The tryptophan salvage pathway is dynamically regulated by the iron-dependent repressor YtgR in *Chlamydia trachomatis*

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Abstract

Mammalian hosts restrict cellular nutrient availability to starve invading pathogens and successfully clear an infection by a process termed “nutritional immunity”. For the obligate intracellular pathogen *Chlamydia trachomatis*, nutritional immunity likely encompasses the simultaneous limitation of the amino acid tryptophan and the essential biometal iron. Unlike other model bacteria, *C. trachomatis* lacks many global stress response systems – such as “stringent response” homologs – adapted to these host insults. However, a physiological response by *Chlamydia* that is common to both stresses is the development of an aberrant, “persistent” state, suggesting that tryptophan and iron starvation trigger a coordinated developmental program. Here, we report that the *trpRBA* operon for tryptophan salvage in *C. trachomatis* serovar L2 is regulated at the transcriptional level by iron. The expression of the tryptophan synthase encoding genes, *trpBA*, is induced following iron limitation while that of the repressor *trpR* is not. We show that this specific induction of *trpBA* expression initiates from a novel promoter element within an intergenic region flanked by *trpR* and *trpB*. YtgR, a DtxR-homolog and the only known iron-dependent transcriptional regulator in *Chlamydia*, can bind to the *trpRBA* intergenic region upstream of the alternative *trpBA* promoter to repress transcription. This binding also likely attenuates transcription from the primary promoter upstream of *trpR* by blocking RNA polymerase read-through. These data illustrate a dynamic and integrated method of regulating tryptophan biosynthesis in an iron-dependent manner, which has not been described in any other prokaryote, underscoring the uniqueness of *Chlamydia*.

Significance Statement

Genital serovars of the obligate intracellular parasite *Chlamydia trachomatis* are the leading cause of bacterial sexually-transmitted infections globally. Proliferation of *C. trachomatis* is likely controlled by simultaneous immunological and environmental restriction of critical nutrients such as tryptophan and iron. However, our understanding of the immediate chlamydial responses to these stimuli is poorly defined. We utilized expression of the stress-responsive *trpRBA* operon in *C. trachomatis* L2 as a proxy for regulatory integration between iron and tryptophan limitation. We identified a unique iron-dependent regulatory mechanism for *trpRBA* in *C. trachomatis*, mediated by the transcriptional repressor YtgR. This distinguishes *Chlamydia* from other bacteria which regulate tryptophan biosynthesis strictly by tryptophan-dependent mechanisms, highlighting a distinct evolutionary adaptation in *C. trachomatis* to integrate stress responses.

/body

Introduction

Nutrient acquisition is critical for the success of pathogenic bacteria. Many pathogenic bacteria must siphon nutrients from their hosts, such as nucleotides, amino acids and biometals (1–4). This common feature among pathogens renders them susceptible to nutrient limitation strategies associated with the host immune response (5). Counteractively, bacterial pathogens have evolved sophisticated molecular mechanisms to counter nutrient deprivation, involving increasingly complex and sophisticated nutrient-sensing regulatory networks. These stress response mechanisms are essential for pathogens to avoid clearance by the immune system. By delineating their function at the molecular level, we can better target aspects of the host-pathogen interface suitable for therapeutic manipulation. However, stress responses in the obligate intracellular bacterium *Chlamydia trachomatis* are relatively poorly characterized, leaving unanswered many fundamental questions about the biology of this pathogen.

Chlamydia trachomatis is the leading cause of bacterial sexually transmitted infections (STIs) and infection-derived blindness worldwide (6–8). Genital infections of chlamydia are associated with serious sequelae in the female reproductive tract such as tubal factor infertility (9). Chlamydiae are Gram-negative bacterial parasites that develop within a pathogen-specified membrane-bound organelle termed the inclusion (10). Chlamydial development is uniquely characterized by a biphasic interconversion of an infectious elementary body (EB) with a non-infectious, but replicative reticulate body (RB) (11). An obligate intracellular lifestyle has led to reductive genome evolution

across chlamydial species; Chlamydiae have retained genes uniquely required for their survival, but have become nutritionally dependent on their hosts by discarding many metabolism-related genes (12). Of note, *C. trachomatis* does not possess genes necessary for eliciting a stringent response to nutrient starvation (*e.g. relA, spoT*), suggesting that this pathogen may utilize novel mechanisms to respond to stress (13).

It is well established, however, that in response to various stressors, Chlamydiae deviate from their normal developmental program to initiate an aberrant developmental state, termed “persistence” (14). This persistent state is distinguished by the presence of viable, but non-cultivable, abnormally enlarged chlamydial organisms that display dysregulated gene expression. Importantly, *Chlamydia* can be reactivated from persistence by abatement of the stress condition. As such, chlamydial persistence at least superficially resembles a global stress response mechanism. Yet the molecular underpinnings of this phenotype are poorly understood, with most published studies focusing on the molecular and metabolic character of the aberrant, persistent form. It is therefore unclear to what extent primary stress responses contribute to the global persistent phenotype in *Chlamydia*.

The best described inducer of persistence is the pro-inflammatory cytokine interferon-gamma (IFN-γ). The bacteriostatic effect of IFN-γ has been primarily attributed to host cell tryptophan (Trp) catabolism, for which *C. trachomatis* is auxotrophic (15–17). Following IFN-γ stimulation, infected host cells up-regulate expression of indoleamine-2,3-dioxygenase (IDO1), which catabolizes Trp to *N*-formylkynurenine via cleavage of the indole ring (18). *C. trachomatis* cannot recycle kynurenines, unlike some other chlamydial species (19), and thus IFN-γ stimulation

effectively results in Trp starvation to *C. trachomatis*. The primary response to Trp starvation in *C. trachomatis* is mediated by a TrpR ortholog, whose Trp-dependent binding to cognate promoter elements represses transcription (20, 21). This mechanism of regulatory control is presumably limited in *C. trachomatis*, as homologs of genes regulated by TrpR in other bacteria (*e.g. trpF, aroH, aroL*) have not been shown to respond to Trp limitation (22).

