The transcript profile of persistent *Chlamyphila (Chlamydia) pneumoniae* in vitro depends on the means by which persistence is induced

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**Abstract**

Expression of specific bacterial genes is differentially regulated during persistent, vs. active, chlamydial infection. Transcript patterns were examined using real-time reverse transcriptase-PCR in four in vitro models of persistence for *Chlamydia pneumoniae* strain CWL 029, using HeLa cells and normal human monocytes as host. Differential expression of genes encoding cell division proteins was variable when persistence was induced by interferon-γ, penicillin G, or deferoxamine mesylate treatment, and in the monocyte model of persistence. Expression of genes encoding hsp60s and those specifying σ-factors also was variable among models. These in vitro observations indicate that chlamydial persistence is not characterizable by a single transcript profile under all circumstances, supporting the idea that persistent infection in vivo is a complex, flexible strategy that promotes long-term survival of these organisms. Each model system studied here can provide information regarding the molecular characteristics of persistent *C. pneumoniae* infection. However, we do not know which aspect(s) of which model correspond to in vivo disease or other contexts.

**Introduction**

*Chlamyphila (Chlamydia) pneumoniae* is an obligate intracellular pathogen that is the etiologic agent for a common form of community-acquired pneumonia (Grayston, 1992; Campbell & Kuo, 2002). Over the last several years, however, infection with *C. pneumoniae* has been associated with a number of severe, chronic diseases, including inflammatory arthritis, atherosclerosis, temporal arteritis, and even neurologic diseases (Campbell & Kuo, 2002; Belland et al., 2004; Whittum-Hudson et al., 2007; Balin et al., 2008). While many of these associations currently are controversial, they remain the focus of active investigation.

*Chlamydia pneumoniae* undergoes an unusual biphasic developmental cycle. In the first phase, the elementary body (EB), the infectious metabolically inactive extracellular form of the organism, attaches to a eukaryotic host cell; these are usually epithelial cells, but many other cell types can be infected (e.g. Gaydos et al., 1996; Dreses-Werringloer et al., 2006). The organism then is brought into a cytoplasmic inclusion within which it reorganizes into the metabolically active growth form of the organism, the reticulate body (RB). RBs undergo several rounds of cell division, after which most reorganize back to the EB form. Newly formed EB can exit the host cell via lysis or exocytosis to further propagate the infection (Hatch, 1999; see also Hybiske & Stephens, 2007).

Studies have shown that under certain conditions and/or within specific host cell types, chlamydiae can alter their biologic state to generate persistent infections. Chlamydiae undergoing such infections are morphologically aberrant and display an unusual transcript profile (e.g. Hogan et al., 2004 for review). Regarding the latter, we and others have reported that expression of many chlamydial genes, including *omp1*, *ftsK*, and others, is severely attenuated during persistence (Gérard et al., 2001, 2002; Ouellette et al., 2006; Mäurer et al., 2007). This alteration in transcriptional activity is thought to be a highly flexible means of...
adjusting the metabolic characteristics of the bacterium to support long-term infection of the host under varying circumstances, as occurs in Mycobacterium tuberculosis and other organisms (Sassetti & Rubin, 2003; Gérard et al., 2006). Importantly, data indicate that the mechanisms of pathogenesis differ between actively and persistently infecting chlamydiae, and that it is in the persistent state that these organisms elicit chronic disease, including inflammatory arthritis (Hogan et al., 2004; Whittum-Hudson et al., 2007). Indeed, it is now accepted that persistent infection is a significant aspect of the overall pathogenic process engendered by these widely distributed bacterial agents (e.g. Beatty et al., 1995; Hogan et al., 2004 for review). Maintenance of the persistent infection state in various disease-related contexts must be a function of host–pathogen interaction, with the bacterium and host cell exchanging signals that lead to a stable state within the host cell. A major question in relation to pathogenesis resulting from chlamydial persistence centers on details of that communication, i.e. to obviate persistent chlamydial infection and its pathogenic consequences, we must understand the details of the communication process.

