RESEARCH ARTICLE

Global gene expression profiling of *Ehrlichia ruminantium* at different stages of development

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Abstract

Ehrlichia ruminantium (*ER*), the causative agent of heartwater on ruminants, is an obligate intracellular bacterium transmitted by ticks of the genus *Amblyomma*. Previous studies have shown that early stages of development may be critical for *Ehrlichia* pathogenicity. To gain insights into the biology of intracellular *ER*, we determined the genome-wide transcriptional profile of *ER* replicating inside bovine aortic endothelial cells using DNA microarrays. At intermediate and late stages of infection (reticulate and elementary bodies, respectively), a total of 54 genes were differentially expressed. Among them, we measured by q-RTPCR the overexpression of 11 of 14 genes. A number of genes involved in metabolism, nutrient exchange, and defense mechanisms, including those involved in resistance to oxidative stress, were significantly induced in *ER* reticulate bodies. This is consistent with the oxidative stress condition and nutrient starvation that seem to occur in *Ehrlichia*-containing vacuoles. During the lysis stage of development, when *ER* is infectious, we showed the overexpression of a transcription factor, *dksA*, which is also known to induce virulence in other pathogens such as *Salmonella typhimurium*. Our results suggest a possible role of these genes in promoting *ER* development and pathogenicity.

Introduction

Ehrlichia ruminantium (*ER*) is a α-proteobacterium in the order *Rickettsiales* that is transmitted by *Amblyomma* ticks. This bacterium is the causal agent of heartwater, a fatal disease in ruminants (Allsopp, 2010). This disease represents a serious problem for livestock productivity in endemic areas such as sub-Saharan Africa and the Caribbean threatening the American continent where indigenous competent ticks are present (Barre *et al.*, 1987).

ER is an obligate intracellular pathogen that infects the endothelium of blood vessels. *ER* has a complex life cycle described as a *Chlamydia*-like developmental cycle (Jongejan *et al.*, 1991). In the early stage of the cycle, elementary bodies (EB), which represent the extracellular and infectious forms of the bacterium, adhere to host target cells and then are engulfed quickly. They remain within intracytoplasmic vacuoles, where they divide by binary fission to produce the vegetative intracellular noninfectious forms, the reticulate bodies (RB), after 2–4 days postinfection, and further intermediate bodies (IB) after 4–5 days. After 5–6 days, the disruption of host cells leads to the release of numerous infective EB, thus initiating a new infectious cycle.

From the sequencing of Gardel and Welgevonden strains, whole-genome *ER* microarrays were designed to analyze the *ER* transcriptome at different development stages (Frutos *et al.*, 2006; Emboule *et al.*, 2009). For *Rickettsia conorii*, the transcriptomic analysis highlighted the overexpressed genes involved in resistance to oxidative and osmotic stress (thioredoxin, *trxB2*, and proline–betaine transporter, *proP*), in DNA repair and recombination, and in some virulence factors (Renesto *et al.*, 2008).

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The aim of this study was to understand the *ER* development and pathogenicity. Sufficient amount of *ER* RNA from noninfectious and infectious forms of *ER* was produced using bovine endothelial cells cultured *in vitro* and infected with virulent strain. First, the upregulated genes between RB and EB stages of development were identified by microarray. Then, the differential gene expression between these stages was evaluated by q-RTPCR on 14 selected genes.

Materials and methods

Production of biological samples

Production of biological samples was performed using bovine aorta endothelial cells (BAE) infected with virulent Gardel strain, as described by Emboule *et al.* (2009).

Four independent experiments were performed using Gardel strain passages p38, p39, p44, and p52. An additional experiment using Gardel p41 was carried out and used for further validation when necessary. For each experiment, *ER* was produced at early (24 hpi = T1 and 48 hpi), intermediate (72 hpi = T2 and 96 hpi), and late development stages (120 hpi = T3) (Fig. 1). At each time postinfection except at 120 hpi, the cell monolayer was harvested by trypsinization and 1/10 of sample was used for DNA extraction and 9/10 for RNA extraction. When 80% cell lysis was observed, at 120 hpi, both supernatant and cellular debris were harvested and divided as described earlier. The number of bacteria per sample was quantified for each time postinfection. For gene expression measurements, only samples collected at 72 hpi, T2 and 120 hpi, T3 were used. The experiment with Gardel p39 was only used for microarray analysis. Selective capture of *ER* transcripts was carried out before hybridization on microarrays as described below. The four other experiments were used for measurements by q-RTPCR.