In many Gram-negative bacteria, such as *Escherichia coli*, *trpR* is monocistronic and distal to the Trp biosynthetic operon. In *C. trachomatis,* TrpR is encoded in an operon, *trpRBA*, which also contains the Trp synthase α- and β- subunits (TrpA and TrpB, respectively), and possesses a 351 base-pair (bp) intergenic region (IGR) that separates *trpR* from *trpBA*. The functional significance of the *trpRBA* IGR is poorly characterized; while a putative operator sequence was identified overlapping an alternative transcriptional origin for *trpBA* (20), the IGR was not shown to be bound by TrpR (21). Based on *in silico* predictions, an attenuator sequence has been annotated within the *trpRBA* IGR (23), but this has not been thoroughly validated experimentally. Regardless, the IGR is more than 99% conserved at the nucleotide sequence level across ocular, genital and lymphogranuloma venereum (LGV) serovars of *C. trachomatis*, indicating functional importance (Fig. S1). Therefore, relative to other model bacteria, the regulation of the *trpRBA* operon remains poorly elucidated.

In evaluating alternative regulatory modes of the *trpRBA* operon, an interesting consideration is the pleiotropic effects induced by IFN-γ stimulation of infected cells. IFN-γ is involved in many processes that limit iron and other essential biometals to intracellular pathogens as a component of host nutritional immunity (5, 24). *Chlamydia*

have a strict iron dependence for normal development, evidenced by the onset of persistence following prolonged iron limitation (25). Importantly, *Chlamydia* presumably acquire iron via vesicular interactions between the chlamydial inclusion and slow-recycling transferrin (Tf)-containing endosomes (26). IFN-γ is known to down-regulate transferrin receptor (TfR) expression in both monocytes and epithelial cells with replicative consequences for resident intracellular bacteria (27–30). However, iron homeostasis in *Chlamydia* is poorly understood, due to the lack of functionally characterized homologs to iron acquisition machinery that are highly conserved in other bacteria (31). Only one operon, represented by *ytgABCD*, has been clearly linked to iron acquisition. The periplasmic metal-binding protein YtgA displays a specific binding affinity for ferric iron over other divalent cations and likely transports iron from the outer membrane to an ABC-3 type inner membrane metal permease formed by the YtgBCD complex (32). Intriguingly, the YtgC (CTL0323) open reading frame (ORF) encodes a N-terminal permease domain fused to a C-terminal DtxR-like repressor domain, annotated YtgR (33, 34). YtgR is cleaved from the permease domain during infection and functions as an iron-dependent transcriptional repressor to autoregulate the expression of its own operon (34). YtgR represents the only identified iron-dependent transcriptional regulator in *Chlamydia.* Whether YtgR maintains a more diverse transcriptional regulon beyond the *ytgABCD* operon has not yet been addressed and remains an intriguing question in the context of immune-mediated iron limitation to *C. trachomatis*. Crucially, simultaneous IFN-γ-mediated iron and Trp starvation raise the possibility that *C. trachomatis* could have evolved an integrated response to multiple stresses.

Consistent with the highly reduced capacity of the chlamydial genome, it is likely that *C. trachomatis* has a limited ability to tailor a specific response to each individual stress. In the absence of identifiable homologs for most global stress response regulators in *C. trachomatis*, we hypothesized that primary stress responses to pleiotropic insults may involve mechanisms of regulatory integration, whereby important molecular pathways are co-regulated by stress-responsive transcription factors such that they can be utilized across multiple stress conditions simultaneously induced by the host. Here, we report on the unique iron-dependent regulation of the *trpRBA* operon in *Chlamydia trachomatis.* We propose a model of iron-dependent transcriptional regulation of *trpRBA* mediated by the repressor YtgR binding specifically to the IGR, which may have implications for how *C. trachomatis* responds to immunological and environmental insults. Such a mechanism of iron-dependent regulation of Trp biosynthesis has not been previously described in any other prokaryote and adds to the catalog of regulatory models for Trp biosynthetic operons in bacteria. Further, it reveals a highly dynamic mode of regulatory integration within the *trpRBA* operon, exemplifying the importance of this pathway to chlamydial stress response.

Results

Brief iron limitation via 2,2-bipyridyl treatment yields iron-starved, but non-persistent *Chlamydia trachomatis.* To identify possible instances of regulatory integration between iron and Trp starvation in *C. trachomatis*, we optimized a stress response condition that preceded the development of a characteristically persistent phenotype. We reasoned that in order to effectively identify regulatory integration, we would need to investigate the bacterium under stressed, but not aberrant, growth

conditions such that we could distinguish primary stress responses from abnormal growth. To specifically investigate the possible contribution of iron limitation to a broader immunological (*e.g.* IFN-γ-mediated) stress, we utilized the membrane-permeable iron chelator 2,2-bipyridyl (Bpdl), which has the advantage of rapidly and homogeneously starving *C. trachomatis* of iron (35). We chose to starve *C. trachomatis* serovar L2 of iron starting at 12 hrs post-infection (hpi), or roughly at the beginning of mid-cycle growth. At this point the chlamydial organisms represent a uniform population of replicative RBs that are fully competent, both transcriptionally and translationally, to respond to stress. We treated infected HeLa cell cultures with 100 μM Bpdl or mock for either 6 or 12 hours (hrs) to determine a condition sufficient to limit iron to *C. trachomatis* without inducing hallmark persistent phenotypes. We stained infected cells seeded on glass coverslips with convalescent human sera and analyzed chlamydial inclusion morphology under both Bpdl- and mock-treated conditions by laser point-scanning confocal microscopy (Fig. 1A). Following 6 hrs of Bpdl treatment, chlamydial inclusions were largely indistinguishable from mock-treated inclusions, containing a homogeneous population of larger organisms, consistent with RBs in mid-cycle growth. However, by 12 hrs of Bpdl treatment, the inclusions began to display signs of aberrant growth: they were perceptibly smaller, more comparable in size to 18 hpi, and contained noticeably fewer organisms, perhaps indicating a delay in RB-to-EB differentiation. These observations were consistent with our subsequent analysis of genome replication by quantitative PCR (qPCR; Fig. 1B.) At 6 hrs of Bpdl treatment, there was no statistically distinguishable difference in genome copy number when compared to the equivalent mock-treated time-point. However, by 12 hrs of treatment, genome copy