Several means have been used to elicit persistence in culture systems, including treatment of C. pneumoniae-infected cells with interferon-γ (IFNγ), penicillin G or several other antibiotics, or limitation of iron (e.g. Gérard et al., 2002; Peters et al., 2005; Ouellette et al., 2006; Mäurer et al., 2007). Persistent chlamydial infection also can be elicited without addition of drugs using normal human monocytic cells as host (Köhler et al., 1997; Gérard et al., 2001, 2002). While the aberrant morphology of persistent C. pneumoniae is similar regardless of the means by which that state is induced, it is not clear whether the transcript pattern in all apparently persistent cultures, and thus presumably the metabolic characteristics of the infecting bacteria, are identical regardless of the eliciting manipulation or conditions. In this article, we demonstrate that the transcript profiles for one strain of C. pneumoniae are not consistent when persistence is elicited by four different means within cultured human host cells.

Materials and methods

Cell culture, C. pneumoniae

HeLa cells were used for passage of the organism in the experiments described here. Culture of these cells, and infection with subsequent preparation of C. pneumoniae strain CWL-029 (from the American Type Culture Collection) EB from them, were performed as described (Byrne et al., 2001). Normal human monocytic cells from three healthy donors were prepared as given (Gérard et al., 2001, 2002).

Induction of persistence

For models of persistence based on infection of HeLa cells, a multiplicity of infection (MOI) of 30 was used; for infection of normal human monocytes, an MOI of 20 was used. Infections of HeLa cell cultures were performed as given (Byrne et al., 2001). Persistence was elicited in C. pneumoniae-infected HeLa cells using 500 U mL⁻¹ of penicillin G (Grüenthal, Aachen, Germany), 250 μg mL⁻¹ deferoxamine mesylate (DAM; Sigma, Taufkirchen, Germany), or 100 U mL⁻¹ of IFNγ (R&D Systems, Wiesbaden, Germany), and confirmed as described (Peters et al., 2005; Eickhoff et al., 2007). No additions to the medium were required for induction of persistence when human monocytes were infected (Gérard et al., 2001, 2002). In the latter model, persistence is fully developed at 3 days postinfection (p.i.; Gérard et al., 2001, 2002; Whittum-Hudson et al., 2007); persistent infection is in effect by 24 h p.i. in the HeLa cell models (Peters et al., 2005; Eickhoff et al., 2007). Infected cultures of all types were harvested at 24 and 96 h p.i. for transcript analyses. Actively growing C. pneumoniae were used as control (see Results), and these were prepared as described (Byrne et al., 2001).

Preparation and analyses of nucleic acids

Total RNA preparations for cDNA analyses were made using Trizol™ according to the manufacturer’s protocol (Invitrogen, Karlsruhe, Germany; Gérard et al., 2001, 2002). cDNA was prepared as described, using random hexamers as primers (Gérard et al., 2001, 2002). Real-time reverse transcriptase-PCR (RT-PCR) analyses to determine relative transcript levels targeted the C. pneumoniae Cpn0612 (polA), Cpn0880 (ftsK), Cpn0903 (ftsW), Cpn0756 (rpoD), Cpn0362 (rpsD), Cpn0771 (rpoN), Cpn0132 (groEL), Cpn0777 (groEL-2), and Cpn0898 (groEL-3) genes. Primer sequences are given in Table 1. Analyses using the Applied Biosystems model 7700 sequence detector with v.1.9 analytical software were conducted as described (e.g. Gérard et al., 2001, 2002, 2006). For each real-time analysis, samples were run in triplicate twice independently. Data for transcript levels for the genes targeted are presented as relative expression level at 24 and 96 h p.i. during normal active infection of HeLa cells, or for each transcript relative to its value at 24 h p.i. in actively growing chlamydiae in HeLa cells. Normalization in all cases is performed to the level of 16S rRNA gene in each sample.

Results

Expression of genes encoding products for DNA replication and cell division

Consistent with previous studies, the data provided in Fig. 1a show that during normal infection of HeLa cells,
Table 1. Primer sequences used in real-time RT-PCR analyses