Quantification of *ER* in biological samples by *map-1* q-PCR

At each time postinfection, 1/10 of sample was centrifuged at 20 000 ***g*** for 5 min. The pellet was dissolved in 200 μL of PBS 1×. Genomic DNA (gDNA) was extracted using QIAamp DNA Mini Kit (QIAGEN, France) according to the manufacturer's instructions.

The number of *ER* per sample was quantified by q-PCR on extracted DNA, at different hours postinfection. The targeted gene, *map1* present as a single copy in the *ER* genome, codes for a major antigenic protein. The primer sequences and hybridization temperature are shown in Table 1. In the PCR mix, the final concentrations of *map1* reverse and forward primers were 900 nM and the final probe concentration was 250 nM. The Taqman master mix (Applied Biosystems, France) was used following the manufacturer's instructions. Four microliters of sample DNA or standard gDNA was added to the mix. The PCR program was as follows: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles with 15 s at 95 °C and 1 min at 60 °C. To quantify the number of bacteria μL^{-1}, a standard curve was established using gDNA of Gardel serially diluted (from 7×10^6 to 7×10^1 copies μL^{-1}).

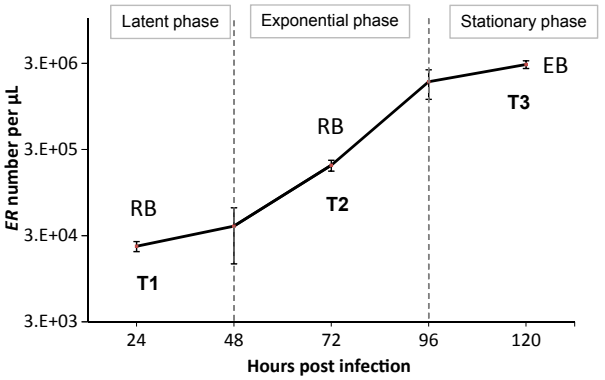

Fig. 1. Growth kinetics of *ER* Gardel strain inside bovine aortic endothelial cells as determined by quantitative PCR assay using *map-1* gene. Specific *ER* concentrations (expressed as number of *ER* per μL) are shown as mean ± SD of number of *ER* per μL obtained for Gardel p38, 41, 44, and 52. The different times postinfection indicated as T1, T2, and T3 correspond to early, intermediate, and late developmental stages of *ER*, respectively. Latent, exponential, and stationary phases of *ER* growth are indicated.

Extraction of total RNA

For each time postinfection, total RNA extraction procedure was carried out on 9/10 of sample as described by Emboule *et al.* (2009). Total RNA quantification was performed by Nanodrop 2000c (Thermo Scientific). For T2 and T3, total RNA samples were pooled in RNase-free water at a final concentration of 0.5 μg μL^{-1} for microarray and at final concentration of 0.15 μg μL^{-1} for qRTPCR.

Reverse transcription of RNA samples

For hybridization on *ER* microarray, RNA samples were reverse-transcribed by random priming with Superscript II (Invitrogen) according to the manufacturer's instructions. The reverse transcription and PCR amplification of corresponding cDNA were carried out using *Kpn*I-primers as previously described (Emboule *et al.*, 2009).

For the validation of gene expression by q-RTPCR, RNA samples were reverse-transcribed using SuperScript VILO cDNA Synthesis kit (Invitrogen) for Gardel p38