number was significantly reduced 4.7-fold in the Bpdl-treated group relative to mock-treatment (p = 0.0033). We then assayed the transcript expression of two markers for persistence by reverse transcription quantitative PCR (RT-qPCR): the early gene *euo*, encoding a transcriptional repressor of late-cycle genes (Fig.1C), and the adhesin *omcB,* which is expressed late in the developmental cycle (Fig. 1D). Characteristic persistence would feature sustained high *euo* expression late into infection, and suppressed *omcB* expression throughout development. We observed that at 6 hrs of Bpdl treatment, there was no statistically distinguishable difference in either *euo* or *omcB* expression when compared to the mock-treatment. Still at 12 hrs of Bpdl treatment, *euo* expression was unchanged. However, *omcB* expression was significantly induced following 12 hrs of Bpdl-treatment (p = 0.00015). This was unexpected, but we note that *omcB* expression has been shown to vary between chlamydial serovars and species when starved for iron (31). Collectively, these data indicated that 6 hrs of Bpdl treatment was a more suitable time-point at which to monitor iron-limited stress responses.

We additionally assayed these same metrics following 6 or 12 hrs of Trp starvation by culturing cells in either Trp-replete or Trp-deplete DMEM-F12 media supplemented with fetal bovine serum (FBS) pre-dialyzed to remove amino acids. We observed no discernable change in inclusion morphology out to 12 hrs of Trp starvation (Fig. S2A), but genome copy numbers were significantly reduced 2.7-fold at this time-point (p = 0.00612; Fig. S2B). The transcript expression of *euo* (Fig. S2C) and *omcB* (Fig. S2D) did not significantly change at either treatment duration, but Trp-depletion did result in a 2.0-fold reduction in *omcB* expression, consistent with a more characteristic

persistent phenotype. These data therefore also indicated that 6 hrs of treatment would be ideal to monitor non-persistent responses to Trp limitation.

We next sought to determine whether our brief 6-hr Bpdl treatment was sufficient to elicit a transcriptional iron starvation phenotype. We chose to analyze the expression of three previously identified iron-regulated transcripts, *ytgA* (Fig. 2A), *ahpC* (Fig. 2B) and *devB* (Fig. 2C), by RT-qPCR under Bpdl- and mock-treated conditions (35, 36). In addition, we analyzed the expression of one non-iron-regulated transcript, *dnaB* (Fig. 2D), as a negative control (37). Following 6 hrs of Bpdl treatment, we observed that the transcript expression of the periplasmic iron-binding protein *ytgA* was significantly elevated 1.75-fold relative to the equivalent mock-treated time-point ($p = 0.0052$). However, we did not observe induction of *ytgA* transcript expression relative to the 12 hpi time-point. We distinguish here between *elevated* and *induced* transcript expression, as chlamydial gene expression is highly developmentally regulated. Thus, it can be more informative to monitor longitudinal expression of genes, *i.e.* their induction, as opposed to elevation relative to an equivalent control time-point, which may simply represent a stall in development. While we did not observe induction of *ytgA*, which would be more consistent with an iron-starved phenotype, we reason that this is a consequence of the brief treatment period, and that longer iron starvation would produce a more robust induction of iron-regulated transcripts. Note that the identification of *ytgA* as iron-regulated has only been previously observed following extended periods of iron chelation (32, 35, 38). Similarly, we observed that the transcript expression of the thioredoxin *ahpC* was significantly elevated 2.15-fold relative to the equivalent mock-treated time-point ($p = 0.038$) but was not induced relative to the 12 hpi time-point. The

transcript expression of *devB*, encoding a 6-phosphogluconolactonase involved in the pentose phosphate pathway, was not observed to significantly respond to our brief iron limitation condition, suggesting that it is not a component of the primary iron starvation stress response in *C. trachomatis.* As expected, the transcript expression of *dnaB*, a replicative DNA helicase, was not altered by our iron starvation condition, consistent with its presumably iron-independent regulation (37). Overall, these data confirmed that our 6-hr Bpdl treatment condition was suitable to produce a mild iron starvation phenotype at the transcriptional level, thus facilitating our investigation of iron-dependent regulatory integration.

Transcript expression of the *trpRBA* operon is differentially regulated by iron in *Chlamydia trachomatis.* Upon identifying an iron limitation condition that produced a relevant transcriptional phenotype while avoiding the onset of persistent development, we aimed to investigate whether the immediate response to iron starvation in *C. trachomatis* would result in the consistent induction of pathways unrelated to iron utilization/acquisition, but nevertheless important for surviving immunological stress. The truncated Trp biosynthetic operon, *trpRBA* (Fig. 3A), has been repeatedly linked to the ability of genital and LGV serovars (D-K and L1-3, respectively) of *C. trachomatis* to counter IFN-γ-mediated stress. This is due to the capacity of the chlamydial Trp synthase in these serovars to catalyze the β synthase reaction, *i.e.* the condensation of indole to the amino acid serine to form Trp (17). In the presence of exogenous indole, *C. trachomatis* is therefore able to biosynthesize Trp such that it can prevent the development of IFN-γ-mediated persistence. Correspondingly, the expression of *trpRBA* is highly induced following IFN-γ stimulation of infected cells (39, 40). These data have

historically implicated Trp starvation as the primary mechanism by which persistence develops in *C. trachomatis* following exposure to IFN-γ. However, these studies have routinely depended on prolonged treatment conditions that monitor the terminal effect of persistent development, as opposed to the immediate molecular events which may have important roles in the developmental fate of *Chlamydia.* As such, these studies may have missed the contribution of other IFN-γ-stimulated insults such as iron limitation.