<table>
<thead>
<tr>
<th>gene</th>
<th>sequence 1</th>
<th>sequence 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>5'-gattgcctcctgatgctg-3'</td>
<td>5'-ctctgtgctactcaacc-3'</td>
</tr>
<tr>
<td>polA (Cpn0612)</td>
<td>5'-tggtgctcctgctgct-3'</td>
<td>5'-agctgagtttctg-3'</td>
</tr>
<tr>
<td>ftsK (Cpn0880)</td>
<td>5'-gtctctcctgctgct-3'</td>
<td>5'-aagcacttccgca-3'</td>
</tr>
<tr>
<td>ftsW (Cpn0903)</td>
<td>5'-ctgctctcctgctgct-3'</td>
<td>5'-accccaaccaagt-3'</td>
</tr>
<tr>
<td>groEL (Cpn0134)</td>
<td>5'-agagctctcctgctgct-3'</td>
<td>5'-agttttgcaagttctg-3'</td>
</tr>
<tr>
<td>groEL-2 (Cpn0777)</td>
<td>5'-gatcctcctgctgct-3'</td>
<td>5'-gcgataaccaagttctg-3'</td>
</tr>
<tr>
<td>groEL-3 (Cpn0898)</td>
<td>5'-gatcctcctgctgct-3'</td>
<td>5'-acctggctcctcagaa-3'</td>
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<tr>
<td>rpoD (Cpn0756)</td>
<td>5'-cgctctcctgctgct-3'</td>
<td>5'-gtctctcctgctgct-3'</td>
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<tr>
<td>rpsD (Cpn0362)</td>
<td>5'-gcgataaccaagttctg-3'</td>
<td>5'-gatcctcctgctgct-3'</td>
</tr>
<tr>
<td>rpoN (Cpn0771)</td>
<td>5'-gcgataaccaagttctg-3'</td>
<td>5'-gcgataaccaagttctg-3'</td>
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</tbody>
</table>

chlamydial genes specifying products involved in DNA replication, such as polA, and those encoding proteins required for cytokinesis, such as ftsK and ftsW, were actively expressed within 24 h p.i. (Byrne et al., 2001; Gérard et al., 2001); the normal time required for completion of the developmental cycle in vitro for C. pneumoniae is about 72 h, and thus by 96 h p.i., transcript levels had declined from each of those genes. Chlamydiae undergoing in vitro infection in normal human monocytes at 3 days p.i., and after about 24 h of treatment with IFNγ, penicillin G, or DAM during infection of HeLa cells, are in the persistent state (Byrne et al., 2001; Gérard et al., 2001; Peters et al., 2005; Eickhoff et al., 2007; Mäurer et al., 2007). In the case of IFNγ treatment, that state is elicited via indoleamine-2,3-dioxygenase (IDO)-mediated tryptophan depletion; DAM treatment accomplishes the induction by limiting iron. It is not entirely clear how penicillin G does so, but it is thought that the drug binds to peptidoglycans that are transiently expressed during the cell division process, thereby attenuating this process. We do not understand the interaction between the organism and the host monocytic cell that elicits the persistent infection state. During persistence, DNA replication-related genes are expressed, but transcripts from ftsK and/or ftsW are attenuated (Whittum-Hudson et al., 2007 for review). The comparative data given in Fig. 1b for polA, ftsK, and ftsW transcript levels generally are consistent with the earlier observations. In all cases of persistence and at both 24 and 96 h p.i. during monocyte infection, polA mRNA levels remained relatively normal compared with those at 24 h p.i. in active infection. Transcripts from ftsW were attenuated by 70% or more in response to IFNγ, penicillin G, or DAM treatment, and at 96 h p.i. in human monocytes; levels of this transcript were normal at 24 h p.i. in monocyte infection, because the initial stages of such infections are relatively normal (Whittum-Hudson et al., 2007). Interestingly, one exception to the overall congruity of our results with previously published observations concerns the level of mRNA from ftsK, which was at levels essentially as those found in actively growing C. pneumoniae during persistence induced via DAM treatment of infected HeLa cells. Thus, one gene specifying a product engaged in DNA replication was expressed as expected during persistence regardless of the means of eliciting that state, but expression of one, but not both, genes encoding cytokinesis-related products was not consistently attenuated under all such circumstances.
Expression of genes encoding the *C. pneumoniae* hsp60 proteins

