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Transcriptional response of *Rickettsia conorii* exposed to temperature variation and stress starvation

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Abstract

Rickettsia conorii is an obligate intracellular bacterium transmitted to humans by *Rhipicephalus sanguineus* ticks. The success of this microorganism at surviving in nature implicates the ability to efficiently adapt to different environments, including the arthropod vector and the mammalian host. Numerous bacterial species possess a highly evolved system for stress adaptation. This so-called stringent response is mediated by guanosine 3′,5′-bispyrophosphate and guanosine 3′-diphosphate 5′-triphosphate which are under *spoT* control in some Gram-negative bacteria. Interestingly, annotation of the *R. conorii* genome evidenced 5 *spoT* paralogs. We hypothesized that these *spoT* genes play a role in adaptation to environmental changes specifically encountered by rickettsiae during their different life cycles. Transcription of the *spoT* paralogs was examined by RT-PCR from infected Vero cells maintained in rich or deficient culture media, from infected C6/36 insect cells cultured at various temperatures and from infected ticks. Our results demonstrated that the 5 *spoT* genes can be transcribed. *SpoT1* (RC0374) is only transcribed upon stringent response. Transcription of *spoT3* (RC0888) was never observed in arthropod cells or ticks, but was specific to *R. conorii* RNA extracted from infected Vero cells. These results indicate that rickettsial *spoT* paralogs are independently transcribed, depending on the different infected hosts and the adaptive capacity of the pathogen. Bioinformatics analysis of these possibly encoded proteins is also reported.

Keywords: Rickettsiae; Transcription; Starvation; (p)ppGpp

1. Introduction

Rickettsia conorii, the causative agent of Mediterranean spotted fever, is a tick-borne pathogen spread by *Rhipicephalus sanguineus*, the brown tick of dogs [20]. During its life cycle, *R. conorii* survival involves adaptation to different environments including physiological and nutrient changes between hosts. Stress conditions encountered by *R. conorii* within the tick include starvation and temperature shifts. These bacteria are probably subjected to periods of starvation within their tick hosts when the ticks molt to the next stage and wait for weeks to months until the next blood meal. During the tick blood meal, rickettsiae undergo a variety of physiological changes and multiply intensively [30]. While the tick is attached to warm skin and imbibes a rich blood meal, the rickettsiae undergo reactivation from a dormant avirulent state to a highly pathogenic one [27]. Exposure to thermal [4] or nutritional factors [31] or both for 24–48 h is required for reactivation of rickettsial pathogenicity.

Molecular mechanisms implicated in adaptation of *R. conorii* to different host conditions and in reactivation of virulence are unknown. As intracellular parasites, rickettsiae have small genomes and an evolutionary tendency towards further genomic reduction [1,17]. Therefore, genes found as multiple copies may outline their specific adaptation. Among these is *spoT* for which 5 copies were identified [17].

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Microarray analysis performed on *Borrelia burgdorferi*, another tick-borne pathogen causing Lyme disease, demonstrated that *spoT* (BB0198) [10] is upregulated approximately 2.0-fold when bacteria are grown under conditions analogous to either those found in fed ticks or during mammalian host adaptation relative to unfed tick experimental conditions [21]. This gene is also expressed in *B. burgdorferi* within 2.5 min after the initiation of serum starvation and during incubation in the saliva of the tick vector *Ixosides scapularis* [8]. These results suggest that *spoT* intervenes in the stringent response encountered by *B. burgdorferi* in unfed ticks and which probably results from limited nutriment availability [29]. Indeed, it is effectively well established that this gene intervenes in the stringent response of various bacteria [6]. This global bacterial stress has been characterized as a short-term response to amino acid and carbon starvation and is brought about by the failure of tRNA aminoacetylation to keep up with the demand for protein synthesis. Stringent response is mediated by the accumulation of the alarmone guanosine 3′,5′-bispyrophosphate and guanosine 3′-diphosphate 5′-triphosphate termed collectively (p)ppGpp [11,13]. As a result of (p)ppGpp accumulation, bacteria shut down processes that consume amino acids and energy reserves, including RNA and DNA synthesis, and this is accompanied by slow growth [7]. In *Escherichia coli* and other Gram-negative bacteria, synthesis of (p)ppGpp involves 2 proteins RelA and SpoT, while in many Gram-positive bacteria only one protein with both RelA and SpoT activities regulates its metabolism. Indeed, SpoT is a bifunctional enzyme capable of both (p)ppGpp synthesis and degradation [11]. The three-dimensional structure of the catalytic domains of the RelA/SpoT homologue from *Strepotococcus dysgalactiae* subsp. *equisimilis* (Rel_{Seq}) has been recently determined [12]. This bifunctional enzyme fragment contains a hydrolase and a synthetase domain at its N and C-terminus, respectively. The structural data revealed precise ligand-residue as well as cofactor–residue interactions.

