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Development of a quantitative gene expression assay for *Chlamydia trachomatis* identified temporal expression of σ factors

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Abstract ***Chlamydia trachomatis*** **is an important human pathogen which possesses a unique bi-phasic developmental cycle. We used lightcycler methodology to quantitatively measure gene transcript levels in *C. trachomatis* strain L2. By measuring 16S rRNA transcript levels, we determined *C. trachomatis* L2 to have a generation time of approximately 3 h and an inclusion burst size of 200–300 particles. The three chlamydial σ factor genes *rpo*D (σ^{66}), *rps*D (σ^{28}) and *rpo*N (σ^{54}) exhibited different patterns of temporal expression. *rpo*D was central to early chlamydial development, whereas *rps*D and *rpo*N were temporally expressed, coinciding with elementary body (EB) to reticulate body (RB) conversion and RB to EB conversion, respectively.**

Key words: Developmental expression; Lightcycler

1. Introduction

The genus *Chlamydia* currently contains four species of obligate intracellular eubacteria responsible for significant disease in human, non-human mammalian and avian hosts [1,2,3]. *Chlamydia* are characterised by a unique bi-phasic life cycle involving the morphogenesis between an extracellular survival form, the elementary body (EB), and an intracellular replicating form, the reticulate body (RB). Whereas the in vitro life cycle for *Chlamydia trachomatis* is well characterised, the true developmental stages for in vivo infections are less well understood [3]. In vitro, chlamydial development occurs within an inclusion membrane derived following the attachment and phagocytosis of the EB into the host cell during the first 6–8 h post-infection (PI). The intracellular EB commences RNA and protein synthesis [4] and subsequently transforms into the metabolically active RB [5]. The RBs subsequently divide by binary fission every 2–3 h and chlamydial development becomes asynchronous from approximately 18 h PI when some RBs transform to EBs and others continue dividing. From 30–48 h PI, the number of transforming RBs increases and inclusions contain an increasing majority of EBs. The resultant infectious EBs are released from 48–72 h PI following lysis of the host cell [5,6].

Despite the importance of chlamydial disease, the developmental expression of relatively few genes has been determined. The major difficulties in quantitative analysis of chlamydial gene expression are the inability to culture *Chlamydia* in a host-free environment, the asynchronous nature of chlamydial development, measuring low level transcripts and difficulty in standardising for the number of chlamydiae within an inclusion. Nevertheless, there have been several studies which have crudely assayed developmental profiles of chlamydial genes using methods such as two-dimensional gel electrophoresis, immunoblot, Northern blot, host-free RNA synthesis, primer extension, S1 nuclease analysis and reverse transcription polymerase chain reaction (PCR). The expression profile of the genes investigated can be loosely grouped into three categories: early, mid to late-specific and non-temporally expressed genes. Only the *euo* gene, which is believed to be a histone Hc1-specific protease, is known to be expressed exclusively during early development [7]. The non-temporal genes include the 16s rRNA [8], ribosomal protein genes [9,10], heat shock protein genes [9], major outer membrane protein gene [11] and RNA polymerase subunit genes *rpo*A [12], *rpo*B [12], *rpo*C [13,14] and *rpo*D [14,15]. The mid to late stage-specific genes include the cysteine rich outer membrane protein (CRP) gene [15,16], macrophage infectivity potentiator-like protein gene [17], histone-like protein homologues, *hct*A and *hct*B [18,19], and *C. trachomatis* plasmid genes encoding the operon reading frame for both integrase [20] and Dna B [21] homologs. Despite the significance of these studies, most of the reported gene expression was not standardised for the number of chlamydial particles and thus, the presence of increased mRNA or protein may simply be the consequence of increased chlamydiae.