In addition to the authentic *groEL*, the chromosomes of both *Chlamydia trachomatis* and *C. pneumoniae* encode two additional paralog genes (e.g. Stephens *et al.*, 1998; Read *et al.*, 2000); in the latter organism the authentic *groEL* gene is Cpn0134, and the additional hsp60-encoding genes are designated Cpn0777 and Cpn0898. An earlier study from this group demonstrated that in *C. trachomatis*, expression from the additional hsp60 genes is at higher levels than those of the authentic hsp60 gene throughout the developmental cycle (Gérard *et al.*, 2004). In contrast, the two paralog genes in *C. pneumoniae* were expressed at considerably lower levels than was displayed by *groEL*-Cpn0134 at both 24 and 96 h p.i. during active infection (Fig. 2a). Interestingly, each of the three genes was expressed at a higher level at 96 h than at 24 h p.i., although this difference was equivocal for the authentic *GroEL*; this differential was relatively large for Cpn0777 and Cpn0898. Expression of these genes showed no consistent pattern at 24 or 96 h p.i. in response to any means of eliciting persistence (Fig. 2b). For example, in some cases Cpn0777 showed either attenuated or relatively normal expression at 24 h p.i., while in others it showed upregulation over levels seen during active growth. Similarly, inconsistent transcript levels for Cpn0134 and Cpn0898 were seen among the treatments used. Transcript levels for each of the *C. pneumoniae* hsp60-encoding genes were higher in infected monocytes than in actively infected HeLa cells at 24 h p.i. or in those cells treated with IFN-γ, penicillin G, or DAM. During *C. trachomatis* infection of human monocytes in *vitro*, expression from one of the additional hsp60-encoding genes is virtually obviated during persistent infection, while expression from the other additional gene is significantly increased (Gérard *et al.*, 2004). This was not the case in *C. pneumoniae* during infection of monocytic cells. Thus, as with the DNA replication- and cytokinesis-related genes, expression of the hsp60-encoding genes in *C. pneumoniae* varied in cells in which persistent infection has been induced by different means.

Expression of genes encoding the *C. pneumoniae* σ-factors

Chlamydial genomes, like those of *Escherichia coli* and other bacteria, encode multiple σ-factors, in the case of *C. pneumoniae* and *C. trachomatis* *rpoD*, *rpsD*, and *rpoN* (Stephens *et al.*, 1998; Read *et al.*, 2000). Studies initially showed that the latter gene is expressed later in the developmental cycle, while the first two are expressed early and relatively continuously throughout the cycle (Mathews *et al.*, 1999). We asked whether these σ-factor-encoding genes would display significant differential expression in *C. pneumoniae* when persistence is induced by the several means examined here. As given in Fig. 3a, expression of *rpoD* during active infection was relatively high at 24 h, but much lower at 96 h p.i., as expected if that time point is beyond the normal developmental cycle duration. *rpsD* was expressed at lower level than was *rpoD* at 24 h p.i., but its expression level was increased at 96 h p.i. *rpoN* was expressed only at low levels in these experiments and showed little difference in transcript level at 96 vs. 24 h p.i. As with expression of the hsp60-encoding genes, expression of the σ-factor genes showed little consistency at 24 or 96 h p.i. in response to any of the means of eliciting persistence (Fig. 3b). In some cases, *rpsD* showed relatively high levels of expression at 24 h p.i., while in others it demonstrated little or no difference over the value seen at that time point in actively growing chlamydiae. Similarly, transcript levels from *rpoD* and *rpoN* were highly variable at both 24 and 96 h time points among...
the various persistent cell cultures. Thus, consistent with data for other genes whose expression was examined here, the genes specifying the *C. pneumoniae* σ-factors did not show a consistent pattern of expression during persistent infection under all circumstances of induction of that state in standard in vitro culture systems.

**Discussion**

As indicated above, a major question in relation to pathogenesis due to chlamydial persistence centers on understanding the molecular details of host-pathogen interaction that support that infection state. Given the obvious difficulties attending dynamic in vivo human studies of chlamydial persistence, and to a lesser extent animal models of that phenomenon, many laboratories have developed in vitro models of persistent chlamydial infection. Chlamydial persistence in vivo is thought to be a metabolically flexible state, and it is not clear which if any of the in vitro model systems represents a reasonable fashion in vivo persistent infection with *C. trachomatis* or *C. pneumoniae* in any particular disease context. Studies from several groups, including this one, have demonstrated that expression of various genes/gene sets on the chlamydial chromosome are expressed differentially in persistent infection vs. that seen during active infection, including genes encoding products required for cytokinesis, energy metabolism, cell surface properties, and others (e.g. Mathews et al., 1999, 2001; Byrne et al., 2001; Gérard et al., 2001, 2002, 2004). In this report, we demonstrate in studies using a constant host cell type and a single strain of *C. pneumoniae* that three sets of example genes known to be differentially regulated in persistence vs. active infection display inconsistent differential regulation among four commonly studied in vitro models of chlamydial persistence.