To decouple Trp limitation from iron limitation and assess their relative contribution to regulating a critical pathway for responding to IFN-γ-mediated stress, we monitored the transcript expression of the *trpRBA* operon under brief Trp or iron starvation by RT-qPCR. When starved for Trp for 6 hrs, we observed that the expression of *trpR*, *trpB* and *trpA* were all significantly induced greater than 10.5-fold relative to 12 hpi (p = 0.00077, 0.025 and 9.7e-5, respectively; Fig. 3B). All three ORFs were also significantly elevated relative to the equivalent mock-treated time-point (p = 0.00076, 0.025 and 9.7e-5, respectively). This result was surprising with respect to the relative immediacy of operon induction in response to our Trp starvation protocol, confirming the relevant Trp-starved transcriptional phenotype. To induce Trp-deprived persistence in *C. trachomatis*, many laboratories rely on compounded techniques of IFN-γ pre-treatment to deplete host Trp pools in conjunction with culturing in Trp-depleted media, among other strategies. While the phenotypic end-point differs here, it is nonetheless interesting to note that only 6 hrs of media replacement is sufficient to markedly up-regulate *trpRBA* expression. This suggests that *C. trachomatis* has a highly attuned sensitivity to even moderate changes in Trp levels.

We next performed the same RT-qPCR analysis on the expression of the *trpRBA* operon in response to 6 hrs of iron limitation via Bpdl treatment (Fig. 3C). While we observed that the transcript expression of all three ORFs was significantly elevated at least 2.1-fold relative to the equivalent mock-treated time-point (p = 0.015, 0.00098 and 0.0062, respectively), we made the intriguing observation that only the expression *trpB* and *trpA* was significantly induced relative to 12 hpi (p = 0.00383 and 0.0195, respectively). The significant induction of *trpBA* expression, but not *trpR* expression, suggested that *trpBA* are specifically regulated by iron availability. This result is consistent with a recent survey of the iron-regulated transcriptome in *C. trachomatis* by RNA sequencing, which also reported that iron-starved *Chlamydia* specifically up-regulate *trpBA* expression in the absence of altered *trpR* expression (37). Our results expand on this finding by providing a more detailed investigation into the specific profile of this differential regulation of *trpRBA* in response to iron deprivation. Taken together, these findings demonstrated that an important stress response pathway, the *trpRBA* operon, is regulated by the availability of both Trp and iron, consistent with the notion that the pathway may be cooperatively regulated to respond to various stress conditions. Notably, iron-dependent regulation of Trp biosynthesis has not been previously documented in other prokaryotes.

Specific iron-regulated expression of *trpBA* originates from a novel alternative transcriptional start site within the *trpRBA* intergenic region. We hypothesized that the specific iron-related induction of *trpBA* expression relative to *trpR* expression may be attributable to an iron-regulated alternative transcriptional start site (alt. TSS) downstream of the *trpR* ORF. Indeed, a previous study reported the presence of an alt.

TSS in the *trpRBA* IGR, located 214 nucleotides upstream of the *trpB* translation start position (20). However, a parallel study could not identify a TrpR binding site in the *trpRBA* IGR (21). We reasoned that a similar alt. TSS may exist in the IGR that controlled the iron-dependent expression of *trpBA.* We therefore performed Rapid Amplification of 5'-cDNA Ends (5'-RACE) on RNA isolated from *C. trachomatis* L2-infected HeLa cells using the SMARTer 5'/3' RACE Kit workflow (Takara Bio). Given the low expression of the *trpRBA* operon during normal development, we utilized two sequential gene-specific amplification steps (nested 5'-RACE) to identify 5' cDNA ends in the *trpRBA* operon. These nested RACE conditions resulted in amplification that was specific to infected-cells (Fig. S3A). Using this approach, we analyzed four conditions: 12 hpi, 18 hpi, 12 hpi + 6 hrs of Bpdl treatment, and 12 hpi + 6 hrs of Trp-depletion (Fig. 4A). We observed three RACE products that migrated with an apparent size of 1.5, 1.1 and 1.0 kilobases (kb). At 12 and 18 hpi, all three RACE products exhibited low abundance, even following the nested PCR amplification. This observation was consistent with the expectation that the expression of the *trpRBA* operon is very low under normal, iron and Trp-replete conditions. However, we note that the 6-hr difference in development did appear to alter the representation of the 5' cDNA ends, which may suggest a stage-specific promoter utilization within the *trpRBA* operon. In our Trp starvation condition, we observed an apparent increase in the abundance of the 1.5 kb RACE product, which was therefore presumed to represent the primary TSS upstream of *trpR*, at nucleotide position 511,389 (*C. trachomatis* L2 434/Bu). Interestingly, the 1.0 kb product displayed a very similar apparent enrichment following Bpdl treatment, suggesting that this RACE product represented a specifically iron-regulated TSS. Both

the 1.5 and 1.0 kb RACE products were detectable in the Trp-depleted and iron-depleted conditions, respectively, during the primary RACE amplification, consistent with their induction under these conditions (Fig. S3B).