Our understanding of chlamydial genetics has been significantly boosted by the recent sequencing of the entire *C. trachomatis* and *Chlamydia pneumoniae* genomes [22]. Of particular interest was the identification of homologues to two alternative σ factor genes, *rps*D (σ^{28}) and *rpo*N (σ^{54}). σ Factors direct RNA polymerase to initiate transcription from a specific promoter sequence [23]. Until the identification of the *C. trachomatis* σ^{28} and σ^{54} genes, the major σ^{70}-like σ factor (σ^{66}) was thought to coordinate transcription from all chlamydial genes. Since σ^{28} and σ^{54} initiate transcription from a range of genes (including those involved in chemotaxis, flagellar synthesis, sporulation and nitrogen fixation) in other eubacteria [23], it is tempting to propose that the *C. trachomatis* homologues initiate transcription of genes required during different stages of chlamydial development. However, before this can be investigated, temporal expression of the *C. trachomatis* σ factors must be determined. With this in mind, we developed a sensitive, PCR-based assay to allow for quantitative analysis of chlamydial gene expression during all stages of the developmental life cycle.

Quantitative analysis of PCR requires that measurements are made during the logarithmic phase of amplification, which

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Abbreviations: PCR, polymerase chain reaction; cDNA, complementary deoxyribonucleic acid; PI, post-infection; LC, lightcycler

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can vary for different amplicons [24]. We chose to utilise lightcycler (LC) technology to monitor continuous real-time PCR using a fluorescent double strand DNA-specific dye. The quantification of low copy transcripts has been achieved with as little as 10 copies of granulocyte/macrophage colony stimulating factor from rat synovial cells being successfully quantified [25]. This should be sensitive enough to allow for low level transcripts from chlamydial infections during the early stages of infection to be assayed. Since the number of chlamydiae increases with respect to the host cell during development, we chose to use 16S rRNA as a reference to standardise for the number of chlamydial particles.

2. Materials and methods

2.1. Chlamydial culture

C. trachomatis strain L2/434/Bu was grown in HEp-2 cell monolayer cultures [26] using DMEM (Gibco BRL Life Sciences) supplemented with 10% FCS (CSL), 2 mM L-glutamine, 1 μg/ml streptomycin and 40 μg/ml gentamicin. Approximately 2.5×10^7 cells in monolayer cultures were infected with *C. trachomatis* and infected cells were harvested at 1, 4, 8, 12, 22, 30, 40 and 48 h PI.

2.2. Nucleic acid isolation

Total RNA was isolated from *Chlamydia*-infected monolayer cultures using the TRIzol procedure (Sigma). Residual DNA was removed by resuspending the RNA in 100 μl DNAse I buffer (10 mM Tris-Cl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTE) and incubating with 20 U RNAse-free DNAse I (Roche) for 30 min at 37°C, followed by purification using RNeasy RNA columns (Qiagen). Total DNA was isolated from parallel cultures using the QIAamp tissue kit (Qiagen).

2.3. Primers for complementary DNA (cDNA) generation, standard PCR and LC assays

Five genes were analysed using the following PCR primer sets: 16S rRNA (ct16s-F: 5′-GGAGAAAAGGGAATTTCACG; ct16sR: 5′-TCCACATCAAGTATGCATCG), *omc*B (ctcrp-F: 5′-GCAATGGTTTCTTACTGTGG; ctcrp-R: 5′-CAAGAAATTTGAGCTCCTGC), *rpo*D (ctsig66-F: 5′-ATGGTCGAATCCAACTTACG; ctsig66-F: 5′-TTTACTCCAGATCGTGTTCG), *rpo*N (ctsig54-F: 5′-CGAAGCAATTGTTTTGCTGC; ctsig54-R: 5′-CATTTCCCTAGATTAGCTCG), *rps*D (ctsig28-F: 5′-GGTAAAGAACCTACTGATGG; ctsig28-R: 5′-GAGTGTATCTGAGAAACTCG).

2.4. Generation of cDNA

For random priming, 5 μg of total RNA was denatured at 70°C with 0.25 μg of random hexamers (Roche) for 5 min before quenching on ice. 1×Superscript II buffer (Gibco BRL Life Sciences), 1 mM dithiothreitol, 0.5 mM dNTPs and 25 U RNAse inhibitor were added to the samples (in a final volume of 20 μl) before incubation at 42°C for 2 min. 200 U Superscript II (Gibco BRL Life Sciences) was added and the reactions were incubated at room temperature for 10 min and 42°C for 50 min before inactivation of the Superscript II at 70°C for 15 min. The resultant cDNA products were purified by treatment with 250 ng DNAse-free RNAse followed by the addition of 270 μl 0.4 M NaCl before phenol/chloroform extraction and ethanol precipitation. The cDNA samples were resuspended in 40 μl TE (10 mM Tris-Cl, 1 mM EDTA pH 8.0).