Microarray analyses have been utilized by several groups to assess expression of all coding sequences on the *C. pneumoniae* and *C. trachomatis* genomes during normal progression through the developmental cycle, and in various models of persistence in vitro. In standard, active growth of *C. trachomatis*, all coding sequences on the bacterial genome are expressed at some point (e.g. Belland et al., 2003b). During IFNγ-mediated persistent infection, genes encoding products for cell division, peptide transport, and other functions were shown to be downregulated, while genes specifying products of the general stress response, phospholipid metabolism, translation, and other systems were upregulated (Belland et al., 2003a; see also Gérard et al., 2006). Removal of IFNγ engendered a more or less ordered return of transcriptional activity to normal. Interestingly, this same group reported a generally dysregulated upregulation of transcriptional activity in *C. pneumoniae*-infected cells treated with IFNγ (Ouellette et al., 2006). It is not clear what this unexpected observation means in terms of pathogenesis in vivo, although it is probably not surprising that an uncoupling of transcription to translation also was observed in these experiments. A large-scale study of gene expression during the normal developmental cycle and during DAM treatment-mediated persistence was recently published for *C. pneumoniae* (Mäurer et al., 2007); that study utilized the same chlamydial strain used in the studies reported here. As with the earlier report for *C. trachomatis*, all genes on the *C. pneumoniae* genome were expressed at some point in the developmental cycle, and they were grouped into early, mid-, late, and ‘tardy’ panels as a function of their time of expression p.i. During the iron limitation-initiated persistent infection, transcriptome data suggested that the transit from active to persistent infection is a midcycle attenuation event rather than a specific program of expression supporting that transit (Mäurer et al., 2007). The proteome response...
to the induction of persistence in *C. pneumoniae* also has been examined in the iron limitation and IFN-γ in vitro model systems (e.g. Mukhopadhyay et al., 2006). Importantly, this study also examined protein expression during both long- and shorter-term heat shock treatment. As with the study presented here and others summarized above, a number of differences were noted between the two models of persistence. Probably the most important observation of that study, however, is that the response of *C. pneumoniae* to the induction of persistence in both models includes a significant translation-level stress response (see also Al-Younes et al., 2001; Molestina et al., 2002).

A comparative study of transcriptional activity in *Chlamydia psittaci* during IFN-γ-, penicillin G-, and DAM-induced persistent infection of a human epithelial cell line indicated that for the 27 genes studied (real-time RT-PCR), those encoding cell division products, membrane proteins, and others were downregulated, as in studies of *C. trachomatis* and *C. pneumoniae* during persistence (Goellner et al., 2006). Contrary to data from the present study, however, expression from genes encoding the *C. psittaci* e-factors was strongly downregulated during persistence. Importantly, consistent with data presented here, the transcript pattern from many of the other genes studied varied from one persistence model to another. The variability shown in the present study, and in the previous studies cited here and others, in terms of gene expression during persistent infection in vitro supports the idea that persistence in vivo is highly flexible in its details in the various disease and other contexts in which it is found. Thus, each model can provide information regarding both the bacterial and host contributions to the interaction process, although we do not know at this point which aspect(s) of which model correspond to any of those in vivo contexts. Importantly, congruent with the divergent transcriptional responses of chlamydiae to the different means of eliciting persistence, divergent patterns of host gene expression were seen when the same four in vitro models of persistence were examined (Peters et al., 2005); those patterns ranged from permanent upregulation to silencing of transcription.

In summary, given our data and those of others, we contend, as have others, that chlamydial persistence is not solely and simply a single fixed default transcriptional response to stress. Rather, it is an evolutionarily determined complex and flexible metabolic strategy designed to enhance long-term survival in the host via evasion of immune surveillance. Importantly, we argue that initiation of that strategy in vivo is dependent on (1) the host cell type and the cellular/tissue context of that host cell, (2) the strain of the infecting organism, and given the relatively limited comparative data available, (3) chlamydial species. As with persistence of *M. tuberculosis*, some general genetically based modifications of metabolic and other processes are required for the transition from active to persistent infection. *Chlamydiae* have evolved a transition to persistence that is genetically determined and reliant on products specified by at least some of the coding sequences of unknown function that populate the various chlamydial genomes (Sassetti & Rubin, 2003; Gérard et al., 2006). More detailed studies will be required to refine our understanding of this aspect underlying chlamydial persistence.

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**References**


those of productive infection and are linked to genes involved in apoptosis, cell cycle, and metabolism. Infect Immun 75: 2853–2863.