The presence of multiple copies of *spoT* in the *R. conorii* genome encouraged us to follow expression of these genes within the major stages of the life cycle of the bacterium which were experimentally mimicked. The sequences of these potentially encoded proteins were then compared with Rel/Spo family homologs among which Rel_{Seq} and analyzed for both (p)ppGpp hydrolase and synthetase activity domain.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A confluent monolayer of African green monkey kidney cells (Vero cell, ATCC C1587) in Eagle's minimum essential medium (MEM, Gibco, Invitrogen, Paisley, UK) supplemented with 4% fetal calf serum (FCS, Gibco) and 2 mM L-glutamine (Gibco) was inoculated with *R. conorii* strain seven (ATCC, VR613) as previously described [23,24]. When the degree of infection was optimal, as estimated by Gimenez staining, rickettsiae were harvested and purified through a 25% sucrose gradient as previously described [22]. The number of bacteria was then estimated by flow cytometry using a microcyte (BioDETECT, Oslo, Norway). Approximately 6.55×10^5 bacteria were used to infect one 25-cm^2 flask of confluent Vero cells. Infected cells were maintained at 32 °C for 48 h, then washed with phosphate buffer saline (PBS, BioMérieux, Marcy–l'Etoile, France) and incubated with MEM supplemented with 4% FCS and 2 mM L-glutamine (rich medium) or with PBS only (nutritional stress). Cells were harvested at time zero (before medium change), 1, 2, 6, 24 and 48 h after medium change. When rickettsiae were exposed to temperature variation, *Aedes albopictus* cells (C6/36) in Leibovitz L-15 (Gibco) supplemented with 10% FCS and 2% Difco tryptose phosphate (Becton Dickinson Microbiology Systems, Sparks, MD) were used as host cells. Confluent C6/36 in 25-cm^2 flasks were inoculated with approximately 4.4×10^5 purified bacteria and maintained at 32 °C for 48 h. Cells were then subjected to a variation in temperature: 10, 32 and 37 °C, and harvested at time zero (before temperature shift), 1, 7, 14, 31 and 38 days after the temperature shift. After 38 days at 10 °C, cells were incubated at 37 °C and harvested at 3 h and 3 days later, respectively. Infected cells harvested at each time point were centrifuged (12 000 g, 10 min) and pellets immediately frozen in liquid nitrogen before storage at –80 °C. In some experiments, RNA-protect bacterial reagent (Qiagen, Hilden, Germany) was added before centrifugation (5000 g, 10 min, 4 °C) to prevent possible RNA degradation. Each protocol, i.e., nutritional stress and temperature variation, was carried out in triplicate and RT-PCR assays were performed twice.

2.2. Infection of ticks

All *R. sanguineus* ticks used in this study were derived from colonies of non-infected ticks maintained since 2002 at the Unité des Rickettsies, Marseille, France. For molecular studies, ticks were artificially infected with *R. conorii* by the capillary feeding method [15]. Briefly, 8 partially fed (day 4 post-attachment) virgin female *R. sanguineus* were forcibly but gently detached from the rabbit host and restrained on double-sided sticky tape in the bottom of a large Petri dish. Modified microcapillary tubes (10 ml) containing a PBS solution of 10^8 rickettsiae/ml were placed over the hypostome of each tick and immobilized as described. Ticks were allowed to feed from microcapillary tubes for up to 24 h in an incubator in a humid chamber at 32 °C. After removing the tube, ticks were placed in an incubator at 25 °C and 80% humidity. At day 6 post-infection, ticks were tested for rickettsial infection by the hemolymph test [5,18]. Hemolymph was obtained by severing the coxatrochanteral joint of leg 2 of ticks previously washed with iodinated alcohol and rinsed with distilled water. A droplet of hemolymph was smeared on a slide glass and stained using Gimenez staining.