Sequence-specific generation of cDNA was done using 100 ng total RNA with 1 μM of each sequence-specific reverse primer in a 20 μl reaction. Basically, the RNA and template were denatured at 65°C for 5 min and quenched on ice before the addition of 1 mM dNTPs, 5 U RNAse inhibitor and 2 U AMV reverse transcriptase (Rosche) and subsequent incubation at 42°C for 1 h. The reverse transcriptase was denatured at 70°C for 15 min.

2.5. LC assay of chlamydial gene transcripts

Standard PCR products for each gene assayed were generated by amplifying 50 ng *C. trachomatis* DNA template with 0.5 μM of each primer pair in a Hybaid thermocycler in 1×PCR buffer with 1.5 mM $MgCl_2$ (Rosche), 200 μM dNTPs and 2.5 U Taq polymerase (Rosche). PCR was performed with a heated lid and the cycling parameters were: initial denaturation at 94°C for 2 min, 15 cycles of 94°C 30 s, 45°C 30 s and 72°C 30 s, 20 cycles of 94°C 30 s, 45°C 30 s and 72°C 30 s with a 5 s increase and a final extension at 72°C for 5 min. PCR products were gel-purified (Bresaclean) before the copy number in the final sample was calculated.

The Idaho Technology LC32 was used to determine the gene copy number. 10 μl PCR reactions were set up with final concentrations: 1×PCR buffer containing 3 mM $MgCl_2$ and 1 mg/ml BSA (Idaho Technology), 0.2 mM dNTPs, 0.5 μM of both forward and reverse primers, 0.3–0.6×SYBR green I (to ensure loading in the LC32 in the range of 1–5) (Molecular Probes), 0.25 U Taq polymerase (Rosche), 0.2 μg TaqStart antibody (Clonetech) and 1 μl of template (cDNA or standard with a known copy number). 5 μl of each PCR reaction was transferred to LC capillary tubes (Idaho Technology). The reactions were cycled in the LC32 with the following parameters: Taq antibody denaturation for one cycle at 94°C for 90 s, 45 cycles (temperature transition of 20°C/s) of 94°C 1 s, 55°C 5 s, 72°C 20 s and fluorescence reading taken at 84°C 1 s, melting curve analysis of 72–94°C (temperature transition of 0.2°C/s) with continuous fluorescence readings.

For each gene assayed, a sequence-specific standard curve was generated using 100-fold serial dilutions (10^2–10^8 copies/μl) of the target gene standard PCR product and the same primers used to amplify the cDNA. The LC32 software generated a standard curve (measurements taken during the exponential phase of the amplification) which enabled the copy number for each gene in each test sample to be determined.

3. Results and discussion

3.1. Standardisation of LC parameters

We compared the use of sequence-specific priming and random priming for generating cDNA for subsequent quantitative LC analysis of chlamydial gene transcripts. Using 16S rRNA as the target, we found that both methods generated a similar relative copy number of cDNA for RNA isolated from *C. trachomatis* infections at 4, 12, 30 and 48 h PI (data not shown). As a result, we used random-primed cDNA in all subsequent analyses, since this significantly reduces the number of reverse transcription reactions required and allows for direct comparison of transcript levels within the same cDNA (hence RNA) sample.

