

Tryptophan Codon-Dependent Transcription in *Chlamydia pneumoniae* during Gamma Interferon-Mediated Tryptophan Limitation

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In evolving to an obligate intracellular niche, *Chlamydia* has streamlined its genome by eliminating superfluous genes as it relies on the host cell for a variety of nutritional needs like amino acids. However, *Chlamydia* can experience amino acid starvation when the human host cell in which the bacteria reside is exposed to interferon gamma (IFN- γ), which leads to a tryptophan (Trp)-limiting environment via induction of the enzyme indoleamine-2,3-dioxygenase (IDO). The stringent response is used to respond to amino acid starvation in most bacteria but is missing from *Chlamydia*. Thus, how *Chlamydia*, a Trp auxotroph, responds to Trp starvation in the absence of a stringent response is an intriguing question. We previously observed that *C. pneumoniae* responds to this stress by globally increasing transcription while globally decreasing translation, an unusual response. Here, we sought to understand this and hypothesized that the Trp codon content of a given gene would determine its transcription level. We quantified transcripts from *C. pneumoniae* genes that were either rich or poor in Trp codons and found that Trp codon-rich transcripts were increased, whereas those that lacked Trp codons were unchanged or even decreased. There were exceptions, and these involved operons or large genes with multiple Trp codons: downstream transcripts were less abundant after Trp codon-rich sequences. These data suggest that ribosome stalling on Trp codons causes a negative polar effect on downstream sequences. Finally, reassessing previous *C. pneumoniae* microarray data based on codon content, we found that upregulated transcripts were enriched in Trp codons, thus supporting our hypothesis.

Chlamydia is an obligate intracellular bacterial pathogen that causes a range of illnesses in humans and animals (1–4). It alternates between functionally and morphologically distinct forms during its developmental cycle (see reference 5 for review). The elementary body (EB) mediates attachment and internalization into a susceptible host cell, whereas the reticulate body (RB) grows and divides within a membrane-bound pathogen-specified parasitic organelle termed an inclusion (6). In evolving to obligate intracellular parasitism, *Chlamydia* has streamlined its genome by eliminating superfluous pathways and genes (7). Conversely, if *Chlamydia* has maintained a set of genes, then it is likely important for the bacterium.

There are two primary species of *Chlamydia* that cause significant disease in humans: *C. pneumoniae* and *C. trachomatis*. Many chlamydial infections are often unrecognized and asymptomatic, and failure to treat these infections can lead to chronic sequelae. For *C. trachomatis*, these sequelae can include pelvic inflammatory disease, tubal factor infertility, and reactive arthritis (8, 9). *C. pneumoniae* has been associated with a number of chronic conditions, including atherosclerosis and adult-onset asthma among others (10, 11). One possible explanation for asymptomatic chlamydial infections may be due to the ability of the organism to enter a nonproductive growth state referred to as persistence.

Chlamydial persistence is a culture-negative but viable growth state characterized by a cessation of cell division and aberrant bacterial morphology (12, 13). Persistence *in vitro* can be induced in cell culture by a variety of methods, including interferon gamma (IFN- γ) treatment of infected human host cells (14), iron starvation via chelation (15, 16), and amino acid starvation (17–19). Of these, IFN- γ -mediated persistence is the most studied and likely the most relevant to *in vivo* infections (20). The means by which IFN- γ induces persistence in *Chlamydia* is well character-

ized (14, 21, 22). IFN- γ induces indoleamine-2,3-dioxygenase (IDO) expression in human cells, and IDO cleaves tryptophan (Trp [W]) (23). *C. trachomatis* and *C. pneumoniae* are Trp auxotrophs and acquire this essential amino acid from the host cell via the free amino acid pools in the cytosol or via uptake of lysosomal degradation products (24). Once starved of Trp, these pathogens stop dividing and become aberrantly enlarged, and their progression through the developmental cycle is blocked. However, removal of IFN- γ from the culture medium will lead to reactivation of productive growth with continued progress through the developmental cycle (25). IDO is the key mediator of the IFN- γ effect in human epithelial cells because (i) chlamydiae grown in IDO mutant cells treated with IFN- γ grow normally, (ii) flooding the culture medium with high levels of trp will allow for normal growth even in the presence of IFN- γ , and (iii) treating cells with an IDO inhibitor in the presence of IFN- γ also allows for normal growth (26–28).

Many laboratories, including our own, have investigated the transcriptional and proteomic changes that lead to IFN- γ -medi-

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ated persistence in *Chlamydia* (29–40). Unfortunately, these studies incorporated two methodological errors: incorrect normalization methods and inappropriate comparisons between samples. The former methodological error was, in retrospect, not surprising given that most bacterial transcriptional studies use so-called “housekeeping” genes to normalize transcript data, with the assumption that these genes accurately reflect bacterial numbers (and, therefore, growth state). However, as *Chlamydia* is a developmentally regulated bacterium that transitions between very different functional forms, there is no bona fide housekeeping gene that is expressed consistently during all developmental stages (41). Rather, the template for transcription—that is the chromosome—is the best means to normalize chlamydial transcript data (41, 42). The latter methodological error stemmed from the comparison of a population of “RBs differentiating to EBs” to the persistent forms (i.e., comparing equivalent time points postinfection of samples that are fundamentally very different). A more useful comparison is to perform longitudinal analyses within the same sample set to determine what happens as the RB transitions to a persistent form. Consequently, most of the previously published transcriptional studies are difficult to interpret, and all assume a specific “regulon” is induced by *Chlamydia* in response to Trp limitation.

In 2006, we investigated *C. pneumoniae* transcription via gene chip microarray during IFN- γ -mediated persistence and used genomic DNA (gDNA) normalization of our transcript data (42). We found that, contrary to expectations that a specific regulon would be induced, chlamydial transcription was globally increased during IFN- γ -mediated persistence, whereas translation was globally decreased. The latter finding is not surprising given that Trp is necessary for the translation of most proteins (~80% in *Chlamydia*, with an average Trp content per protein of 0.96% for *C. pneumoniae*). We hypothesized that one explanation for the unusual observation of increased transcription may be that *Chlamydia* increases the transcription of genes that contain Trp codons in a futile attempt to make the protein. To test this, we measured the transcript levels of a subset of *C. pneumoniae* genes that were either rich or poor in Trp codons. Our results indicate that transcription of Trp codon-rich genes is generally increased, whereas transcription of Trp-free or Trp codon-poor genes is generally decreased or unchanged during IFN- γ -mediated Trp starvation. Some exceptions were noted in regard to operons and large genes, but a closer analysis revealed that these exceptions could be rationalized based on the overall Trp codon content of the operon/gene where upstream Trp codons lead to destabilization of downstream transcripts. Finally, we reexamined our earlier microarray data as a function of codon content and found that the most “upregulated” transcripts were significantly enriched in Trp codons, whereas the “downregulated” transcripts showed Trp codon levels that were average for the proteome. Additionally, the most downregulated transcripts corresponded to the largest genes, in keeping with a general defect in translation leading to transcript destabilization. These findings are in keeping with a model in which ribosome stalling on Trp codons feeds back to increase the transcription of the given gene.

