and tick host cells. Our results enable identification of additional *Rp*_sR candidates uniquely expressed during infection of tick cells and suggest differential expression of select *Rp*_sRs in tick vis-à-vis human host cells. In addition, a comprehensive analysis of encoding rickettsial transcriptome during infection of human and tick vector host cells yields the first evidence for the utilization of multiple transcription start sites depending on the host niche.

2. Materials and methods

2.1. Rickettsia prowazekii and cell culture

Stocks of *R. prowazekii* strain Breinl were prepared by infecting Vero cells cultured in DMEM supplemented with 2% fetal bovine serum in an atmosphere of 95%O_2: 5% CO_2 at 35 °C following standard protocols (Rydkina et al., 2005). Rickettsiae were purified by differential centrifugation, titered by a quantitative PCR-based assay, and stored at −80 °C as aliquots until use (Labruna et al., 2004). Considering that repeat freeze-thaw cycles may alter rickettsial viability and transcriptome, all experiments were performed using *R. prowazekii* stocks gently thawed on ice for the first time. Human dermal microvascular endothelial cells (HMECs) were cultured at 37 °C with 5% CO_2 in MCDB131 medium supplemented with 10% fetal bovine serum, 10 mM L-glutamine, 1 μg/ml hydrocortisone, and 10 ng/ml epidermal growth factor as previously described (Schroeder et al., 2015, 2016). The use of human cell lines in our study was exempt by the University of Texas Medical Branch (UTMB) Institutional Review Board (IRB), but approved by the UTMB Institutional Biosafety Committee (IBC). *Amblyomma americanum* tick cells (AAE2) were grown in L–15B complete medium (pH 7.5) at 34 °C to ~90% confluence. Approximately 24 h prior to infection, the medium in each flask was replaced with L–15B infection medium (pH 7.5) containing 25 mM sodium bicarbonate and HEPES (Munderloh and Kurtti, 1989). *In vitro* infection of HMECs with *R. prowazekii* stocks was carried out at 37 °C or 34 °C according to our standard protocols and procedures (Rydkina et al., 2005; Narra et. al., 2016). To achieve a comparable level of infection, AAE2 cells were infected with different stocks of *R. prowazekii* and incubated at 34 °C. At 24 h, the cells were gently scraped and pelleted by centrifugation at 400*g* for 5 min. The cell pellet was washed twice with sterile phosphate buffered saline (PBS) and processed for total DNA extraction using DNeasy Blood & Tissue kit (Qiagen). The MOI was estimated by absolute quantification using gene specific primers (Supplementary Table 1) targeting tick calreticulin and *R. prowazekii* citrate synthase (*gltA*) genes. For RNA-Seq experiments, HMECs were infected with *R. prowazekii* at an MOI of 5:1 (~6×10^4 pfu of rickettsiae per cm^2) in minimal volume of MCDB131 medium and incubated at room temperature for 15 min with gentle, intermittent rocking to enhance adhesion and invasion. The inoculation medium was then replaced with fresh medium and the cells were incubated for 24 h at 37 °C, 5% CO_2 (Schroeder et al., 2015, 2016). For AAE2 cells, the L–15B medium (containing viable, semi-adherent cells) from the culture flask was collected to pellet the non-adherent cells by centrifugation at 400*g* for 5 min. The pellet was then suspended in 1 mL of L–15B infection medium and added back to the adherent cells within the same flask. The cells were then infected with *R. prowazekii* at an MOI of 5:1 based on the estimation of infectivity titers as described above and gently rocked at room temperature for 15 min, at which time the medium containing rickettsiae was replaced with fresh medium as described above and the flasks were placed in an incubator at 34 °C for 24 h. At the end of incubation, the medium was completely removed and total RNA was extracted by the Tri-Reagent method detailed below. Each RNA-Seq experiment was performed on two independent biological replicates.

2.2. RNA isolation and sequencing

Isolation of total RNA from HMECs and AAE2 cells infected with *R. prowazekii* was carried out at 24 h using our standard Tri-Reagent (Molecular Research Center) protocol for deep sequencing. Total RNA was treated with DNaseI (Zymo Research) to remove any contaminating genomic DNA, and the samples were further processed using Dynabeads® Oligo $(dT)_{25}$ (ThermoFisher Scientific) and Ribo-Zero (Epicentre) to remove any interfering eukaryotic mRNAs and ribosomal RNAs, respectively. The enriched total RNA preparations thus obtained were quantified using the MultiSkan Go Microplate Spectrophotometer (ThermoScientific) and assessed for their quality on an Agilent 2100 Bioanalyzer (Agilent Technologies).

Subsequently, the total RNA from each biological replicate was divided into two equal aliquots. One aliquot was treated with Terminator 5′-Phosphate-dependent Exonuclease (TEX) (Epicentre) resulting in the degradation of processed RNA transcripts containing the 5′ monophosphate (+TEX) but not the primary transcripts with 5′ triphosphate. The other aliquot served as untreated control and contained both processed and primary transcripts (-TEX). Independent cDNA libraries for each aliquot were generated using the TruSeq RNA Sample Prep Kit (Illumina) as per manufacturer's directions. Strand-specific sequencing on non-size selected cDNA libraries was performed on an Illumina HiSeq 1500 at our institutional Next Generation Sequencing Core facility. The sequencing libraries were comprised of 100 base long reads in a FASTQ format. The quality of each read was assessed and any base with a PHRED score < 15 was excluded from the analysis. The first 14