For most stages of the chlamydial development, infected cells contain a mixture of EBs and RBs. Since the ratio of RNA to DNA differs between EBs and RBs, it was important to confirm that the number of 16S rRNA cDNA copies is a

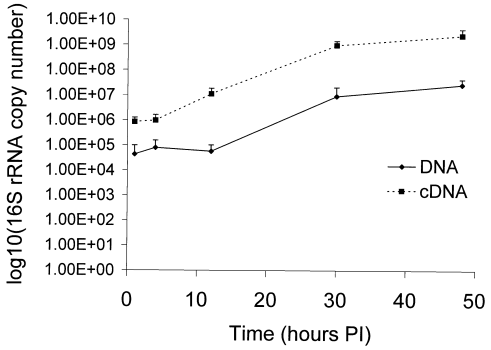

Fig. 1. Comparison of the 16S rRNA copy number from DNA and random-primed cDNA isolated from *C. trachomatis*. Total DNA and RNA was isolated from *C. trachomatis* L2 infections at 1, 4, 12, 30 and 48 h PI and the RNA was random-primed. 16S rRNA copy numbers were determined by a LC assay for 50 ng cDNA and 50 ng DNA. LC assays were repeated at least twice and error bars determined for two S.D.s.

relevant way to standardise for the number of chlamydial particles within a sample. DNA and RNA were therefore prepared from the same *C. trachomatis* cell culture at 1, 4, 12, 30 and 48 h PI and cDNA was generated from the RNA using random primers. The number of copies of 16S rRNA in 100 ng of both cDNA and DNA was determined using the LC assay. As can be seen in Fig. 1, similar curves were obtained for the number of 16S rRNA gene copies in both the DNA and cDNA samples. The lower proportion of 16S rRNA DNA copies compared to 16S rRNA cDNA copies during early infection can be explained since *C. trachomatis* inclusions during 4–12 h PI would contain entirely RBs and therefore a higher proportion of RNA. Thus, standardising the number of chlamydial particles within a *C. trachomatis* infection can be successfully achieved using the number of 16S rRNA cDNA copies.

In order to determine that our estimates of the gene transcript number were accurate, 16S rRNA levels were used as an internal control since they were shown to reflect the number of chlamydial particles (Fig. 1). Since the level of transcription of *omc*B (60 kDa CRP gene, also known as *omp*2) relative to 16S rRNA has previously been determined by a Northern blot [16], we decided to validate the LC assay with *omc*B. The cDNA copy number of both 16S rRNA and *omc*B was determined for 1, 4, 8, 12, 30 and 48 h PI of *C. trachomatis* development. The relative level of *omc*B per chlamydial particle was determined by dividing the total *omc*B copy number by the total 16S rRNA copy number and multiplying by 10^5 (Fig. 2). As can be seen, there was no *omc*B expressed at the 1, 4 or 8 h PI time points but after 12 h PI, significant *omc*B expression was observed. Previous studies have shown that the CRP is only required in the EB [27] and we know that from 12 h PI, some RBs have begun transformation into EBs [5]. Our data from the LC therefore confirms previous Northern blot analysis of the CRP gene [15] and validate the use of the LC for chlamydial gene transcript analysis. The LC was sensitive to both product size and primer quality. We obtained best results using primers generating a product less than 250 bp and we noticed batch to batch variation in column-purified primers. We also found that even though all our primers contained 9/20 G plus C and were anchored by either G or C at each end, there were significant differences in the sensitivity of the LC assay (data not shown). Analysis of the sequence for possible stem-loop structures failed to reveal reasons for gene to gene variation. As the use of LC technology becomes more extensive, the nuances of the assay will be removed.

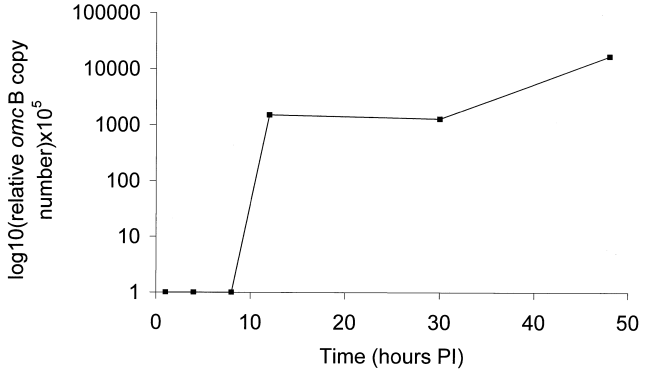

Fig. 2. Relative developmental expression of *omc*B. Total *C. trachomatis* RNA isolated at 1, 4, 8, 12, 30 and 48 h PI was random-primed and assayed using the LC for 16S rRNA and *omc*B copy number. Relative *omc*B expression was determined by dividing the *omc*B copy number by the 16S copy number at the corresponding time points ($\times 10^5$). LC assays were repeated at least twice and error bars determined for two S.D.s.