MATERIALS AND METHODS

Organisms and cell culture. *Chlamydia pneumoniae* AR39 EBs were harvested from infected HEP-2 cell cultures at 35°C with 5% CO₂, and their titers were determined for infectivity by determining inclusion-forming

units (IFU) on fresh cell monolayers. The human epithelial cell line HEP-2 was routinely cultivated at 37°C with 5% CO₂ in Iscove’s modified Dulbecco’s medium (IMDM) containing GlutaMAX, glucose, HEPES, and sodium bicarbonate (Gibco-Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Sigma). The HEP-2 cells were a kind gift from H. Caldwell (Rocky Mountain Laboratories, NIAID). Recombinant human interferon gamma (IFN- γ) was purchased from Cell Sciences (Canton, MA) and resuspended to 100 $\mu\text{g ml}^{-1}$ in 0.1% bovine serum albumin (BSA) diluted in water. Aliquots were frozen at -80°C and used once only to avoid freeze-thawing. IFN- γ was titrated for its effect to induce persistence without killing and in our experiments was used at 2 ng ml^{-1} and added at the time of infection.

Nucleic acid extraction and RT-qPCR. For transcript analyses, HEP-2 cells were plated in 6-well plates at a density of 10^6 per well and infected at a multiplicity of infection (MOI) of 2 with *C. pneumoniae* AR39. In some wells, cells were plated and infected on coverslips to monitor the infection and progression to persistence (see “Immunofluorescence analysis”). Assays to quantify the indicated transcripts were performed essentially as described previously (41, 42). Briefly, total RNA was collected from infected cells at the indicated times using TRIzol (Invitrogen, Thermo) and rigorously treated with Turbo DNasefree (Ambion, Life Technologies) to remove contaminating DNA, according to the manufacturer’s guidelines. One microgram of DNA-free RNA was reverse transcribed with random nonamers (New England BioLabs, Ipswich, MA) using SuperScript III reverse transcriptase (RT) (Invitrogen, Life Technologies) according to the manufacturer’s instructions. The RT reaction mixtures were diluted 10-fold with water and aliquoted and stored at -80°C . Equal volumes of cDNA were used in 25- μl quantitative PCRs (qPCRs) with SYBR green (Quanta Biosciences, Gaithersburg, MD) and measured on an ABI 7300 system (Applied Biosystems, Life Technologies) using the standard amplification cycle with a melting curve analysis. Results were compared to a standard curve generated against purified *C. pneumoniae* genomic DNA. Duplicate DNA samples were collected from the same experiment using the DNeasy tissue kit (Qiagen). Chlamydial genomes were quantified from equal amounts of total DNA by qPCR using the *euo* primer set as described above and used to normalize transcript data as described previously (41, 42). RT-qPCR results were corrected for efficiency (typically above 90% with r^2 values above 0.999). Primer sequences are listed in Table S1 in the supplemental material and were designed using the PrimerQuest tool (Integrated DNA Technologies, Coralville, IA) based on the *C. pneumoniae* CWL029 sequence available from the STD sequence database (<http://stdgen.northwestern.edu>). Results were graphed using GraphPad Prism 6 for Mac OS X (version 6.0f; GraphPad Software, Inc., La Jolla, CA). Student’s *t* test was used for determination of significance of differences between the 48-h-postinfection (hpi) IFN- γ -treated sample and the 24-hpi sample. Spearman’s rank correlation analysis was performed for the data in Table 1 to determine the significance of fold change as a function of Trp codon content.

Immunofluorescence analysis. For culture on coverslips, the cells were fixed in methanol at 48 h postinfection and stained with a mouse primary antibody targeting the *C. pneumoniae* major outer membrane protein (MOMP) GZD1E8, a kind gift from H. Caldwell) and a goat anti-mouse secondary antibody conjugated to Alexa Fluor 488 (Molecular Probes, Life Technologies). Images were collected on an Olympus Fluoview 1000 laser scanning confocal microscope. Images were processed equally for brightness and contrast in Adobe Photoshop.

Western blotting. HEP-2 cells were infected with *C. pneumoniae* and treated or not with IFN- γ as described above. At 24 or 48 hpi, cells were lysed in 50 mM Tris-HCl (pH 8.0) with 1% FBS, 150 μM clastolactacystin (Cayman Chemical, Ann Arbor, MI), and 1 \times HALT protease inhibitor cocktail (Pierce, ThermoFisher). Protein concentrations were determined using the EZQ protein concentration kit (Molecular Probes, ThermoFisher). Samples were equilibrated by taking known protein concentrations and diluting them based on genome equivalents, using 24-hpi samples as the baseline. Samples were resolved by SDS-12% PAGE and

TABLE 1 Fold change in transcript levels of the genes analyzed in Fig. 2 and 3

Gene name	Function	No. of Trp codons	Fold change in transcript in IFN- γ -treated cells from 24 to 48 hpi ^a
<i>mreB</i>	Cell division	0	0.63 \pm 0.26
<i>rodZ</i>	Cell division	5	5.91 \pm 0.47
<i>nusA</i>	Transcription	0	0.75 \pm 0.19
<i>nusG</i>	Transcription	4	2.34 \pm 0.47
<i>dksA</i>	Transcription	0	0.70 \pm 0.34
<i>rho</i>	Transcription	0	1.11 \pm 0.03
<i>obg</i>	Translation	2	8.57 \pm 3.29
<i>ubiA</i>	Metabolism	5	7.90 \pm 1.90
<i>groEL_1</i>	Chaperone	0	0.16 \pm 0.12
<i>groEL_2</i>	Chaperone	2	1.96 \pm 0.29
<i>groEL_3</i>	Chaperone	1	1.74 \pm 0.41
<i>clpP_1</i>	Protein degradation	3	1.65 \pm 0.05
<i>clpP_2</i>	Protein degradation	0	0.35 \pm 0.09
<i>rsbV_1</i>	Transcription	0	0.95 \pm 0.22
<i>rsbV_2</i>	Transcription	0	1.23 \pm 0.37

^a $P = 0.0001$ and $r = 0.8560$ by Spearman rank correlation analysis for fold change versus Trp content.

transferred for 60 min at constant 0.04 A using the Trans-Blot SD semidry transfer cell (Bio-Rad, Hercules, CA). Western blots were developed using a mouse primary antibody against chlamydial Hsp60 (a kind gift from Rick Morrison, University of Arkansas for Medical Sciences) and goat anti-mouse IRDye-800CW secondary antibody (LiCor, Lincoln, NE). The Western blot was imaged using the LiCor Odyssey CLx and subsequently processed, including densitometric analysis, with LiCor Image Studio (version 5.2.5).