If iron depletion was inducing *trpBA* expression independent of *trpR,* we reasoned that we would observe specific enrichment of *trpB* sequences in our 5'-RACE cDNA samples relative to *trpR* sequences. We again utilized RT-qPCR to quantify the abundance of *trpB* transcripts relative to *trpR* transcripts in the 5'-RACE cDNA samples (Fig. 4B). In agreement with our model, only under iron starved conditions did we observe a significant enrichment of *trpB* relative to *trpR* ($p < 0.01$). Additionally, we observed that at 12 and 18 hpi in iron-replete conditions, the ratio of *trpB* to *trpR* was approximately 1.0, suggesting non-preferential basal expression across the three putative TSSs. Another factor contributing to this ratio is the synthesis of the full-length *trpRBA* polycistron. In support of this, the *trpB* to *trpR* ratio remained near 1.0 under the Trp-starved condition, which would be expected during transcription read-through of the whole operon. The apparent lack of preferential promoter utilization as described above could be attributed to the relatively low basal expression of the operon at 12 and 18 hpi under Trp- and iron-replete conditions, thus precluding quantitative detection of differential promoter utilization in this assay.

To determine the specific location of the 5' cDNA ends within the *trpRBA* operon, we isolated the 5'-RACE products across all conditions by gel extraction and cloned the products into the pRACE vector supplied by the manufacturer. We then sequenced the ligated inserts and BLASTed the sequences against the *C. trachomatis* L2 434/Bu genome to identify the location of the 5'-most nucleotides (Fig. 4C). These data are

displayed as a statistical approximation of the genomic regions most likely to be represented by the respective 5'-RACE products in both histogram (semi-continuous) and density plot (continuous) format (See Dataset S1 for a description of all mapped 5'-RACE products). As expected, the 1.5 kb product mapped in a distinct and tightly grouped peak near the previously annotated *trpR* TSS, with the mean and modal nucleotide being 511,388 and 511,389, respectively (Fig. S4A). Surprisingly, we found that neither the 1.1 or 1.0 kb RACE product mapped to the previously reported alt. TSS in the *trpRBA* IGR, at position 511,826. Instead, we observed that the 1.1 kb product mapped on average to nucleotide position 511,878, with the modal nucleotide being found at 511,898 (Fig. S4B). The 1.0 kb product mapped with a mean nucleotide position of 512,013, with the modal nucleotide being 512,005 (Fig. S4C), only 35 bases upstream of the *trpB* coding sequence. Interestingly, the 1.0 kb product mapped to a region of the *trpRBA* IGR flanked by consensus σ^{66} -10 and -35 promoter elements, found at positions 512,020-5 and 511,992-7, respectively (41). These data collectively pointed toward the 1.0 kb 5'-RACE product representing a novel, iron-regulated alt. TSS and *bona fide* σ^{66}-dependent promoter element that allows for the specific iron-dependent expression of *trpBA*.

YtgR specifically binds to the *trpRBA* intergenic region in an operator-dependent manner to repress transcription of *trpBA*. As the only known iron-dependent transcriptional regulator in *Chlamydia*, we hypothesized that YtgR may regulate the iron-dependent expression of *trpBA* from the putative promoter element we characterized by 5'-RACE. Using bioinformatic sequence analysis, we investigated whether the *trpRBA* IGR contained a candidate YtgR operator sequence. By local sequence alignment of

the putative YtgR operator sequence (33) and the *trpRBA* IGR, we identified a high-identity alignment (76.9% identity) covering 67% of the putative operator sequence (Fig. 5A). Interestingly, this alignment mapped to the previously identified palindrome suspected to have operator functionality (20). By global sequence alignment of the YtgR operator to the palindromic sequence, an alignment identical to the local alignment was observed, which still displayed relatively high sequence identity (43.5% identity). We hypothesized that this sequence functioned as an YtgR operator, despite being located 184 bp upstream of the *trpBA* alt. TSS.

To investigate the ability of YtgR to bind and repress transcription from the putative *trpBA* promoter, we implemented a heterologous two-plasmid assay that reports on YtgR repressor activity as a function of β-galactosidase expression (14). In brief, a candidate DNA promoter element was cloned into the pCCT expression vector between an arabinose-inducible pBAD promoter and the reporter gene *lacZ*. This plasmid was co-transformed into BL21 (DE3) *E. coli* along with an IPTG-inducible pET151 expression vector with (pET151-YtgR) or without (pET151-EV) the C-terminal 139 amino acid residues of CTL0323 (YtgC). Note that we have previously demonstrated that this region is a functional iron-dependent repressor domain (34). To verify the functionality of this assay, we determined whether ectopic YtgR expression could repress pCCT reporter gene expression in the presence of three candidate DNA elements: a no-insert empty vector (pCCT-EV), the putative promoter element for *C. trachomatis lpdA* (pCCT-*lpdA*), and the promoter region of the *ytgABCD* operon (pCCT-*ytgABCD*; Fig. 5B). As expected, from the pCCT-EV reporter construct, ectopic YtgR expression did not significantly reduce the activity of β-galactosidase. Additionally,

reporter gene expression from pCCT-*lpdA*, containing the promoter of iron-regulated *lpdA* (37), which is not known to be YtgR-regulated, was not affected by ectopic expression of YtgR. This demonstrated that the assay can discriminate between the promoter elements of iron-regulated genes and *bona fide* YtgR targeted promoters. Indeed, in the presence of pCCT-*ytgABCD*, induction of YtgR expression produced a significant decrease in β-galactosidase activity (p = 0.03868) consistent with its previously reported auto-regulation of this promoter (34).