Ticks (7/8) which tested positive by Gimenez were immediately put in RNA*later*–RNA stabilization reagent (Qiagen). Following 12 h incubation at 4 °C samples were stored at −80 °C.

2.3. RNA extraction

Total RNA was isolated from an aliquot of frozen *R. conorii*-infected Vero or C6/36 cells using the RNeasy mini-kit (Qiagen) according to the manufacturer's instructions. Ticks infected as described above and stored at −80 °C were resuspended in lysis buffer and lysed 2 × 2 min using the Mixer Mill MM 300 (Qiagen) before extraction of homogenized samples with the RNeasy mini-kit (Qiagen). All RNA samples were treated with the RNase-free DNase set (Qiagen) for 30 min during the extraction procedure. The concentration and quality of isolated RNA were determined with an Agilent 2100 bioanalyzer using the RNA 6000 Nano Labchip kit (Agilent Technologies, Englewood, NJ, USA). Aliquots of the DNase-treated total RNA samples were stored at −80 °C until use.

2.4. RT-PCR

The RNA obtained was used for two separate reactions, one to synthesize cDNA and the other to check for possible DNA contamination. RT-PCR was performed from 2 µl of RNA (25 µl final reaction volume) with the Superscript one-step RT-PCR with platinum *Taq* (Invitrogen, Paisler, UK) and DNA contamination was assessed with the Expand high fidelity polymerase (Roche, Mannheim, Germany). Cycling conditions were 30 min at 50 °C, 5 min at 95 °C, and 40 cycles at 30 s at 95 °C, 30 s at 50 °C, 1 min at 72 °C followed by a final extension cycle of 7 min at 72 °C. The RT-PCRs were conducted on the PTC-100 thermocycler (MJ Research, Inc.). The primers used for RT-PCR are described in Table 1. From the *R. conorii* genome database (http://www.igs-server.cnrs-mrs.fr/mgdb/Rickettsia, [17]), we designed forward and reverse primers amplifying a segment with no more than 500 bp and with an annealing temperature of 50 °C. To confirm the specificity of these primers, their sequences were compared with other sequences included in the GenBank database with BLASTN through the National Center for Biotechnology Information server. The gene *rpsL* was used as control. Aliquots of PCR and RT-PCR amplification products were run on 2% (wt/vol) agarose gels, and the DNA was stained with ethidium bromide. The size of the PCR product was determined by comparison with DNA molecular weight marker VI (Boehringer Mannheim).

2.5. Sequencing

The amplicons obtained were purified (PCR purification kit, Qiagen) and their sequence was checked by DNA sequencing (ABI 310 automated sequencer, Perkin–Elmer, Warrington, UK) using the same primers as for RT-PCR.

2.6. Bioinformatic analysis of protein sequences

The multiple sequence alignment of *spoT*-encoded proteins was constructed with the use of T-Coffee [16].

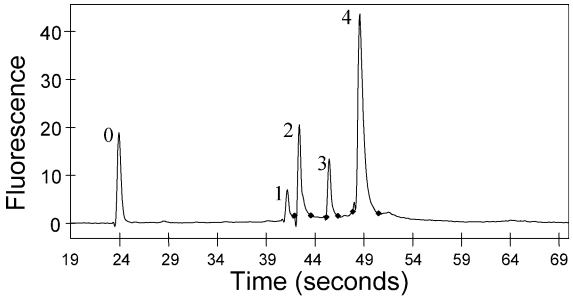
3. Results

3.1. Assessment of the quality and amount of RNA

RNA obtained from infected Vero cells was analyzed with a Bioanalyzer 2100. Resulting electrophoretograms showed intact 16S and 23S prokaryotic RNA with important host cell RNA contamination (Fig. 1). Thus, bacterial RNA corresponded to only 11% of the total RNA. The amount of bacterial RNA recovered from a 25-cm^2 flask of Vero cells infected with *R. conorii* for 48 h at 32 °C was estimated to be 4.1 ± 0.5 µg ($n = 3$). The concentration of rickettsiae present in the inoculum was estimated by flow cytometry counting, as indicated in Section 2. Their replication was not quantified in the course of infection. Therefore, the quantity of RNA purified was close for each time point, testifying to the reproducibility of bacterial growth kinetics. No DNA contamination was detected. For all extracted samples, negative