Table 1. *ER* primers and PCR conditions for q-PCR and q-RTPCR

Primer name	Primer sequence (5′→3′)	Hybridization temperature (°C)	Product size (bp)	Target gene
map1 gardF *map1* gardR *map1* gard-ex3	CACTTGAAGGAATGCCAGTTTCTC CTTAGGATTTGTAGCATTGATTACTGACACT 6-FAM-ATGCCTGCACACACATAT-MGB	60	85	*map1*
F-*map1-6* R-*map1-6*	ATACACCAACATTCCAGAACA CAGGGATTTCTGCATCGA	60	120	*map1-6*
F-*secF* R-*secF*	TGGCCCACAAGTAGGGTATAAGCA ACCAAGCAAGCTAATAAGACCCAATGT	60	175	*secF*
F-*sppA* R-*sppA*	TGGCGTACTTTAACATTCCTTATGGTT ACCAGTAACTGCACCACCTGGACT	60	231	*sppA*
F-*dapA* R-*dapA*	AGGCATATTTTGCCGGTGATGGGT TGCTCATGGTGGTTCTGGATGC	63	182	*dapA*
F-*dksA* R-*dksA*	GCTGACACAGATCTAACAGACATGGCA TGGGTTTGCTTTGAGTCGTGCT	60	189	*dksA*
F-*lolD* R-*lolD*	TGTTCAGCTACAACTTCAGATCGAGA CGACCCGTTGTCTTTCTCCTCCA	60	250	*lolD*
F-*proP1* R-*proP1*	TGGTGGTGAAGCAGGTGCAA GGAACTCTCCACCCCCAAACA	63	183	*proP1*
F-*trx* R-*trx*	TGGGCTCCGTGGTGTGGACC AGTGGGTACTGCACTAACACCATACTG	63	150	*trx*
F-*resol* R-*resol*	TCAATGGTAATTGGGTTGCCACTTGAT TTTGCTACCCTAGTAGCCATAGCAGT	63	158	*resol*
F-*ccmB* R-*ccmB*	TGCACTGGGTTTATGTTGGAATACCTG AGCCAATGCATGACCTACAGCAGA	60	161	*ccmB*
F-*cytoC1* R-*cytoC1*	ACGTGATGTTGGGTTTTCAGAGGATG AGCAGCTACAGCAGCCTCCTT	60	160	*cyto C1*
F-*atpB* R-*atpB*	AGGCACACCAATGTGGTTAGCACC TGGCCAGCTATCATATTAGCAGCGAGT	60	111	*atpB*
F-*hypo* R-*hypo*	CCCAGCGTACAACAGCTAAGGC CCATGCTGTTTTGTTTCAGATGTTTCA	63	163	*hypo*
F-*folK* R-*folK*	TGGCTTACTCACCTGGCAATGAT GCATTTTCTGGTAATAGTGCCATGCTT	63	157	*folK*

F, forward primer; R, reverse primer.

and p52 samples and with First Strand cDNA Synthesis kit (GE Healthcare) for Gardel p41 and p44. The conversion conditions were performed according to the manufacturer's instructions.

The bacterial gDNA contaminant in RNA samples was evaluated by PCR 25 cycles targeting *pCS20* gene using primers AB128 and AB129 as described previously (Martinez *et al.*, 2004). In RNA samples, no signal was obtained by *pCS20* PCR. Moreover, the efficiency of conversion was checked using q-RTPCR targeting *ER* 16S gene by processing simultaneously RNA and cDNA samples (Emboule *et al.*, 2009). The difference in Ct between RNA and cDNA samples was always higher than 5 cycles, indicating low contamination by gDNA in RNA samples.

Measure of differential gene expression by microarray

Specific cDNAs of *ER* were selected using the SCOTS method as described by Emboule *et al.* (2009). Two

successive SCOTS captures were generated for the sample used for microarray analysis.

ER microarray used in this study was previously used in the study on SCOTS method validation (Emboule *et al.*, 2009). Microarray results were analyzed using GENEANOVA software (Didier *et al.*, 2002). Genes differentially expressed between T2 and T3 had a $\log_2$-fold change (FC) > 1 with a *P*-value < 0.1 and variance > 0.5.

The functions of genes differentially expressed and of proteins were checked on NCBI and KEGG databases, respectively.

Measure of differential expression by q-RTPCR

On the genes differentially expressed identified by microarrays, a selection of genes for validation by q-RTPCR was made. The design of the primers was carried out with PRIMER3PLUS software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) using conventional parameters (Table 1). Primers' specificity was verified by BLAST and by the use of DNA from BAE cells as negative control during PCR validation steps. The final concentration of forward and reverse primers in the PCR mix was identical and varied from 150 to 300 nM depending on the targeted gene. SyberGreen Master Mix (Applied Biosystems) was used following the manufacturer's instructions with 2 μL of cDNA or standard gDNA. The PCR program was 10 min at 95 °C, and 40 cycles with 30 s at 95 °C, 30 s at the optimal hybridization temperature (Table 1), and 1 min at 72 °C. An absolute quantification was carried out to measure the gene expression. A range of 7×10^6 to 7×10^1 copies μL^{-1} of gDNA Gardel was used for standard calibration and processed simultaneously with *ER* cDNA samples.