Using this same assay, we then inserted into the pCCT reporter plasmid 1) the *trpR* promoter element (pCCT-*trpR*), 2) the putative *trpBA* promoter element represented by the IGR (pCCT-*trpBA*), and 3) the same putative *trpBA* promoter element with a mutated YtgR operator sequence that was diminished for both palindromicity and A-T richness, two typical features of prokaryotic promoter elements (pCCT-*trpBA*ΔOperator; Fig. 5C) (42, 43). When YtgR was ectopically expressed in the pCCT-*trpR* background, we observed no statistically distinguishable change in β-galactosidase activity. However, in the pCCT-*trpBA* background, ectopic YtgR expression significantly reduced β-galactosidase activity at levels similar to those observed in the pCCT-*ytgABCD* background (p = 0.01219). This suggested that YtgR was capable of binding to the *trpBA* promoter element specifically. Interestingly, this repression phenotype was abrogated in the pCCT-*trpBA*ΔOperator background, where we observed no statistically meaningful difference in β-galactosidase activity. We subsequently addressed whether the region of the *trpRBA* IGR containing the YtgR operator site was sufficient to confer YtgR repression in this assay (Fig. S5). Therefore, we cloned three fragments of the *trpRBA* IGR into the pCCT reporter plasmid: the first

fragment represented the 5'-end of the IGR containing the operator site at the 3'-end (pCCT-IGR1), the second fragment represented a central region of the IGR containing the operator site at the 5'-end (pCCT-IGR2), and the third fragment represented the 3'-end of the IGR and did not contain the operator site (pCCT-IGR3). Surprisingly, we observed that none of these fragments alone were capable of producing a significant repression phenotype in our reporter system. This finding indicated that while the operator site was necessary for YtgR repression, it alone was not sufficient. Together, these data indicated that YtgR could bind to the *trpBA* promoter element and that this binding was dependent upon an intact AT-rich palindromic sequence, likely representing an YtgR operator, but that further structural elements in the *trpRBA* IGR may be necessary for repression. Nonetheless, we demonstrated the existence of a functional YtgR binding site that conferred iron-dependent transcriptional regulation to *trpBA*, independent of the major *trpR* promoter.

Iron limitation promotes transcription read-through at the *trpRBA* YtgR operator site. If YtgR binds to the operator sequence within the intergenic region under iron-replete conditions, does it influence RNA polymerase (RNAP) read-through from the major *trpR* promoter to synthesize the polycistronic *trpRBA* mRNA? We hypothesized that the presence of YtgR at the *trpRBA* operator may disadvantage the processivity of RNAP initiating transcription at the upstream *trpR* promoter. Similar systems of RNAP read-through blockage have been reported; the transcription factor Reb1p "roadblocks" RNAPII transcription read-through in yeast by a mechanism of RNAP pausing and subsequent labelling for degradation (44). To investigate this question, we first identified transcription termination sites (TTSs) in the *trpRBA* operon in *C. trachomatis.* We

utilized 3'-RACE to map the 3'-ends of transcripts using gene-specific primers within the *trpR* CDS (Fig. 6A; bottom). We again utilized two RACE amplification cycles to generate distinct, specific bands suitable for isolation and sequencing (Fig. S6B-C). By gel electrophoresis of the 3'-RACE products, we observed the appearance of four distinct bands that migrated with an apparent size of 0.55, 0.45, 0.40 and 0.20 kb. In our Trp-depleted condition, we observed only a very weak amplification of the 2.5 – 3 kb full-length *trpRBA* message by 3'-RACE (Fig. S6A). However, we did observe it across all replicates. To confirm that the full-length product was relatively specific to the Trp-deplete treatment, we amplified the *trpRBA* operon by RT-PCR from the 3'-RACE cDNA (Fig. 6A; top). As expected, only in the Trp-deplete sample did we observe robust amplification of the full-length *trpRBA* message. We note however that image contrast adjustment reveals a very weak band present in all experimental samples.

To identify the specific TTS locations, we gel extracted the four distinct 3'-RACE bands across all conditions and cloned them into the pRACE sequencing vector as was done for the 5'-RACE experiments. We then sequenced the inserted RACE products and mapped them to the *C. trachomatis* L2 434/Bu genome (Fig. 6B). This revealed a highly dynamic TTS landscape contained almost exclusively within the *trpRBA* IGR, which has not previously been investigated (For a full description of mapped 3'-RACE products, see Dataset S2). The 0.20 kb RACE product mapped tightly to the 3'-end of the *trpR* CDS, with a mean nucleotide position of 511,665 and a modal nucleotide position of 511,667 (Fig. S7A). Contrastingly, the other three RACE products did not map in such a way so as to produce specific, unambiguous modal peaks. Instead, their distribution was broader and more even, with only a few nucleotide positions mapping

more than once. Accordingly, the 0.45 kb product mapped with an average nucleotide position of 511,889, just downstream of the 1.1 kb 5'-RACE product (Fig. S7C), while the 0.55 kb product mapped with an average nucleotide position of 511,986, upstream of the 1.0 kb 5'-RACE product (Fig. S7D). Interestingly, the 0.40 kb product mapped to a region directly overlapping the putative YtgR operator site, with a mean nucleotide position of 511,811 (Fig. S7B). We therefore reasoned that this putative TTS may have an iron-dependent function.

We next aimed to quantitatively analyze the possibility that iron-depletion, and thus dissociation of YtgR from this region, may facilitate transcription read-through at the operator site. Working from the 3'-RACE generated cDNAs, we utilized RT-qPCR to monitor the abundance of various amplicons across the *trpRBA* operon in relation to a "read-through" normalization amplicon that should only be represented when the full-length *trpRBA* message is transcribed (Fig. 6C). Therefore, as each amplicon is increasingly represented as a portion of the full-length, read-through transcript, the representation ratio of the specific amplicon to the normalization amplicon should approach 1.0. We first analyzed an amplicon from nucleotide 511,416 – 531 to monitor transcript species associated with transcription initiating at the *trpR* promoter. We observed that the representation of this amplicon was not significantly altered following iron limitation relative to 12 hpi, suggesting that the depletion of iron was not affecting initiation of transcription at the *trpR* promoter. Interestingly, at 18 hpi, the representation ratio of this amplicon significantly shifted further away from 1.0 (p = 0.00358), indicating that at 18 hpi this amplicon is represented less as a component of read-through transcription relative to 12 hpi. As expected, under Trp-deplete conditions, the

representation ratio shifted significantly closer to 1.0 (p = 0.00064), consistent with read-through transcription of the full-length *trpRBA* message.