Table 1
Primers designed for *R. conorii* genes targeted in this study

Gene	Function	Sequence	Nucleotidic position (bp)	Length (bp)
SpoT1 (RC0374)	(p)ppGpp 3-pyrophosphorylase	Forward primer: 5′-ACTTTTATGGAAGATTTAAGCTC-3′ Reverse primer: 5′-ATAATCGCTACTTCAATCGGGTG-3′	374375–374596	221
SpoT2 (RC0426)	(p)ppGpp 3-pyrophosphorylase	Forward primer: 5′-AGCTGTAAATATTCAGATCAATTAATC-3′ Reverse primer: 5′-TTCTAGTTAGACCCGCTACATGCC-3′	422215–422556	293
SpoT3 (RC0888)	(p)ppGpp 3-pyrophosphorylase	Forward primer: 5′-AATCCTTATTATTCGCACCCG-3′ Reverse primer: 5′-AAAGTCGCTTCAATAATTTTTCTAGC-3′	836759–837128	370
SpoT4 (RC1098)	(p)ppGpp 3-pyrophosphorylase	Forward primer: 5′-AAGTTTGAAATTTGTGTTTACGC-3′ Reverse primer: 5′-AGTATAATCGCTGCTTCGATCG-3′	1025221–1025410	169
SpoT5 (RC1099)	(p)ppGpp 3-pyrophosphorylase	Forward primer: 5′-ATGCTGCTCTACTTCCACTATAC-3′ Reverse primer: 5′-TTGCTTAATTAATAGGTTTAAG-3′	1025465–1025628	164
rpsL (RC0172)	30S ribosomal protein	Forward primer: 5′-ATATAATCAATTAGTACGTTTTG-3′ Reverse primer: 5′-ACGAGTACGATATGATATTTC-3′	171388–171679	291



Peak number	rRNA	Total surface (%)
1	16S	3.90
2	18S	12.5
3	23S	7.2
4	28S	38.4

Fig. 1. Representative profile of total RNA extracted from *R. conorii* infected Vero cells. Total RNA was extracted after 48 h infection at 32 °C and analyzed with the Bioanalyzer 2100 (Agilent). The amount of RNA analyzed corresponds to 1/400 of the total RNA extracted. Peak 0: standard; peaks 1 and 3: rickettsial rRNA; peaks 2 and 4: eukaryotic rRNA.

RT-PCR controls were processed in the absence of reverse transcriptase, while successful PCR amplification with DNA from purified *R. conorii* as template was considered as a positive control for the sensitivity and specificity of the gene-specific primer pairs listed in Table 1.

3.2. *Transcriptional profiles of* spoT *paralogs during starvation of* R. conorii *in host cells*

When Vero cells infected with *R. conorii* were submitted to nutritional stress (incubation in PBS), the signal intensity of *rpsL* (RC0172, S12 ribosomal protein) decreased from the 24th h and disappeared at the 48th h (Fig. 2A). Transcription of this gene involved in protein synthesis is expected to reflect the growth of rickettsiae. Transcription of the 5 paralogs of *spoT* was also measured from the same samples. Concerning four of these genes, namely, *spoT2* (RC0426), *spoT3* (RC0888), *spoT4* (RC1098) and *spoT5* (RC1099), the same transcription profiles as that described for *rpsL* were observed (not shown). Only *spoT1* (RC0374) presented variable transcription. As illustrated in Fig. 2A, *spoT1* (RC0374) was not transcribed under normal growth conditions. When cell cultures were subjected to starvation, this gene was up-regulated earlier from the first hour. When infected cells were maintained in a rich but unchanged medium this transcription was delayed at the 6th h. For all samples, identity of the obtained amplicon was confirmed by sequencing.