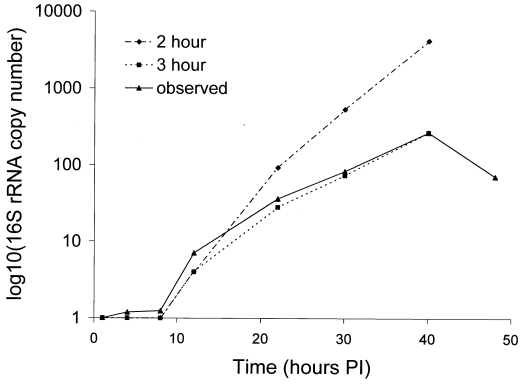

Fig. 3. Modelling of *C. trachomatis* development. Total *C. trachomatis* RNA isolated at 1, 4, 8, 12, 22, 30 and 48 h PI was random-primed and assayed using the LC for the 16S rRNA copy number. The observed 16S rRNA copy numbers are expressed assuming one starting EB. Growth profiles for both 2 and 3 h RB doubling times were determined as described in the text.

3.2. Modelling of C. trachomatis development based on the level of 16S rRNA

Previous studies investigating the developmental stages of the chlamydial life cycle have relied primarily on microscopic observations and biochemical and infectivity assays [6]. By using the sensitive and quantitative characteristics of the LC, we have been able to determine an in vitro chlamydial life cycle based on 16S rRNA units (Fig. 3). During the first 4 h, when attachment and entry of the elementary body into the new host cell is occurring, relatively little new 16S rRNA is being synthesised. These data correlate with previous studies showing that RNA synthesis during the first 8 h PI increases slowly compared to RNA synthesis during the RB multiplication phase 8–24 h PI [5]. The fact that relatively little 16S rRNA synthesis occurs during the first few hours PI might indicate that the process of attachment and entry involves only a few chlamydial genes and that early translation events rely upon 16S rRNA which is previously stored within the EB.

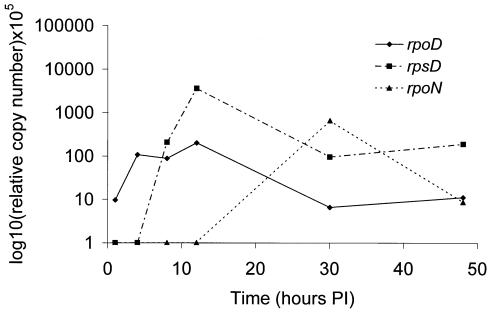

Fig. 4. Relative developmental expression of *rpo*D, *rps*D and *rpo*N. Total *C. trachomatis* RNA isolated at 1, 4, 8, 12, 22, 30 and 48 h PI was random-primed and assayed using the LC for the 16S rRNA, *rpo*D, *rps*D and *rpo*N copy number. *rpo*D, *rps*D and *rpo*N transcript levels were expressed relative to 16S rRNA levels at the same time points (average of three separate experiments).

After 8 h PI, there is a rapid increase in 16S rRNA which parallels RB multiplication. We used the period from 8 to 40 h PI to determine the doubling time and RB to EB conversion rate for *C. trachomatis*. Assuming that during 8–18 h PI, the entire chlamydial population contains RBs, we determined the population size based on both 2 and 3 h doubling times and found that our 16S rRNA LC data (standardised to a single 16S rRNA copy at 1 h PI) matched the 3 h generation curve (Fig. 3). Between 12 and 18 h PI, some RBs start to convert to EBs and hence, we used our LC data to determine the rate at which RB to EB conversion occurs during the period 18–40 h PI. The equations $RB^x = 2/3 \times RB^{x-1}$ and $EB^x = 1/4 \times RB^{x-1} + EB^{x-1}$ (where RB^x and EB^x are the number of RBs and EBs in generation x) generate a curve for *C. trachomatis* development (using a 3 h doubling time) that mirrors the observed data. The interpretation of these data would simply be: for 8–18 h PI, chlamydial RBs multiply every 3 h, solely producing more RBs; from 18 to 40 h PI, RB multiplication continues at the same rate, however, one quarter of the progeny of RB division are destined to differentiate into EBs (but remain counted as RBs in generation x). The RB doubling and RB to EB conversion rates alter after 40 h PI when inclusions contain an increasing proportion of EBs. The validity of these measurements was confirmed by repeating the assay with a second *C. trachomatis* cell culture, which was again random-primed to generate cDNA and analysed for the 16S rRNA transcript number by a LC assay. The same growth profile was observed (data not shown).