Microarray analysis. The *C. pneumoniae* microarray data set from Ouellette et al. (42), a measure of transcription at 24 and 48 h postinfection in the presence and absence of IFN- γ , was analyzed based on codon content by merging the array data into an in-house-generated file listing the amino acid content for each open reading frame (ORF) (see Table S2 in the supplemental material). Data were then sorted based on fold change in transcript levels from 24 to 48 hpi during the different treatment conditions. The individual codon content for those genes that were at least 2-fold increased during IFN- γ , with the exception of late genes (described below), were then averaged. The same analysis was performed for those genes that were 2-fold decreased. The data file also included protein length, which was used to calculate the average size of the gene products from the up- and downregulated data sets. Spearman rank correlation analysis was performed on the data sets to determine the correlation between amino acid content and fold change in transcription in GraphPad Prism 6.

RESULTS

Chlamydial transcriptional responses are highly reproducible.

Our prior experiments assessing chlamydial transcription during IFN- γ -mediated persistence focused primarily on *C. pneumoniae* as a model system, although we measured responses of *C. trachomatis* as well (42). Here, we decided to continue using *C. pneumoniae* with the rationale that its longer developmental cycle would lead to more reproducible results in response to IFN- γ added at the time of infection, as opposed to *C. trachomatis*, where we typically have to add IFN- γ prior to infection to ensure that tryptophan (Trp) pools are sufficiently diminished (43). We previously observed that it takes approximately 24 h for IFN- γ -activated human epithelial cells to deplete their Trp pools, and this is consistent with the findings of others (44, 45).

Chlamydial transcription can be broadly categorized based on the developmental cycle (46). Early genes are transcribed during EB-to-RB differentiation. Mid-cycle genes are transcribed during RB growth and division. Late genes are transcribed during RB-to-EB differentiation. We initiated these studies by measuring transcript levels of two genes: *euo*, an early gene, and *omcB*, a late gene (47–49). We and others have observed that *euo* transcription is highly elevated during IFN- γ -mediated persistence (20, 35, 42). Unusually, we previously observed that late gene transcription is also activated during IFN- γ -mediated persistence (42), likely owing to the inability to translate a negative regulator of late gene transcription. Importantly, late gene transcription is not accompanied by either late proteins or the production of EBs (42). We collected RNA and DNA samples from seven separate experiments and analyzed transcripts from multiple technical and/or biological replicates to assess the reproducibility of chlamydial transcriptional responses. We assayed two time points: 24 h postinfection (hpi), corresponding to peak mid-cycle/RB growth, and 48 hpi, a late time when RBs are converting to EBs. Monitoring chlamydial growth and morphology in IFN- γ -treated cells at 48 hpi, we observed typically aberrant forms in smaller inclusions (Fig. 1A) and noted that DNA replication effectively ceases (Fig. 1B) (42).

From our multiple sample measurements, we made several key observations. First, in keeping with its taking approximately 24 h to deplete Trp pools, there was no statistical difference in gene transcription or DNA replication in untreated or IFN- γ -treated *C. pneumoniae* cultures at 24 hpi (Fig. 1B to D; see Fig. S1 in the supplemental material). The transcript levels within the IFN- γ -treated cultures at 24 hpi were statistically indistinguishable from, and within 1.5-fold of, those of the untreated sample for all transcripts we have measured (42) (see Fig. S2 in the supplemental material), and thus we use this cutoff to determine changes in transcription by RT-qPCR. Second, *euo* and *omcB* transcription is highly reproducibly elevated during IFN- γ treatment as previously observed (42). Whether comparing the raw transcript data (i.e., nanograms of cDNA/gDNA) across experiments or normalizing within each experiment to the 24-h value (i.e., fold change [data not shown]), both transcripts are significantly ($P \leq 0.05$) and reproducibly increased (Fig. 1C and D). Thus, we conclude that the IFN- γ -mediated transcriptional changes in *C. pneumoniae* are reproducible across experiments when persistence is confirmed by other means (e.g., genomic DNA levels, aberrant morphology, etc.).

Chlamydial transcription during IFN- γ -mediated Trp limitation correlates with Trp codon content. Interestingly, *euo* contains three Trp residues in *C. pneumoniae* (versus two in *C. trachomatis*), and its transcript levels are highly elevated during IFN- γ -mediated persistence. To begin testing our hypothesis that chlamydial transcription during IFN- γ -mediated Trp limitation is dependent on the Trp codon content of the gene being transcribed, we measured the transcript levels of eight genes, with various Trp codon contents, representing diverse functions from cell division (*mreB* and *rodZ*) (50, 51), to transcription (*nusA*, *nusG*, *dksA*, and *rho*), to translation (*obg*) (52), to metabolism (*ubiA*). *MreB*, *DksA*, *NusA*, and *Rho* contain no Trp. *RodZ* (5 Trp codons), *NusG* (4 Trp codons), *UbiA* (5 Trp codons), and *Obg* (2 Trp codons) contain various numbers of Trp codons. Consistent with our hypothesis, transcript levels for all genes lacking Trp codons were either unchanged or decreased and showed no statis-

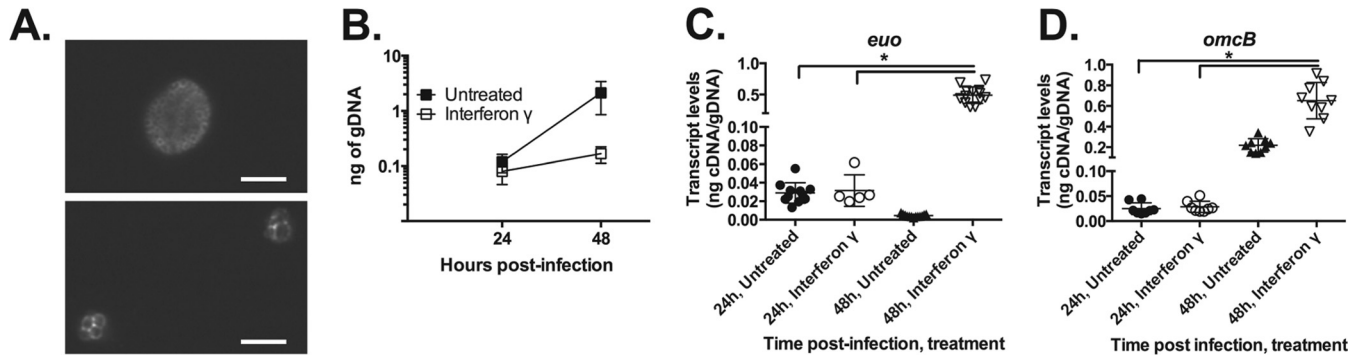


FIG 1 Interferon gamma (IFN- γ) induces a highly reproducible persistent phenotype in *C. pneumoniae*. (A) Characteristic immunofluorescent images of *C. pneumoniae* cells from untreated or IFN- γ -treated cultures. HEp-2 cells were infected with *C. pneumoniae* and treated or not with IFN- γ . At 48 h postinfection, infected cells were fixed and stained for the major outer membrane protein. IFN- γ -induced persistence results in smaller inclusions containing aberrantly enlarged RB forms. (B) Quantification of *C. pneumoniae* genomic DNA (gDNA) by qPCR from untreated (solid symbols) and IFN- γ -treated (open symbols) cultures at 24 and 48 h postinfection. (C and D) Quantification of *C. pneumoniae euo* (C) and *omcB* (D) transcript levels from untreated and IFN- γ -treated cultures at 24 and 48 h postinfection. Individual data points (themselves averages from three measurements) are from all technical and biological replicates from all experiments ($n = 7$, except 24-hpi IFN- γ , where $n = 4$). *, $P \leq 0.05$ for 48-hpi IFN- γ versus 24-hpi IFN- γ .

tically significant differences from the 24-hpi samples (Fig. 2A). Conversely, transcripts for those genes containing Trp codons were significantly ($P \leq 0.05$) increased in IFN- γ -treated cells (Fig. 2B). The fold change in transcript levels between 24 and 48 hpi during IFN- γ -mediated persistence can be seen in Table 1. Overall, these data support the hypothesis.