3.3. *Transcriptional profiles of* spoT *paralogs in* R. conorii *submitted to temperature variation*

These experiments were carried out using insect cells (C6/36) which, in contrast to Vero cells, can be maintained within a large range of temperatures. We thus kept C6/36 infected by *R. conorii* for 38 days at 10 °C. Under these conditions, bacteria were still viable, as demonstrated by *rpsL*

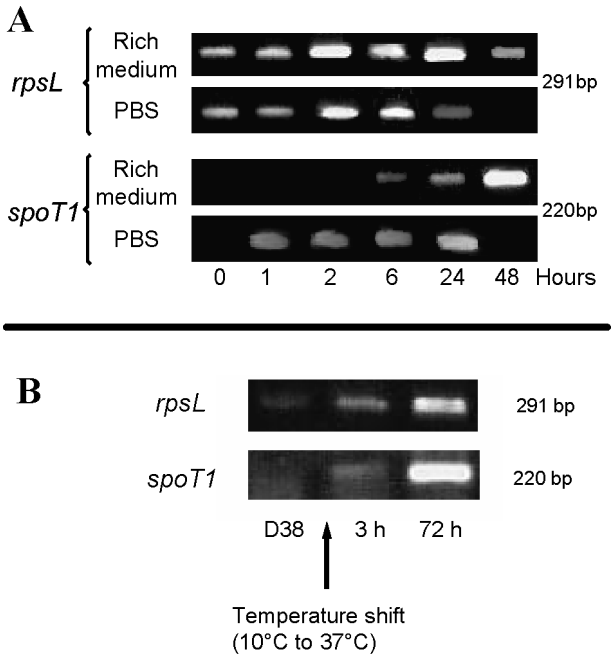


Fig. 2. RT-PCR analysis of *rpsL* and *spoT1* from *R. conorii* submitted to (A) nutritional stress or (B) temperature variation. (A) Vero cells infected for 48 h at 32 °C were washed and incubated either in a rich medium or in PBS up to 48 h. (B) Infected C6/36 cells were maintained for 38 days at 10 °C (D38) and then placed at 37 °C for 72 h. Size of the RT-PCR amplification products, i.e., 291 bp (*rpsL*) and 220 bp (*spoT1*), was checked by using marker VI (Boehringer) as standard. The same pattern was obtained from three distinct RNA extractions and RT-PCR was carried out twice on each sample.

transcription for which a signal was detected, though of a lower intensity than that measured at day 1 (Fig. 2B and Table 2). Concerning the *spoT* gene family, *spoT2, spoT4 and spoT5* were always transcribed and their transcription followed the same pattern as *rpsL*. In contrast, neither *spoT1* nor *spoT3* transcription was detected. In order to mime in part the reactivation process observed when *R. conorii* are injected from the vector into host cells, we induced a temperature shift from 10 to 37 °C. Rickettsiae were shown to be highly sensitive to such a variation, since *spoT1* transcription was observed early from the 3rd h with a strong intensity signal at 72 h (Fig. 2B). Gimenez staining of cells before and after the temperature shift showed that bacteria were far more numerous after the temperature shift to 37 °C, demonstrating intensive and rapid multiplication (not shown). Major transcription changes observed under the different experimental conditions tested and designed to mime different life stages of the bacteria are summarized in the Table 2.

3.4. *Transcriptional profiles of* spoT *paralogs in infected ticks*

A total of 7 ticks were confirmed to have been infected by *R. conorii* using capillary feeding, and their RNA was purified. Only three out of 5 *spoT* paralogs examined by RT-PCR

Table 2
Schematic representation of the environmental conditions encountered by *R. conorii* within the ticks or after human infection

	Diapause (Starvation)		Blood meal reactivation	Infected vertebrate host
Rickettsia growth	None or little		Intense multiplication	Intense multiplication
Temperature (°C)	5–37		From 5–10 to 32–37	37
Experimental model	Infected Vero cells grown at 32 °C in rich medium and then depleted in nutriments (PBS)	Infected C6/36 maintained at 10 °C for 38 days	Infected C6/36 shifted at 37 °C maintained at 10 °C for 38 days and then shifted at 37 °C	Infected Vero cells maintained at 37 °C in rich medium
Genes	Time of transcription profile determination			
Time-point of RT-PCR	Kinetics from 0 to 48 h after nutriment removal	On day 38	Kinetics for 7 days	48 h after infection
spoT1	Negative to upregulated in course of time	Negative	Rapidly upregulated in the course of time	Negative
spoT2	Positive at to downregulated in the course of time	+[a]	++[b]	++
spoT3		Negative	Negative	++
spoT4		+	++	++
spoT5		+	++	++
rpsL		+	++	++

During their life, *R. conorii* theoretically survive in 3 different situations described above. The experimental model was determined according to the different parameters (temperature, medium) of each of these stages. Transcription of *spoT* paralogs and *rpsL* was determined at different time points as described in Section 2. Each point was performed at least in duplicate.
[a] (+) detectable level of expression;
[b] (++) strong level of expression.