We then preformed the same analysis on an amplicon from nucleotide 511,639 – 764, immediately upstream of the TTS at the YtgR operator site. We again observed that at 18 hpi, the representation ratio was significantly increased (p = 0.01046), and following Trp-depletion, the ratio was significantly decreased (p = 0.00023), as expected. Notably, and consistent with our hypothesis, we observed that the representation ratio of this amplicon was also significantly closer to 1.0 following iron limitation (p = 0.00407), suggesting that transcription read-through was increased at this site under iron limited conditions. If YtgR is dissociating from the operator site during iron depletion, a greater proportion of transcripts would be expected to read-through this locus.

Finally, we analyzed an amplicon from nucleotide 513,856 – 968, at the very 3'-end of *trpA* to monitor terminal transcription under our experimental conditions. At 18 hpi, we observed a significant increase in the representation ratio of this amplicon (p = 0.00476), which is likely attributable to both basal levels of alternative transcription from the IGR as well as poor transcription read-through of the full-length message. Following 6 hrs of Bpdl treatment, we also observed a significant increase in the representation ratio of this amplicon (p = 0.01510), which supports the finding that *trpBA* is being preferentially transcribed under this condition, distinct from the full-length *trpRBA* transcript. We were only able to detect a marginal decrease in the representation of this amplicon under Trp-depleted conditions (p = 0.07942), which may suggest that the very 3'-end of *trpRBA* is relatively under-represented than our normalization amplicon, which

falls within the middle of the operon. In fact, recent work has reported on the relatively poor representation of 3'-end mRNAs in *Chlamydia* (45). In sum, this set of experiments provides evidence that iron-depletion specifically alters the representation of particular mRNA species across the *trpRBA* operon. Additionally, they implicate iron-dependent YtgR DNA-binding as the mediator of these effects. By alleviating YtgR repression via iron depletion, transcription is allowed to proceed through the operator site, albeit at basal levels. Concomitantly, transcription is specifically activated at the downstream alt. TSS for *trpBA*.

Discussion

In this study, we report a mechanism of stress adaptation that integrates responses to iron and Trp starvation. Specifically, we demonstrated that the *trpRBA* operon is transcriptionally regulated by the iron-dependent repressor YtgR. We determined that iron-dependent expression of *trpBA* initiates from a novel internal promoter in an IGR of the *trpRBA* operon and that this IGR also contains an YtgR operator site necessary to confer a transcriptional repression phenotype. We suggest that the dual promoter configuration of *trpRBA* presents the opportunity for YtgR to block transcription read-through from the *trpR* promoter; transcripts terminate at the YtgR operator site and iron depletion facilitates read-through of the operon at this locus. This is the first time an iron-dependent mode of regulation has been shown to control the expression of tryptophan biosynthesis in prokaryotes, which is a reflection of the uniquely specialized nature of *C. trachomatis*.

The distance separating the YtgR operator site from the *trpBA* alt. TSS indicate the involvement of a regulatory mechanism more complex than simple steric hindrance

of RNAP by YtgR. One possible explanation is that YtgR functions similarly to other prokaryotic transcription factors that repress “at a distance” by a mechanism of DNA-bending (46, 47). In this scenario, YtgR binding simultaneously to an additional operator site may facilitate a conformational bend in the double-helix DNA such that RNAP no longer has access to the alternative *trpBA* promoter site. This would be consistent with the observation that a truncated *trpRBA* IGR containing the candidate YtgR operator is insufficient to confer transcriptional repression. The topological alteration induced by DNA-bending could also feasibly contribute to diminished RNAP read-through from the *trpR* promoter. *In silico* identification of additional putative YtgR operator sites was unsuccessful, which could be due to the lack of enough validated binding sites to generate a robust consensus sequence. Thus, a more unbiased approach (*e.g.* ChIP-Sequencing) will be required to identify additional YtgR binding events. We also note that the YtgR operator upstream of *ytgA*, while only 21 bp from the predicted -35 element, rests within a 660-bp IGR, raising the possibility that other cryptic YtgR operators are present in this sequence. Another possibility is that YtgR functions in concert with additional transcription factors more proximal to the *trpBA* promoter elements. In *E. coli*, the repressor Fis binds 135 bp upstream of the *nir* promoter TSS, controlling co-activation of *nir* expression by proximally-bound Fnr and NarL/NarP (48). While we have no evidence to suggest other transcription factors are controlling *trpBA* expression, this does not preclude the possibility of their involvement.

While we demonstrate here that iron-dependent *trpBA* expression originates from a novel promoter element immediately upstream of the *trpB* CDS, this is not the first description of an alt. TSS within the *trpRBA* IGR. Carlson, et al. identified an alt. TSS

within the IGR which they suggested was responsible for *trpBA* expression (20). Interestingly, this TSS was observed to originate from within the palindromic sequence that we have identified here as a functional YtgR operator site. In these studies, we were unable to confirm the presence of the previously identified alt. TSS by 5'-RACE. This is likely because Carlson, et al. examined the presence of transcript origins following 24 hrs of Trp starvation whereas here we monitored immediate responses to stress following only 6 hrs of treatment. Prolonged Trp depletion would result in a more homogeneously stressed population of chlamydial organisms that may exhibit the same preferential utilization of the promoter identified by Carlson, et al. Population heterogeneity in response to brief stress may explain the observation of multiple T(S/T)Ss across the *trpRBA* operon in our studies. However, the contribution of such a Trp-dependent alt. TSS to the general stress response of *C. trachomatis* remains unclear. Akers & Tan were unable to verify TrpR binding to the *trpRBA* IGR by EMSA, suggesting that some other Trp-dependent mechanism may control transcription from this site (21). Ultimately, our approach of investigating more immediate responses to stress revealed previously unreported mechanisms functioning to regulate Trp biosynthesis in *C. trachomatis*, underscoring the value of transient as opposed to sustained induction of stress.