Paralogous genes are transcribed in a pattern correlated with Trp codon content. *Chlamydia* contains several sets of paralogous genes, and we reasoned that these genes may provide a unique opportunity to directly test our hypothesis of the effect of Trp codons on the transcription of a given gene during Trp limitation. However, many of these paralogs have potential confounding factors, such as (i) the same or similar numbers of trp codons, (ii) transcriptional patterns reflecting different developmental stages (e.g., *lcrH_1* and *lcrH_2*) (41), and (iii) positions within operons that might impact their transcript abundance (see “Exceptions” below). Therefore, we focused on two sets with different Trp

codon contents in *C. pneumoniae* and one set in which neither gene encoded Trp codons: *groEL_1* to -3, *clpP_1* and -2, and *rsbV_1* and -2. We then measured their transcript levels during IFN- γ -mediated Trp limitation. Each of these gene sets provided us with a unique opportunity to directly test our hypothesis. Additionally, each gene is located distally from its paralog and is thus regulated distinctly.

The *groEL* genes encode putative chaperones (53). Hsp60_1, encoded by *groEL_1*, contains no Trp residues, whereas Hsp60_2 and Hsp60_3 contain two Trp residues and one Trp residue, respectively. Interestingly, *groEL_1* transcripts were decreased approximately 6-fold (0.16 ± 0.12) during Trp limitation and were statistically indistinguishable from the untreated sample at the 48-h time point. Conversely, *groEL_2* (1.96 ± 0.29) and *groEL_3* (1.74 ± 0.41) transcripts were increased by roughly 2-fold (Fig. 3A; Table 1). Of note, the *groEL_2* primer set was located downstream of both Trp residues, whereas the *groEL_3* primer set was

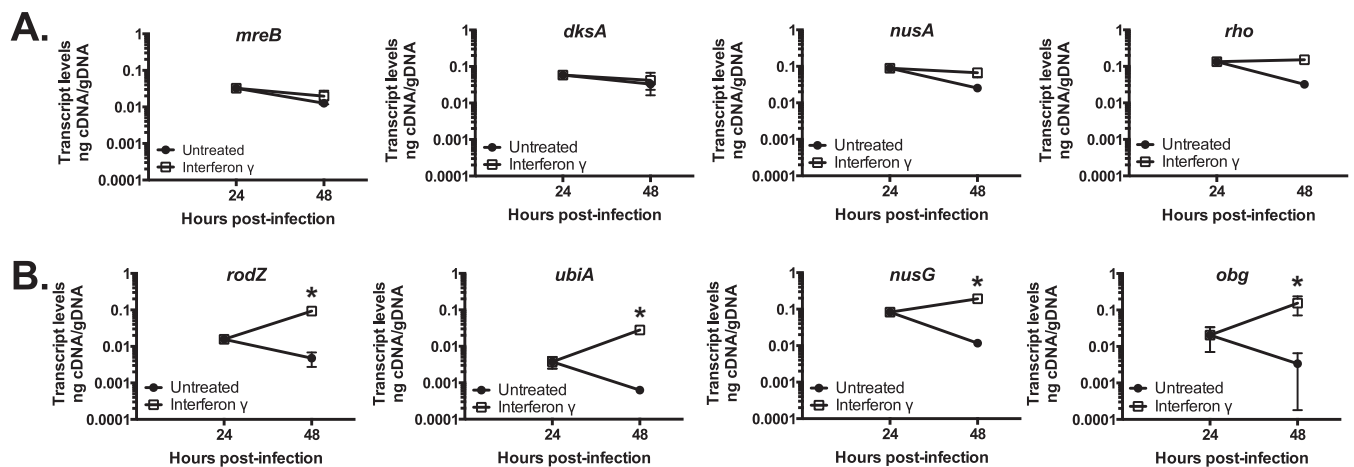


FIG 2 The tryptophan codon content of a gene influences its transcription in *C. pneumoniae* during IFN- γ -mediated persistence. Nucleic acid samples were collected from untreated (solid symbols) and IFN- γ -treated (open symbols) *C. pneumoniae*-infected HEp2 cells at 24 and 48 h postinfection. Transcript levels for the indicated genes were measured by RT-qPCR and normalized to genomic DNA (nanograms of cDNA/gDNA). (A) Quantification of transcript levels for genes containing no Trp codons: *mreB*, *dksA*, *nusA*, and *rho*. (B) Quantification of transcript levels for genes containing various numbers of Trp (W) codons: *rodZ* (5 codons), *ubiA* (5 codons), *nusG* (4 codons), and *obg* (2 codons). Data are averages from at least 2 experiments with standard deviations displayed. *, $P \leq 0.05$ for 48-hpi IFN- γ versus 24-hpi IFN- γ .