Table 3
Summary of RT-PCR results obtained from *R. conorii* infected ticks

Tick number	*rpsL* (RC0172)	*SpoT1* (RC0374)	*SpoT2* (RC0426)	*SpoT3* (RC0888)	*SpoT4* (RC1098)	*SpoT5* (RC1099)
1	–[a]	–	+[b]	–	+	+
2	–	–	+	–	–	+
3	–	–	++[c]	–	+	+
4	–	–	+	–	–	–
5	+	–	+	–	+	–
6	+	–	+	–	+	–
7	–	–	++	–	++	–

[a] (–) undetectable level of expression;
[b] (+) detectable level of expression;
[c] (++) strong level of expression.

were found to be transcribed by *R. conorii* in infected ticks. The expression of *spoT2* and *spoT4* was effective and did not differ significantly between the different samples when *spoT5* transcription was only observed in half of them. In contrast, *spoT1* and *spoT3* were never transcribed in ticks. Results obtained are presented in Table 3.

3.5. Sequence analysis of proteins likely to be encoded by spoT homologs

No homology was evidenced between the rickettsial-encoded SpoT proteins and residues 200–375 of the Rel_{Seq} protein (not shown). In contrast, as illustrated in Fig. 3, three SpoT sequences (SpoT1 to 3) from *R. conorii* were readily aligned with other hydrolase domain sequences including Rel_{Seq} (PDB 1VJ7) [12].

4. Discussion

In this study we have investigated transcription of the 5 rickettsial *spoT* paralogs from bacteria maintained either in eukaryotic cells submitted to various temperature and medium conditions or in ticks. The strict intracellular nature of these bacteria indicated a high background of host cell mRNA in samples; we first optimized RNA extraction and purification conditions to yield the highest RT-PCR sensitivity with the least background.

The natural environments of bacteria are often defined by changes in nutrient availability. When bacterial cells are deprived of an amino acid or carbon source, changes in numerous processes occur within the cells. In order to determine whether *spoT* expression can be regulated by such a stringent response, infected Vero cells were incubated for