To normalize the gene expression, the number of cDNA copies from the targeted *ER* gene is divided by the number of *ER* (number of gDNA copies) present in the biological sample, giving *R* at one time postinfection (T):

R_T = (number of cDNA copies)/(number of *ER*)

The differential gene expression (FC) between different development stages is measured by the following formula:

$FC = R_{T3}/R_{T2}$

Results are expressed in $\log_2$-FC between T3 and T2. Positive $\log_2$-FC corresponds to overexpression at T3 compared with T2. Negative $\log_2$-FC corresponds to overexpression at T2 and is expressed as absolute value of $\log_2$-FC. All qPCR were performed on the ABI Prism 7500 (Applied Biosystems).

Results

Quantification of the number of bacteria by quantitative PCR using *map-1* gene

The number of *ER* per sample was measured by q-PCR using *map-1* gene. The growth curve of *ER* had a classical profile of bacterial growth with three phases: a latent phase between 24 and 48 hpi with $2.26–3.87 \times 10^4$ bacteria μL^{-1}, an exponential phase between 48 and 96 hpi with a 2 $\log_{10}$ increase of *ER* concentration, and a stationary phase between 96 and 120 hpi before the lysis of the cells (Fig. 1). The final mean concentration was 3.10^6 bacteria μL^{-1}. The *ER* development was synchronized because of standardized inoculum and its development was observed by optical microscopy at each time postinfection. The stage of *ER* between 24 (T1) and 72 (T2) hpi corresponded to the RB and the 96 hpi stage represented the IB. At 120 hpi (T3), *ER* is on its infective EB form and induces host cell lysis.

Functional category classification of genes differentially expressed during *ER* cycle

Fifty-four genes (5% of *ER* predicted genes) were found differentially expressed between T2 (RB) and T3 (EB) after microarray analysis. Depending on the gene, the $\log_2$-FC of overexpression varied from 1.5 to 6.1 for RB stage and from 3 to 8.2 for EB stage (Table 2). They were classified according to their COG (clusters of orthologous groups of proteins) function and by the stage of development. The proportion of overexpressed gene categories at EB stage and RB stage is shown in Fig. 2a and b, respectively. Hypothetical genes represented the highest percentage of overexpressed genes. There were 51.4% and 26.3% of hypothetical genes overexpressed at EB and RB stages, respectively. Between the two stages, there was similar percentage of overexpressed genes (5.7% vs. 5.3%) involved in nucleotide transport and metabolism. The percentage of genes involved in (1) post-translational modification, protein turnover, and chaperones and (2) carbohydrate, AA, and inorganic ion transport and metabolism was higher for EB than for RB stages. *Rne* and *rsme* belonging to the same translation, ribosomal structure and biogenesis category were overexpressed at RB and EB, respectively. For coenzyme transport and metabolism function, there was a higher proportion of overexpressed genes at RB compared with EB stage (15.8% vs. 5.7%). Similarly, the proportion of genes belonging to replication and DNA repair and energy production and conversion groups was twice higher at RB stage (10.5% vs. 5.7%). The genes of five function