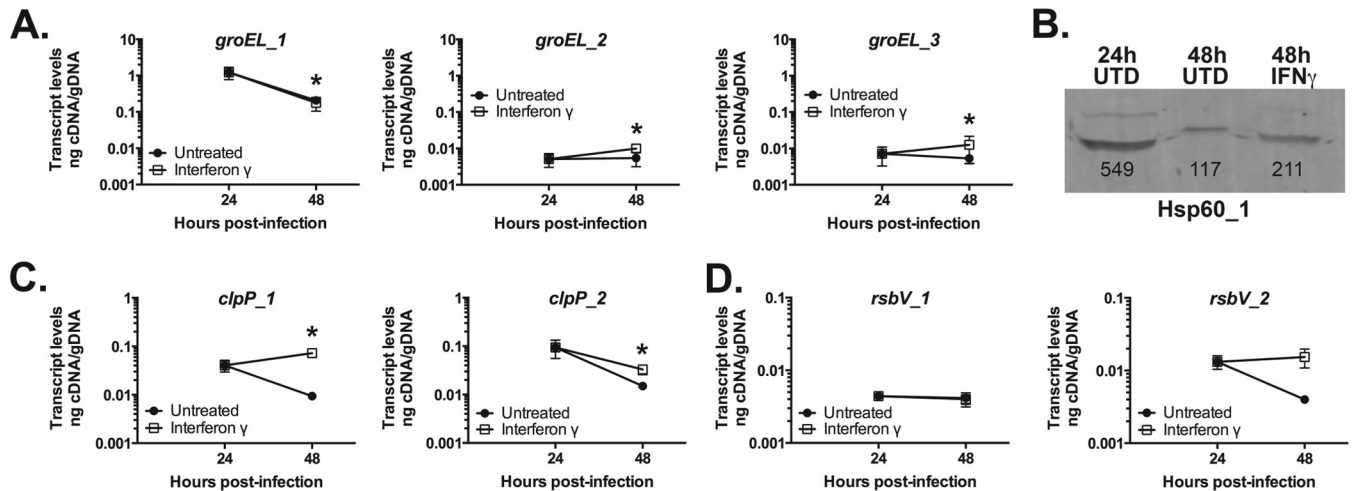


FIG 3 The presence of different tryptophan codon contents within paralogous genes in *C. pneumoniae* affects their transcription during IFN- γ -mediated tryptophan limitation. Nucleic acid samples were collected from untreated (solid symbols) and IFN- γ -treated (open symbols) *C. pneumoniae*-infected HEP2 cells at 24 and 48 h postinfection. Transcript levels for the indicated genes were measured by RT-qPCR and normalized to genomic DNA (nanograms of cDNA/gDNA). (A) Quantification of transcript levels for *groEL* paralogs, where *groEL_1* encodes 0 Trp codons, *groEL_2* encodes 2 Trp codons, and *groEL_3* encodes 1 Trp codon. (B) Western blot measurement of chlamydial Hsp60_1 (i.e., GroEL_1) from untreated and IFN- γ -treated *C. pneumoniae*-infected cultures. Protein loading was normalized for *C. pneumoniae* genomic DNA levels. Densitometry readings are displayed below the bands. (C) Quantification of transcript levels for *clpP* paralogues where *clpP_1* encodes 3 Trp codons and *clpP_2* encodes 0 Trp codons. (D) Quantification of transcript levels for *rsbV* paralogues where neither encodes Trp codons. All transcript data are the average from at least 2 experiments with standard deviations displayed. *, $P \leq 0.05$ for 48-hpi IFN- γ .

located upstream of the single Trp residue (see Table S1 in the supplemental material). Hsp60_1 protein was also decreased compared to the 24-h sample, as measured by Western blotting, with protein loading normalized for chlamydial genome content, and its levels were less than 2-fold different from those of the 48-h untreated sample (Fig. 3B). The decrease in Hsp60_1 is consistent with a decreased need for protein chaperone function when less translation is occurring (42).

The ClpP proteins function in the Clp protease system (54). ClpP_1 contains three Trp residues, whereas ClpP_2 contains none. As with the *groEL* paralogs, *clpP_1* transcripts were significantly increased 1.65 ± 0.05 -fold, whereas *clpP_2* transcripts decreased 3-fold (0.35 ± 0.09) during IFN- γ treatment (Fig. 3C, Table 1). Given the role of ClpP in degradation of proteins, it is perhaps not surprising, given the decrease in translation we previously observed (42), that *clpP_2* transcripts are decreased. Although not dramatically increased, the *clpP_1* transcripts have the opposite profile consistent with the presence of Trp codons in the gene.

The RsbV proteins function within the Rsb anti-sigma factor signaling pathway to regulate, in *Chlamydia*, the function of the major housekeeping sigma factor (σ^{66}) (55). Both RsbV_1 and RsbV_2 contain no Trp residues; transcripts for both were unchanged during Trp limitation (0.95 ± 0.22 and 1.23 ± 0.37 , respectively) (Fig. 3D; Table 1). The transcription of these paralogous genes varied in a manner consistent with their Trp codon content and lends further support to the hypothesis.

The heat shock regulon is maintained during IFN- γ -mediated persistence. Our *groEL_1* data were consistent with our prior observations (42) and prompted us to look more closely at the heat shock regulon, which serves an important chaperone function. We hypothesized that the global decrease in translation efficiency during IFN- γ -mediated persistence (42) would result in a

decreased need for chaperone function. We also noted that, like *groEL_1*, the heat shock genes *dnaK*, *dnaJ*, and *hrcA* lack Trp codons; thus, we predicted that their transcript levels would be unchanged or decreased. Of note, *dnaK* is the terminal gene in the *hrcA-grpE-dnaK* operon, although it also encodes its own promoter (56, 57). As expected, *dnaK* followed the predicted pattern (0.2 ± 0.13) (Fig. 4A). However, *hrcA* transcripts increased, although with some variability (2.82 ± 2.15), which rendered the change statistically insignificant (Fig. 4B). HrcA is a negative regulator of the heat shock response that recognizes a consensus DNA-binding site called CIRCE; thus, an increase in *hrcA* transcripts (and presumably HrcA protein) that leads to downregulation of the other genes makes intuitive sense. The differences between *dnaK* and *hrcA* may reflect an unstable polycistronic transcript (e.g., see below) or differential regulation via the *dnaK* P2 promoter, which is not regulated by HrcA (57). *dnaJ* transcripts showed a consistent 2-fold increase (2.06 ± 0.16) (Fig. 4C). This increase of *dnaJ* transcripts is more difficult to rationalize and represents a potential exception to our overall hypothesis. It further suggests that *dnaJ* may not be regulated by HrcA, which is consistent with the lack of an identifiable CIRCE element upstream of *dnaJ*.

Exceptions: position within an operon or gene can affect transcript levels in a manner dependent on Trp codon content. We anticipated that there would be two primary exceptions to our hypothesis and reasoned that these would most likely occur in (i) operons where the first gene was either rich or poor in Trp codons and (ii) large genes with multiple Trp codons. Our rationale was that ribosome stalling at Trp codons would lead to destabilization of the downstream transcript. To test this, we measured the transcript levels at the 5' and 3' ends of the *ytg* and *rnhB_1* operons as well as the large gene *greA* (encoding 11 Trp residues within 722 amino acids).

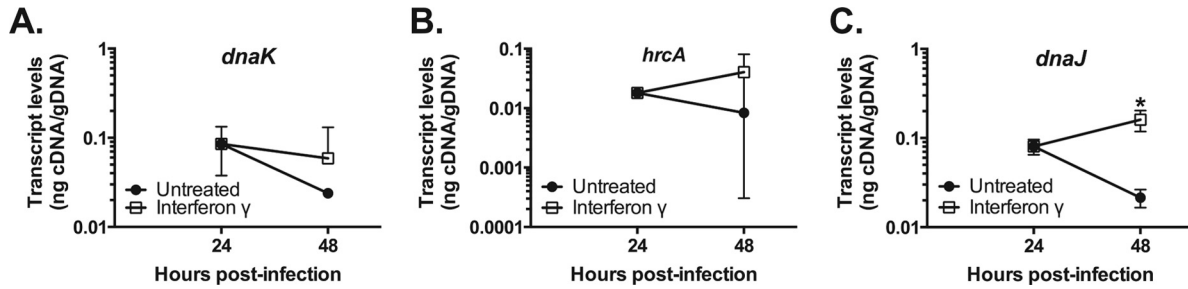


FIG 4 Measurement of *C. pneumoniae* transcript levels for genes associated with the heat shock response during IFN- γ -mediated persistence. Nucleic acid samples were collected from untreated (solid symbols) and IFN- γ -treated (open symbols) *C. pneumoniae*-infected HEp2 cells at 24 and 48 h postinfection. Transcript levels for the indicated genes were measured by RT-qPCR and normalized to genomic DNA (nanograms of cDNA/gDNA) for (A) *dnaK*, (B) *hrcA*, and (C) *dnaJ*. All transcript data are the average from at least 3 experiments with standard deviations displayed. *, $P \leq 0.05$ for 48-hpi IFN- γ versus 24-hpi IFN- γ .