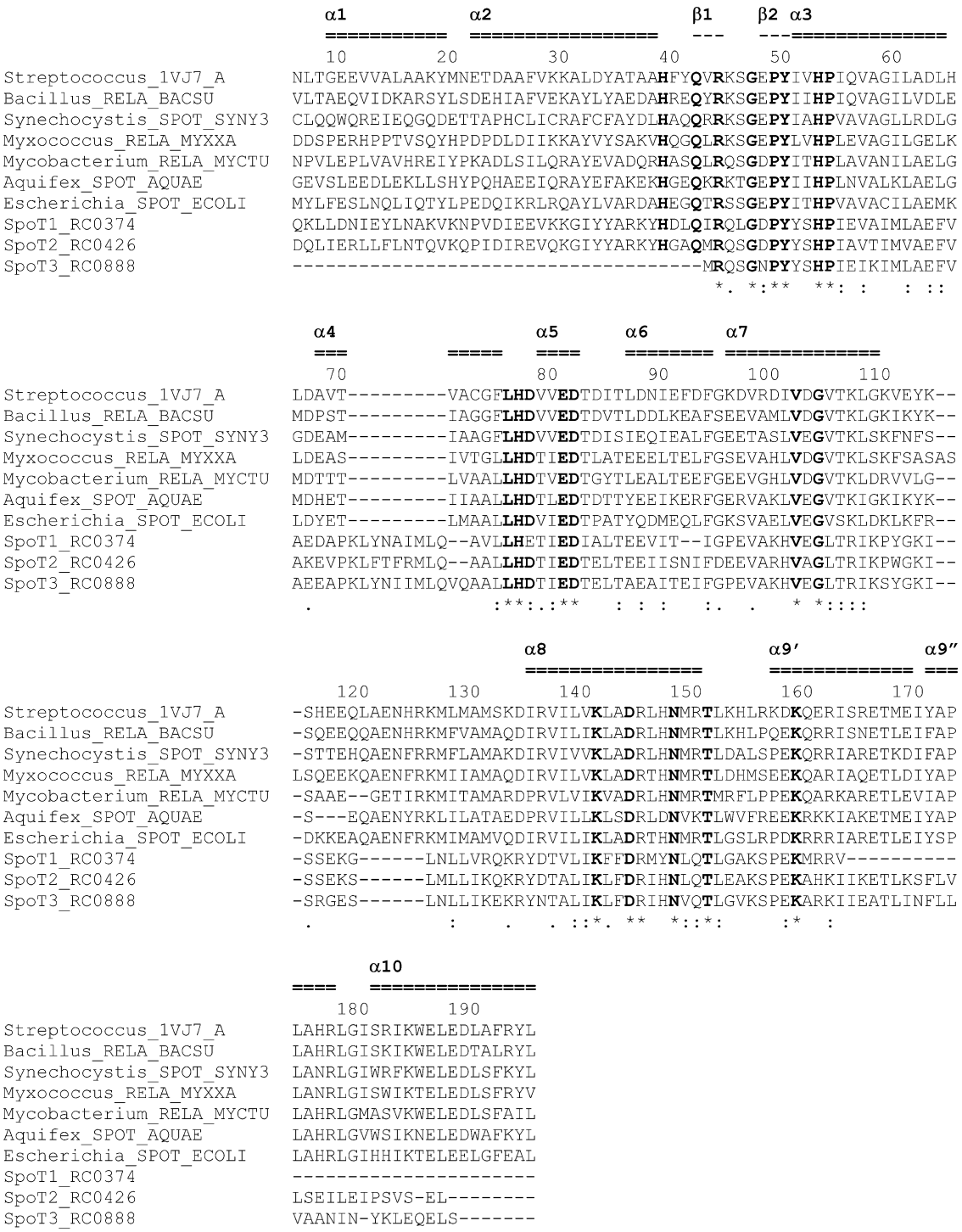


Fig. 3. Multiple sequence alignment of 3 *R. conorii* SpoT sequences and hydrolase domain sequences of the Spo/Rel family homologues from other species. The hydrolase domain sequence of *Strepotococcus dysgalactiae* subsp. *equisimilis* Rel_{Seq} is shown at the top with its residue numbers and secondary structure assignment (PDB 1VJ7). Four residues interacting with the catalytic Mn^{2+} ion are highlighted in red. Residues composing a cleft for the guanine base binding are highlighted by yellow. Other conserved residues are highlighted with gray background.

up to 48 h in PBS. Rickettsiae were also submitted to temperature variations using C6/36 (mosquitos) infected cells which can survive at 10 °C. Under such stress conditions, it was essential to control viability of rickettsiae. In theory, mRNA analysis should provide a good indication of bacterial viability, as mRNA persists for only short periods of time in actively metabolizing bacterial cells [2]. Caution must be exercised in the interpretation of negative signal data, as injured, stressed or otherwise viable but non-culturable bacterial cells [3] containing very low levels of mRNA can

persist but may not be detectable by the assay. Otherwise, the persistence of mRNA may vary greatly depending on the environmental conditions [26], the conditions of cell death [9,25] and the physiological state of the cell population before killing. The target choice can significantly affect the correlation with viability. The use of ubiquitously expressed sequences should ensure that the transcript is present under most assay conditions, and highly expressed targets may provide more sensitive analysis. In this respect, the transcription of the ribosomal protein gene *rpsL* was also investigated in this work. Positive *rpsL* transcription confirmed that *R. conorii* RNA was present in sufficient quantity and quality to allow for detection. Therefore, the *rpsL* signal was not observed in infected eukaryotic cells maintained for 38 days at 10 °C as in 5/7 infected ticks. This probably results from a low number of bacteria since, when eukaryotic cells were shifted to 37 °C the signal appeared, confirming their viability.