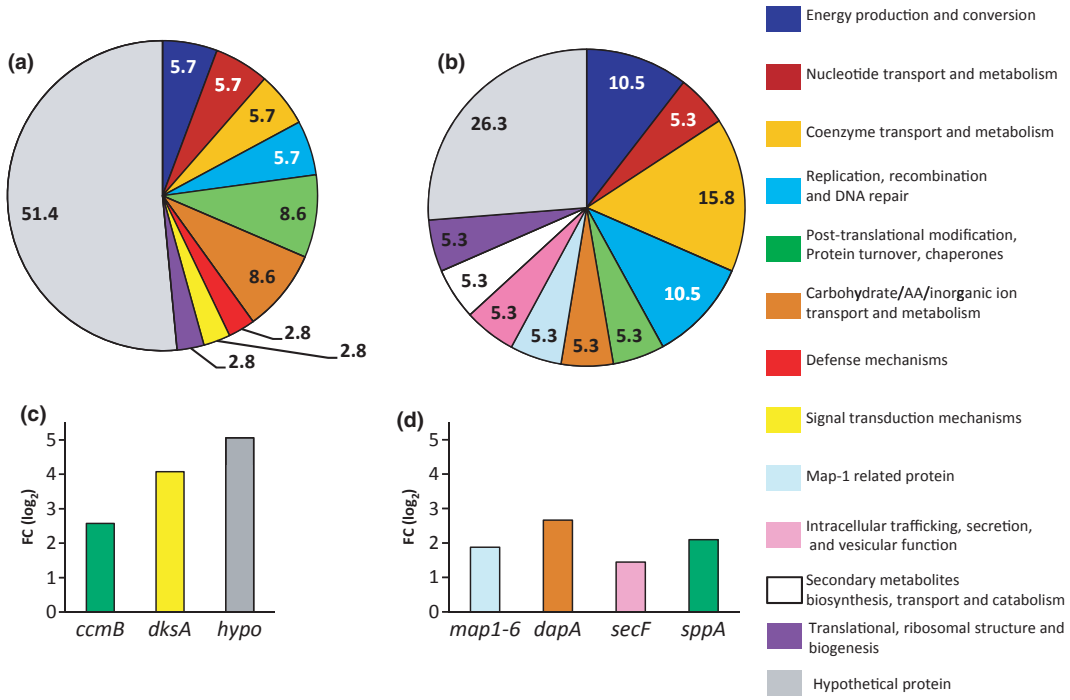

Fig. 2. Gene overexpression analysis at EB and RB stages by microarrays and q-RTPCR. Pie charts showing functional category classification of genes identified as overexpressed by microarray analysis during *ER* developmental cycle (a and b). The figure represents the proportion of genes overexpressed according to the COG functional classification for the EB (a) and RB (b) stages. Differential gene expression measured on Gardel p44 by q-RTPCR at EB (c) and RB stages (d). Experiments were repeated at least three times.

categories were specifically overexpressed at one stage. The *map1-6* gene codes for a MAP-1-related protein, and *secF* is associated with intracellular trafficking, secretion, and vesicular function at RB stage. *FabG*, involved in the secondary metabolite biosynthesis transport and catabolism, was also upregulated at RB. At EB stage, *dksA* is involved in signal transduction mechanisms and *lolD* is taking part in defense mechanisms (Fig. 2a and b and Table 2).

Gene expression profiling of selected genes by q-RTPCR

Fourteen of 54 overexpressed genes identified after microarray analysis were selected for further gene expression validation: (1) *map1-6* ($\log_2$-FC = 5.9) and CDS_003900 (*hypo*) ($\log_2$-FC = 8.2) for their strong FC expression and (2) *ccmB*, *dksA*, *dapA*, *secF*, *sppA*, *atpB*, *cytoC1*, *trx*, *lolD*, *resolvase* (*resol*), *proP1*, and *folK*, mainly for their biological functions and involvement in the pathogenicity, the nutrient and protein transports, and the metabolism of the bacteria (Table 2). A typical profile of gene expression measured by q-RTPCR was shown for one experiment with Gardel p44 (Fig. 2c and d). The overexpression of *ccmB* ($\log_2$-FC = 2.57), *dksA* ($\log_2$-FC = 4.07), and *hypo* ($\log_2$-FC = 5.06) at EB stage and *map1-6* ($\log_2$-FC = 1.88), *dapA* ($\log_2$-FC = 2.66), *secF* ($\log_2$-FC = 1.45), and *sppA* ($\log_2$-FC = 2.09) at RB stage measured by q-RTPCR confirmed microarrays data (Fig. 2c and d, Table 2). *CcmB*, *dksA*, *hypo*, and *map1-6* were found overexpressed also in the two other experiments with Gardel p38 and p52. The strongest FCs were observed for *dksA* ($\log_2$-FC = 8.79 and 5.58) and *hypo* ($\log_2$-FC = 6.45 and 6.37) (data not shown).

For the other genes, an additional experiment with Gardel p41 was used either because there was a slight discrepancy in the gene expression trend or because there was no difference in expression between RB and EB stages for one of the three replicates. The overexpression of *dapA*, *secF*, and *sppA* genes was confirmed on this fourth experiment (data not shown). The expression of *resol*, *proP1*, and *folK* genes measured by q-RTPCR with four biological replicates did not confirm the overexpression observed by microarrays (data not shown). However, three independent replications of the time course showed that the remaining genes, *atpB*, *cytoC1*, *trx*, and *lolD*, were overexpressed at RB stage (data not shown). The mean $\log_2$-FC obtained by q-RTPCR was 2.9 for *atpB*, 2.6 for *cytoC1*, 2.7 for *trx*, and 3 for *lolD* (data not shown).