The organization of the *ytg* operon is such that the 5' gene, *ytgA*, encodes six Trp residues, whereas *ytgD* encodes one (Fig. 5A). Conversely, the putative *rhbB_1* operon is organized such that the 5' gene *rhbB_1* lacks Trp, whereas the 3' gene *metG* encodes eight Trp residues (Fig. 5B). One would presume that the

operon transcript levels should follow the pattern of the 5' gene, given the single promoter controlling expression. Our earlier microarray data suggested that *ytgA* transcripts were increased 5.8-fold, with the other open reading frames (ORFs) showing dramatic decreases toward the 3' end of the operon (Fig. 5A). When

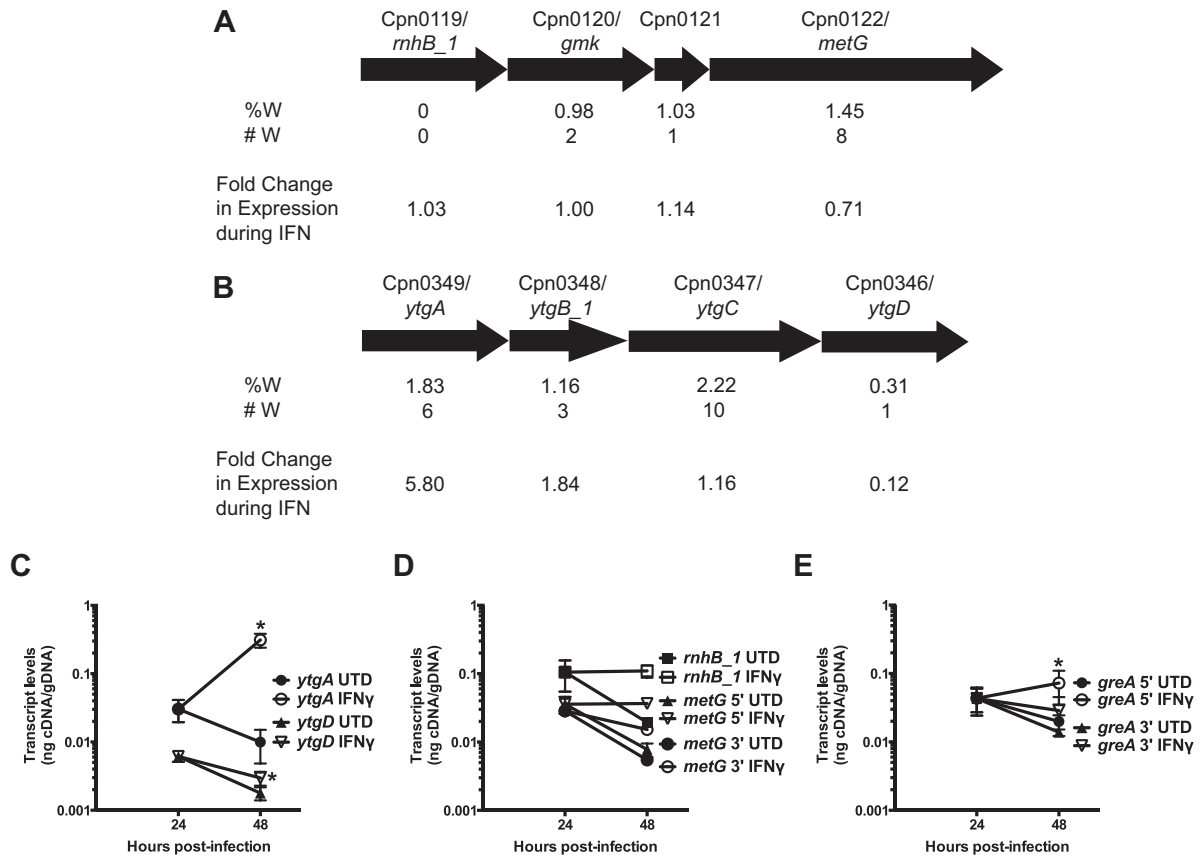


FIG 5 Tryptophan codons have destabilizing effects on downstream transcripts. Shown is determination of the effect of Trp (W) codons on transcript levels within operons and large genes in *C. pneumoniae* during IFN- γ -mediated persistence. (A and B) Schematic representation of the (A) *ytg* and (B) *rhbB_1* operons, including the percentage (%W) and number (#W) of Trp codons within the open reading frames. Arrow sizes represent the relative sizes of the genes in the operons. Also shown is the fold change in transcript levels during IFN- γ persistence between 24 and 48 h postinfection, as measured by microarray (42). (C to E) Nucleic acid samples were collected from untreated (solid symbols) and IFN- γ -treated (open symbols) *C. pneumoniae*-infected HEp2 cells at 24 and 48 h postinfection. Transcript levels for the indicated genes were measured by RT-qPCR and normalized to genomic DNA (nanograms of cDNA/gDNA). (C) Quantification of transcript levels of *ytgA* and *ytgD*. (D) Quantification of transcript levels of *rhbB_1* and *metG*. Two different primer sets were used to quantify *metG* transcripts: one targeting the 5' end of the gene and another the 3' end. (E) Quantification of transcript levels of *greA* using primer sets designed against the 5' and 3' ends of the gene. (See the text for more detail.) All transcript data are the average from 3 experiments (except *metG* 3', where $n = 2$) with standard deviations displayed. *, $P \leq 0.05$ for 48-hpi IFN- γ versus 24-hpi IFN- γ .