Results obtained showed that the only *spoT* gene presenting variable transcription according to nutritional stress and temperature conditions was *spoT1*. RT-PCR results obtained with specific primers designed to amplify this gene were negative when RNA was purified from rickettsiae in the logarithmic growth phase within Vero cells (48 h post-infection in rich medium). However, after nutriment removal, this gene was immediately transcribed (within the first h). When rickettsiae were maintained in the rich medium, *spoT1* transcription appeared later (from the 6th h). This could be a reaction to exhaustion of the medium. We then looked for *spoT1* transcription following 38 days at 10 °C. Under such conditions, which correspond to the temperature that *R. conorii* infected ticks may encounter in nature, this gene was not transcribed. According to *rpsL* transcription, bacteria were still viable. To mimic the reactivation phenomenon, these cells were then shifted to 37 °C. Under these conditions, *spoT1* transcription was effective and was paralleled by intensive bacterial replication. Indeed, temperature shifts and nutritional stress are tightly linked events, since growth of rickettsiae depends on temperature, and bacterial growth requires nutriment consumption. *spoT1* transcription under such experimental conditions should be a signal for limiting bacterial multiplication, thus allowing their long-term survival. In both experimental protocols, 3 other *spoT* paralogs were transcribed, namely, *spoT2*, *spoT4* and *spoT5*. Their pattern was shown to be similar to that of *rpsL*. Both *spoT4* and *spoT5* are split by insertion of the internal stop codon, fragmenting the intact ortholog in *Ureaplasma urealyticum* [17]. In a previous paper [17], we proposed the designation 'split genes' instead of 'pseudogenes', which are usually considered as inactive sequences of genomic DNA with sequences similar to known functional genes. *SpoT3* transcription seems to reflect a difference in adaptation of *R. conorii* to different hosts. This gene was consistently transcribed when rickettsiae were cultivated at 37 °C in Vero cells (mammalian epithelial cells), while no amplification signal was obtained from C6/36-infected cells (insect cells). This hypothesis was reinforced by data obtained from infected ticks from which *spoT3* transcription was negative, as for mosquito cells, suggesting that the response observed in arthropod cells might be specific.

While these 5 *spoT* paralogs can be transcribed, their translation as functionally active proteins remains to be demonstrated. From a preliminary proteomic analysis (bidimensional gel analysis coupled with mass spectrometry) of *R. conorii* grown within Vero cells, we identified the SpoT2 protein (unpublished data). This is an accordance with the transcription of the *spoT2* gene observed under "normal" culture conditions. Other SpoT proteins were not yet detected. Expression of these proteins does not mean that active sites are functional. In fact, topological analyses suggest that in contrast to Rel_{Seq}, and other Rel/Spo family homologues, none of these putative rickettsial SpoT proteins seem to possess (p)ppGpp synthetase activity. From the crystal structure of Rel_{Seq}, the synthetase domain of this 750 amino acid long protein was localized between residues 200–375 [12]. This region shares no homology with rickettsial-encoded SpoT proteins. Alternative sources of (p)ppGpp production may exist within rickettsiae, as evoked for *Streptococcus pyogenes* [28] and *Streptococcus mutans* [14]. From bioinformatics analysis it can be speculated that at least 3 *spoT*-encoded proteins might possess (p)ppGpp activity. These SpoT sequences (SpoT1 to 3) from *R. conorii* were readily aligned with other hydrolase domain sequences including Rel_{Seq}. Four residues binding to the essential Mn^{2+} cofactor are conserved in all three *R. conorii* SpoTs, except for SpoT1 that exhibits a glutamic acid residue instead of a conserved aspartic acid residue at position 78 of the Rel_{Seq} sequence. Residues composing a deep cleft for the binding of the guanine base are also highly conserved, including two of 4 residues (Lys45, Leu155, Arg44, Asn148, according to the numbering of Rel_{Seq}) directly interacting with the base. No homology was evidenced concerning SpoT4 and SpoT5. As previously mentioned, *spot4* and *spoT5* are contiguous ORFs interrupted by a STOP codon and classified as split genes [17]. It is conceivable that these genes are undergoing degradation but their transcription may have biological significance.

In summary, these preliminary experiments showed that environmental stress conditions are accompanied by variable *spoT1* transcription in *R. conorii*, a phenomenon which could intervene in adaptation of these bacteria to unfed ticks and in reactivation phenomena.

More work will be required to understand the function of *spoT* paralogs within rickettsiae and to characterize the stringent response of these pathogens. While genetic manipulation of rickettsiae has remained impossible for years [32], the first stable knock-out mutants of *R. prowazekii* were isolated a few months ago [19]. Such an approach could open the way for future investigations concerning the precise function of *spoT* genes within *Rickettsia*.

Acknowledgements

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