Table 2. Overexpressed genes and functions determined by microarray analysis

	RB stage overexpression			EB stage overexpression		
Gene function	Gene id	Name	log_2-FC	Gene id	Name	log_2-FC
Energy production and conversion	CDS_05590	*nuoM*	2.6	**CDS_05160**	***cytoC1***†	**4.6**
	CDS_01340	*lpd*	6.1	**CDS_08780**	***atpB***†	**4.6**
Nucleotide transport and metabolism	CDS_07260	*dcd*	4.6	CDS_08290	*purK*	3.7
				CDS_05880		6.7
Coenzyme transport and metabolism	CDS_06730	*bioB*	1.44	CDS_03490	*coaD*	5.7
	CDS_06560	*dfp*	1.6	**CDS_06750**	***folK***†	**6.2**
	CDS_02710	*nadE*	4.3			
Replication, recombination, and DNA repair	CDS_07910		1.8	CDS_00420	*recF*	4.1
	CDS_08960	*recJ*	1.8	**CDS_05820**	***resol***†	**4.7**
Post-translational modification, protein turnover, chaperones	**CDS_06350**	***sppA******	**1.7**	**CDS_07850**	***trx***†	**3.2**
				CDS_00340	***ccmb******	**4.9**
				CDS_05090		5.5
Carbohydrate/AA/inorganic ion transport and metabolism	**CDS_02670**	***dapA******	**2.0**	**CDS_02740**	***proP1***†	**5.3**
				CDS_07250		6.5
				CDS_04690	*tal*	5.0
Defense mechanisms				**CDS_01120**	***lolD***†	**5.5**
Signal transduction mechanisms				**CDS_00330**	***dksA******	**8.2**
Map1-related protein	**CDS_09090**	***map1-6******	**5.9**			
Intracellular trafficking, secretion, and vesicular transport	**CDS_00550**	***secF******	**5.2**			
Secondary metabolites' biosynthesis, transport, and catabolism	CDS_03920	*fabG*	2.0			
Translational, ribosomal structure, and biogenesis	CDS_05680	*rne*	3.3	CDS_05020	*rsme*	4.5
Hypothetical protein	CDS_00780		1.5	CDS_05770		3.0
	CDS_03690		2.2	CDS_01370		3.5
	CDS_04730		3.3	CDS_06790		3.6
	CDS_00080		5.1	CDS_09310		3.8
	CDS_08320		5.4	CDS_04750		3.9
				CDS_09210		4.2
				CDS_01730		4.4
				CDS_09340		4.5
				CDS_02510		5.2
				CDS_00640		5.8
				CDS_04560		5.9
				CDS_07620		6.0
				CDS_06420		6.8
				CDS_06710		6.9
				CDS_07360		7.0
				CDS_03620		7.3
				CDS_05100		7.6
				CDS_00390	***hypo******	**8.2**

Bold text: selected genes for validation by q-RTPCR; overexpression *Confirmed by q-RTPCR. †Not confirmed by q-RTPCR
FC: fold change.

Discussion

Up to now, few studies have been conducted on *Rickettsiales* transcriptomes (Leroy & Raoult, 2010). Moreover, functional studies of genes involved in the bacterial development and pathogenesis are challenging for obligate intracellular pathogens. Microarrays provide detailed knowledge of bacterial pathogenesis by high-throughput whole-genome analysis (Leroy & Raoult, 2010).

We assumed that the analysis of gene expression profiles from *ER* replicating inside bovine endothelial cells would give clues on critical genes for *Ehrlichia* development and pathogenicity. We first used DNA microarrays to identify the genes differentially expressed during *ER* development. In a second time, we analyzed the expression profiles on selected genes by q-RTPCR.

Obtaining *ER* synchronized and standardized cultures, as previously described by Marcelino *et al.* (2005) was crucial to ensure a good reproducibility between biological replicates. Despite our previous analysis on several genes described in the literature as internal reference genes for other pathogens (*16S*, *ffh*, *recA*, *rpoD*, and *proC*), we could

not identify constitutively expressed *ER* genes to use as normalizers for relative quantification of gene expression (data not shown). In this context, the gene expression was measured first by cDNA quantification for each targeted gene and then normalized by the number of bacteria per sample. Such method of normalization constitutes an optimal option for intracellular organisms (Vandecasteele *et al.*, 2002; Borges *et al.*, 2010).