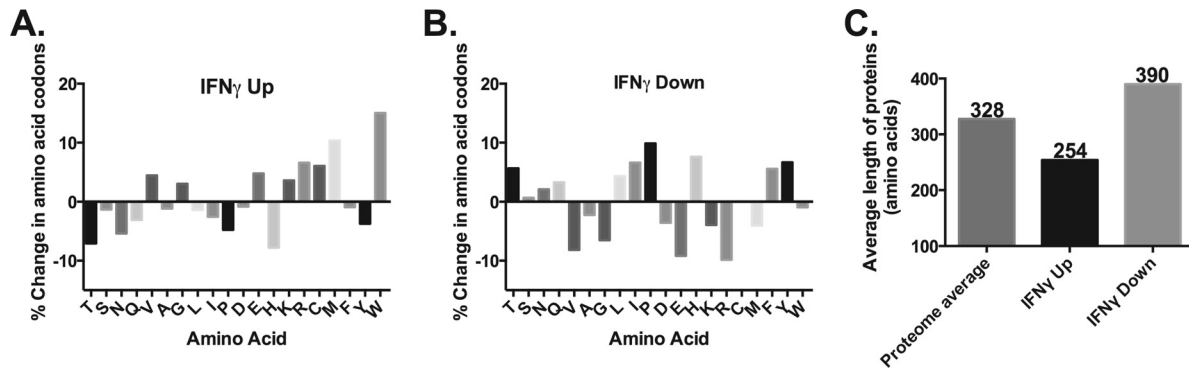


FIG 6 Codon content analysis of *C. pneumoniae* transcript data during IFN- γ -mediated persistence reveals an enrichment of tryptophan codon-containing genes in the upregulated data set. Microarray data from IFN- γ -treated *C. pneumoniae* were analyzed based on the codon content of the genes that were at least 2-fold increased (A [IFN γ Up]) or 2-fold decreased (B [IFN γ Down]). The percentage change in the codon content is reflected on the y axis, whereas the individual single-letter code for the amino acid is listed on the x axis. The 0 value reflects the average *C. pneumoniae* codon content of the 24-h postinfection (i.e., RB profile) transcript levels. (C) For the data in panels A and B, the average length of the proteins encoded by the differentially transcribed genes was plotted and compared to the proteome average (328 amino acids).

we measured *ytgA* transcript levels during IFN- γ treatment by RT-qPCR, we observed an increase of roughly 10-fold. There was also a notable difference between the 5' end and 3' end of *ytgA* itself, with the 5' end displaying an approximately 25% increase over the 3' end compared to untreated cultures, where the 3' end is detected within 10% of the 5' level (data not shown). Remarkably, transcript levels of *ytgD* were more than 2-fold decreased during IFN- γ treatment (Fig. 5C). This indicates a destabilization of the 3' end of the *ytg* operon that appears dependent on upstream Trp codons. In keeping with our prior observations, *rnhB_1* transcript levels were unchanged during IFN- γ compared to untreated cultures (Fig. 5B and D). Somewhat surprisingly but in keeping with the operon regulation, *metG* transcript levels were also unchanged when using a primer set designed at the 5' end of the gene upstream of all Trp codons. However, when we used a primer set toward the 3' end (and downstream of 5 of 8 total Trp codons), *metG* transcripts were 2-fold decreased (Fig. 5D) in keeping with a negative polar effect of Trp codons on downstream sequences. Overall, the *metG* example illustrates how a high-Trp gene may behave transcriptionally like a low-Trp gene.

To determine if the polar effect of Trp codons would apply within large monocistronic units, we examined *greA* transcription using primer sets designed near the ends of the gene. The GreA protein contains 11 Trp residues. We observed that the 5' end of *greA*, downstream of 3 Trp codons, was increased approximately 2-fold in IFN- γ -treated cultures (Fig. 5E). Conversely, the 3' end of the gene, downstream of 8 Trp codons, was decreased approximately 2-fold in IFN- γ treatment, reflecting an overall 4-fold decrease compared to the 5' end. In untreated cultures, the difference between the ends of *greA* was less than 10%. Overall, the transcript levels of *greA* would appear to be unchanged or even decreased, in apparent opposition to our hypothesis, yet the Trp codons clearly affect the transcript levels at each end of the gene.

A reanalysis of the microarray data supports a role for Trp codon content in increased transcription. To test our hypothesis on a larger scale, we revisited our previous microarray data (42) and normalized the changes in transcription based on codon content (see Table S2 in the supplemental material). The average percentage of Trp codons within *C. pneumoniae* genes is 0.96%, or roughly 1 Trp codon per 100 amino acids. In Fig. 6, the RB profile

at 24 hpi is represented by the 0 value on the y axis. Importantly, we excluded all late genes, those transcribed during RB-to-EB differentiation, from the analysis (85 total) as their increased transcription (63 of 85) during IFN- γ -mediated persistence is almost certainly due to the inability to effectively regulate their expression; no late gene protein products are present, and no EBs are produced (42). In addition, late genes are typically poor in Trp codons (average of 0.84%), and thus this artificially skews the analysis.

In total, 226 genes displayed increased transcription of more than 2-fold between 24 hpi and 48 hpi during IFN- γ -mediated persistence, and these genes had an average Trp codon content of 1.1% (versus 1.05% if late genes are included), which corresponds to an overall Trp enrichment of +15% ($P < 0.05$, $r = 0.1371$, by Spearman rank correlation) (Fig. 6A). These genes also showed an enrichment of +10% in methionine (Met), but this was not statistically significant. All other amino acids showed changes of less than 10%. Intriguingly, of the 15 genes that showed an increase of transcripts of at least 10-fold, these had an average Trp enrichment of +51% compared to +9.7% for Met (data not shown). Conversely, for the 296 genes that displayed decreased transcription of more than 2-fold, the average Trp content was unchanged (0.95%), corresponding to a net enrichment of -1% that was not statistically significant (Fig. 6B). Several amino acids approached a 10% change in codon abundance in the downregulated genes, including proline (+9.9%), valine (-8.2%), glutamate (-9.2%), and arginine (-9.8%), but none of these changes were significant.

Because of our findings with operons and large genes where the 3' ends appeared to be destabilized, we next asked the question whether there was a correlation between the levels of transcripts and the size of the encoded protein. Our hypothesis was that larger gene transcripts would be less abundant. The average size of *C. pneumoniae* proteins is 328 amino acids (Fig. 6C). Here, we found that the most upregulated transcripts were for smaller gene products with an average size of 254 amino acids. The opposite profile was found for the downregulated transcripts, where these gene products had an average size of 390 amino acids. Overall, the microarray data show a trend that is consistent with our hypothesis and our RT-qPCR analysis, which is remarkable given the

caveats we have previously characterized concerning operons and large genes.

DISCUSSION

Most bacteria respond to amino acid limitation by enacting the stringent response (58). This global regulatory response is evolved to effectively shut down anabolic pathways and activate catabolic processes that result in an increase in the free amino acid pool such that the organism can continue growing. These effects are mediated by a polyphosphorylated guanine analog, (p)ppGpp, the levels of which are controlled by the enzymes RelA and SpoT (59). During amino acid starvation, an increase in uncharged tRNAs will lead to ribosomes' stalling on transcripts, and this activates RelA to produce (p)ppGpp. ppGpp in turn acts as a global regulator by binding RNA polymerase, among other targets, and modifying its activity in a promoter-dependent manner (60). This global regulatory "program" is highly conserved in bacteria.