Using the 16S rRNA copy number data (Fig. 3) standardised to one infectious EB equals one rRNA copy, our data can predict the maximum number of chlamydial particles per inclusion, which, for *C. trachomatis* L2 at least, is 200–300 particles. Previous estimates of numbers of particles per chlamydial inclusion, based on the number of infectious progeny per host cell, have ranged from 100 to more than 1000 [6].

3.3. Temporal expression of C. trachomatis σ factor genes

The three *C. trachomatis* σ factor genes exhibited different patterns of temporal expression (Fig. 4). The major σ factor (σ^{66}) gene, *rpo*D, was present as early as 1 h PI, suggesting that it might also be present in the EB and therefore be the σ factor responsible for transcription of early chlamydial genes. The level of *rpo*D increased significantly from 1 to 4 h, the time at which EB to RB conversion is primarily occurring, again highlighting its important role in early gene regulation. Neither of the alternate chlamydial σ factors was detected at these early time points. *rpo*D levels were maintained at high levels throughout the RB replication phase, slightly decreasing towards the end of the cycle (30 and 48 h PI). The constitutive expression of *rpo*D corresponds with the essential function of the major, σ^{70}-like, σ factor.

σ^{28} (*rps*D) was the next σ factor to be switched on during chlamydial development. We were unable to detect any *rps*D transcripts at 1 or 4 h, suggesting that σ^{28} is probably not involved in the processes of attachment or invasion. However, there was a significant increase in *rps*D activity after 4 h PI, strongly suggesting a role for this σ factor in either EB to RB conversion or RB multiplication. Since the level of *rps*D transcripts remained high through the mid-late stages of *C. trachomatis* development and the population of chlamydial inclusions between 8–12 h PI consists entirely of RBs [5], this supports σ^{28} involvement in RB replication. In other eubacteria, σ^{28} is involved in expression of a range of coordinately regulated genes, including those involved in chemotaxis, late flagellar synthesis genes and early sporulation [23].

σ^{54} (*rpoN*) was the last of the three *C. trachomatis* σ factors to be switched on. We could not detect *rpo*N transcripts at 1, 4, 8 or 12 h PI, suggesting that σ^{54} is not involved in the processes of attachment, invasion or EB to RB conversion. There was a marked increase in *rpo*N transcription at 30 h PI, coinciding with significant RB to EB conversion. The marked reduction in *rpo*N expression from 30 to 48 h PI strengthens the hypothesis of involvement in RB to EB conversion since the proportion of converting RBs decreases during this time as the EB population increases. Of the eubacteria investigated, expression of *rpo*N is constitutive, except *Caulobacter cresentus* where *rpo*N is regulated temporally during the cell cycle [23].

3.4. Conclusions

The ability to monitor real-time amplification of DNA provides the means to calculate the starting copy number of any DNA template. Since *Chlamydia* is a very difficult organism to propagate and determining the level of gene expression per chlamydial particle has eluded many investigators, we decided to utilise LC technology to investigate the temporal expression of the *C. trachomatis* σ factor genes. By determining the level of 16S rRNA in a random-primed cDNA sample, we were able to (a) confirm that random priming of total RNA generated cDNA representative of the RNA levels, (b) predict the doubling time (3 h) and RB to EB conversion rate for *C. trachomatis* L2 and (c) determine the relative mRNA level of the three *C. trachomatis* σ factor genes. The use of a quantitative transcript assay, such as described in this study, combined with the recent sequencing of the entire *C. trachomatis* and *C. pneumoniae* genomes, should significantly increase our understanding of chlamydial growth and gene regulation.

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