Only 5% of CDS were identified as differentially expressed by microarrays between RB and EB stages. Hypothetical proteins represent the main functional category of overexpressed genes. It would be interested to study the function of some of these *ER*-specific genes. We showed a differential expression of genes belonging to energy production and conversion, coenzyme transport and metabolism, replication, recombination, and DNA repair functional categories, with a higher proportion of genes at RB stage. *fabG* implicated in secondary metabolites' biosynthesis, transport, and catabolism and *rne* participating in translational, ribosomal structure and biogenesis, were also overexpressed at RB stage. These results are in accordance with the division phase of the bacteria, with a major transcriptional switch that correlated with the RB to EB transition (Leroy & Raoult, 2010). We found that genes involved in the carbohydrate, amino acid, inorganic ion, nucleotide, and coenzyme transports and metabolisms are differentially expressed at both RB and EB stages. However, *ER* was thought to have a condensed genome with a reduced gene expression at EB stage like *Chlamydia* (Nicholson *et al.*, 2003). On the contrary, our results suggest interestingly that EB of *Ehrlichia* could be metabolically actives.

A differential expression was observed by q-RTPCR on 11 of 14 targeted genes, among which seven had different trend from microarrays data. This discrepancy could be due to the hybridization default of some microarrays probes. Similar expression profiles for the 11 genes were obtained between three biological replicates strengthening the accuracy of the q-RTPCR results, which were therefore considered to be the gold standard reference for further analysis.

After q-RTPCR analysis, the three genes, *atpB*, *cytoC1*, and *dapA* essential for the energy production and for the lysine biosynthesis, were found upregulated at RB stage (Domigan *et al.*, 2009). These data confirm the high metabolic activity, typical of RB of *ER* development.

At RB stage, we also showed an overexpression of *map1-6*, *secF*, *sppA*, and *lolD* genes that code for proteins involved in nutrient and protein exchanges and transports, indicating a possible important role in *ER* growth and division. *Map1-6* is essential for *ER* host adaptation and intracellular survival (Postigo *et al.*, 2008). The Sec system represents the major route in *Rickettsia typhi* for protein secretion including the secretion of virulence factors (Ammerman *et al.*, 2008). SppA is the enzyme responsible for cleaving the signal peptide of Sec-dependent proteins. It is also described as a protease IV (Kim *et al.*, 2008; Golde *et al.*, 2009). Further study of this secretion system will give information of its involvement in *ER* virulence. LolD is a part of LolCDE protein complex that belongs to the ABC transporter superfamily and initiates the lipoprotein sorting to the outer membrane by catalyzing their release from the inner membrane. LolCDE complex is well conserved in various Gram-negative bacteria and thought to be essential for their growth (Narita & Tokuda, 2006).

The implementation of defense mechanisms against reactive oxygen species produced by host cells could be supported by the overexpression of *trx* we observed at RB stage. This gene contributes to the resistance to oxidative stress (Arner & Holmgren, 2000), and its overexpression has also been reported for *R. conorii* (Renesto *et al.*, 2008).

Three genes, *hypo*, *ccmB*, and *dksA*, were strongly overexpressed at EB stage. The *hypo* gene is identified in both *Ehrlichia canis* and *chaffeensis* and codes for an outer membrane protein, which is unique to the genus *Ehrlichia* (Miura & Rikihisa, 2007). The overexpression of this protein at EB stage suggests that it could be involved in the bacterium–host cell interaction and that a membrane reorganization could occur as described previously for *Chlamydia trachomatis* (Nicholson *et al.*, 2003). *CcmB* and *dksA* are localized on the same operon, thus suggesting that they could be coregulated. *CcmB* is known as a component of an ABC transporter involved in cytochrome C maturation (Richard-Fogal & Kranz, 2010). In *Salmonella typhimurium* and enterohemorrhagic *Escherichia coli*, *dksA* is involved in the virulence factor regulation, especially at late stage (Nakanishi *et al.*, 2006). In our model, EB may express virulence factors as *dksA* to improve new host cell invasion. Further investigations into the *dksA* function will be developed for *ER* model.

In conclusion, our study shows that several genes of *ER* are differentially expressed during the development stages. Here, we identified a number of known pathways as well as new genes that could be important for various aspects of *Ehrlichia* development and pathogenicity. Further analysis of these functions, in association with comparative genomic and proteomic approaches, will give us a better view of mechanisms of infection of *ER*.

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Authors' contribution

L.P., L.E., D.F.M. and N.V. contributed equally to this work.

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