Interestingly, no functional stringent response has been described in obligate intracellular bacteria like *Chlamydia*. Why these highly adapted organisms would eliminate this pathway is not clear and may reflect the relatively stable nutritional environment within a host cell. However, *Chlamydia* can experience amino acid starvation. IFN- γ , an immune cytokine produced during chlamydial infections (61, 62), can induce a Trp-limiting environment in human epithelial cells by activating expression of the enzyme IDO, which cleaves the indole ring of Trp (23). Thus, IFN- γ treatment leads to an enzymatically controlled Trp starvation state. Importantly, *Chlamydia* is a Trp auxotroph and acquires this essential amino acid from the host cell. By analogy with other bacteria, when *Chlamydia* is exposed to amino acid starvation, an increase in uncharged tRNAs will lead to ribosome stalling on transcripts. However, what occurs after this at a regulatory level is not known. Descriptively, it leads to a persistent growth state characterized by a cessation in cell division and aberrantly enlarged organisms that remain viable (14, 25). Thus, understanding how *Chlamydia* responds to Trp starvation will facilitate our understanding of chlamydial persistence more generally.

Stringent response (so-called "relaxed") mutants have been created in other bacteria to study the effects of this system. The main characteristic of these mutants is that they do not downregulate stable RNA synthesis in response to amino acid starvation (e.g., see references 63–66). Some mutants have been examined by large-scale transcriptomic analyses but not in the context of codon influence on transcription. Also not clear is whether these studies used appropriate normalization methods given the tendency to rely on 16S rRNA or relative expression to another gene as a normalizer (e.g., see references 67–70). We previously observed that *Chlamydia* does not downregulate stable RNA synthesis during IFN- γ -mediated Trp limitation (42), and as such, this would qualify *Chlamydia* as a relaxed mutant.

Why would *Chlamydia* eliminate the stringent response when it is clearly affected by nutrient limitation? Interestingly, different chlamydial species have adapted to respond to the IFN- γ effectors of their host species. For example, in the mouse, IFN- γ does not induce IDO (27); thus, *Chlamydia muridarum* does not encode mechanisms for responding specifically to Trp. In the guinea pig, IFN- γ does induce IDO, but *Chlamydothrix caviae* contains an operon that recycles kynurenine, the metabolic product of IDO activity, to Trp (71). In humans, as described, IFN- γ induces IDO.

C. trachomatis has eliminated most Trp biosynthetic genes, with the exception of the terminal genes (*trpBA*) in the operon that use indole to produce Trp (45). One hypothesis is that *C. trachomatis* will use indole generated by normal flora (in the female genital tract) to withstand the IDO-mediated Trp-limiting environment, and thus, it would never be exposed to Trp starvation (72, 73). Thus, for all these species, it is easy to envision the advantage in eliminating the now superfluous stringent response. However, once *C. trachomatis* ascends the genital tract and reaches areas that are devoid of normal flora producing indole, it will become persistent. In this scenario, persistent *C. trachomatis* could contribute to chronic sequelae without producing infectious EBs. *C. pneumoniae*, on the other hand, does not carry *trpBA*, suggesting that the respiratory tract is an unlikely source of indole and may explain why *C. pneumoniae* becomes persistent more readily.

We previously observed that chlamydial transcription was globally increased even though translation was generally decreased during IFN- γ -mediated persistence (42). That a bacterium would globally increase transcription in response to a stress is difficult to rationalize. We hypothesized that the Trp codon content of a given gene would determine its transcription during Trp limitation. We analyzed the transcription of 25 genes (2.3% of the genome) by RT-qPCR and observed patterns of transcription that were generally consistent with the hypothesis. Furthermore, an analysis of prior microarray data based on codon content supported a role for increased abundance of Trp codon-containing transcripts during IFN- γ -mediated persistence. We also made the surprising observation that there was a differential pattern of expression based on the size of the gene, with a correlation between larger genes and downregulation and vice versa.

Not surprisingly, we did find exceptions to our hypothesis. In the first instance, *dnaJ* transcripts were significantly increased 2-fold, even though DnaJ lacks Trp. Why this should be is not clear. DnaJ is a cochaperone of DnaK that functions in proteostasis to limit misfolded proteins (74, 75). Given that Trp limitation leads to a decrease in global translation rates (42), one would anticipate a decreased need for chaperone function under these conditions. Further work will be necessary to understand the promoter elements that lead to the increased transcription of *dnaJ* during Trp limitation in *Chlamydia* as well as what the significance of this finding is.

In a second instance, we observed apparent exceptions to our hypothesis in the context of operons and large genes. For example, *metG* transcripts (when measured at the 5' end upstream of all Trp codons) were unchanged even though MetG encodes eight Trp residues. However, the 5' gene of the operon, *rnhB_1* (*rnhB_1* encodes 0 Trp residues), was also unchanged. These data suggest that the regulatory information controlling transcript levels of operons is encoded within the promoter and determined by the Trp codon content of the 5' gene.

We also analyzed the *ytg* operon because of the Trp content of its proteins and because of our initial microarray data (Fig. 5A). *ytgA* transcripts were dramatically increased, as expected given its high Trp codon content, whereas transcripts for the 3' gene *ytgD* were significantly decreased. Similarly, we observed that the 3' end of *greA* was significantly decreased in abundance compared to the 5' end during IFN- γ -mediated Trp limitation. *greA* encodes 11 Trp residues. Taken together, these data indicate an apparent destabilization of transcripts downstream of Trp codons and are supported by the microarray data showing larger gene transcripts

being less abundant. We hypothesize this is due to one of two factors associated with ribosome stalling at Trp codons: either the 3' end of the transcript is exposed for degradation by RNases or the transcription terminator Rho is activated to stop transcription (i.e., Rho-dependent polarity). We are examining these possibilities.

In further support of the effect of Trp on downstream sequences, we did not observe a positive correlation between the amount of Trp codons within a gene and its overall increased transcript level. For instance, transcripts for *obg* (2 W codons) were more than 8-fold increased compared to those for *nusG* (4 W codons), which were less than 3-fold increased (Table 1). However, these differences may also be explained by the location of the primer sets used. That for *obg* was downstream of a single Trp codon, whereas that for *nusG* was downstream of two Trp codons. Thus, even within a smaller gene, Trp codons may have an effect on overall transcript abundance.

Is this transcriptional response unique to *Chlamydia*, or does it represent a typical relaxed (e.g., *relA*) mutant? Interestingly, *Chlamydia* has maintained the gene *dksA*, which is a cofactor or facilitator of the stringent response in other bacteria that possess this system. In some instances, overexpression of DksA can compensate for the lack of a stringent response (77). However, we observed a decrease in *dksA* transcripts; thus, this is not its likely function in *Chlamydia*. Rather, DksA in *Chlamydia* may be critical for transcription elongation and mediating conflicts between the replication and transcriptional machineries (78). Further work will be necessary to determine the role of DksA in *Chlamydia*.

The specific mechanism that controls this unusual response of *Chlamydia* to amino acid starvation remains to be determined. Comparing and contrasting the responses of *C. pneumoniae* and *C. trachomatis* may aid in this. How does the Trp content of a gene feed back to influence transcription? Is it a sequence-encoded event, or is there a global regulator controlling this response? Is this response the consequence of the inability to translate key transcription factors? Recent advances in chlamydial genetic approaches should allow these questions to be addressed (79), and we are currently implementing such strategies.

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We have no conflict of interest to declare.

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