Another mechanism of regulation reported to control the chlamydial *trpRBA* operon is Trp-dependent transcription attenuation. Based on sequence analysis, a leader peptide has been annotated within the *trpRBA* IGR (23). Presumably, this functions analogously to the attenuator in the *E. coli trpEDCBA* operon; Trp starvation causes ribosome stalling at sites of enriched Trp codons such that specific RNA

secondary structures form to facilitate RNAP read-thru of downstream sequences – in this case, *trpBA* (49). However, robust experimental evidence to support the existence of attenuation in *C. trachomatis* is lacking. To date, the only experimental evidence that supports this model was reported by Carlson, et al., who demonstrated that transcript expression of *trpBA* is increased following 24 hr Trp-depletion in a *trpR*-mutant strain of *C. trachomatis*, suggesting that an additional level of Trp-dependent regulation controls *trpBA* expression (20). However, this could be attributable to an alternative Trp-dependent mechanism controlling *trpBA* expression at the alt. TSS identified by Carlson, et al. None of the data presented here point conclusively to the existence of a Trp-dependent attenuator; while we acknowledge that the additional termination sites identified in our 3'-RACE assay may represent termination events mediated by an attenuator, without more specific analysis utilizing mutated sequences we cannot draw definitive conclusions about the functional relevance of those termination sites. Additionally, it is unlikely that we would be able to observe Trp-dependent attenuation under our brief stress conditions given that attenuation has a much higher tolerance for Trp-depletion than TrpR-mediated transcriptional repression in *E. coli* (50).

Interestingly, in *Bacillus subtilis,* Trp-dependent attenuation of transcription takes on a form markedly different from that in *E. coli*. Whereas attenuation functions in *cis* for the *E. coli trp* operon, *B. subtilis* utilize a multimeric Tryptophan-activated RNA-binding Attenuation Protein, TRAP, which functions in *trans* to bind *trp* operon RNA under Trp-replete conditions, promoting transcription termination and inhibiting translation (51). This interaction is antagonized by anti-TRAP in the absence of charged $tRNA^{Trp}$, leading to increased expression of TRAP regulated genes. We suggest that YtgR may

621 represent the first instance of a separate and distinct clade of attenuation mechanisms:
622 iron-dependent *trans*-attenuation. We have demonstrated that transcription terminates
623 in the *trpRBA* operon at the YtgR operator site, and that read-thru of the operon is
624 facilitated under iron-deplete conditions, which is consistent with the idea that relief of
625 iron-activated YtgR DNA-binding at this site would permit RNAP to pass through the
626 YtgR operator site. This mechanism may function independently of specific RNA
627 secondary structure, relying instead on steric blockage of RNAP processivity, but
628 ultimately producing a similar result. Possible regulation of translation remains to be
629 explored. The recent development of new genetic tools to alter chromosomal
630 sequences and generate conditional knockouts in *C. trachomatis* should enable a more
631 detailed analysis of *trpRBA* regulation, including possible *trans*-attenuation (52, 53).
632 As a Trp auxotroph, what might be the biological significance of iron-dependent
633 YtgR regulation of the *trpRBA* operon in *C. trachomatis*? We have already noted the
634 possibility that iron-dependent *trpBA* regulation in *C. trachomatis* may enable a
635 response to simultaneous Trp and iron starvation, such as that likely mediated by IFN-γ.
636 However, this mechanism also presents the opportunity for *C. trachomatis* to respond
637 similarly to distinct *sequential* stresses, where a particular stress primes the pathogen to
638 better cope with subsequent stresses. To reach the female upper genital tract (UGT),
639 where most significant pathology is identified following infection with *C. trachomatis*, the
640 pathogen must first navigate the lower genital tract (LGT). *Chlamydia* infections of the
641 female LGT are associated with bacterial vaginosis (BV), which is characterized by
642 obligate and facultative anaerobe colonization, some of which catalyze the production of
643 indole (54, 55). This provides *C. trachomatis* with the necessary substrate to salvage

644 tryptophan via TrpBA. Interestingly, the LGT is also likely an iron-limited environment.
645 Pathogen colonization and BV both increase the concentration of mucosal lactoferrin
646 (Lf), an iron-binding glycoprotein, which can starve pathogens for iron (56, 57). Lf
647 expression is additionally hormone-regulated, and thus the LGT may normally
648 experience periods of iron limitation (58, 59). Moreover, the expression of TfR is
649 constrained to the basal cells of the LGT stratified squamous epithelium (60), which
650 likely restricts iron from *C. trachomatis* infecting the accessible upper layers of the
651 stratified epithelia. In fact, it was recently demonstrated that *C. trachomatis*
652 development is attenuated in the terminally differentiated layers of an *in vitro*-generated
653 stratified squamous epithelium (61). Collectively, LGT conditions that favor *C.*
654 *trachomatis* infection may be marked by concomitant iron limitation and indole
655 accessibility. For *C. trachomatis*, iron limitation may therefore serve as a critical signal in
656 the LGT, inducing the expression of *trpBA* such that Trp is stockpiled from available
657 indole, allowing the pathogen to counteract oncoming IFN-γ-mediated Trp starvation.
658 We propose the possibility that iron limitation in the LGT may be a significant predictor
659 of pathogen colonization in the UGT.
660 Finally, and of note, the expression of the ribonucleotide diphosphate-reductase
661 encoding *nrdAB* was also recently shown to be iron-regulated in *C. trachomatis* (37).
662 The regulation of *nrdAB* is known to be mediated by the presumably
663 deoxyribonucleotide-dependent transcriptional repressor NrdR, encoded distal to the
664 *nrdAB* locus (62). As NrdR activity is not known to be modulated by iron availability, this
665 raises the intriguing possibility that here too a unique iron-dependent mechanism of
666 regulation may integrate the chlamydial stress response to promote a unified response

across various stress conditions. Future studies may require more metabolomics-based approaches to thoroughly dissect the integration of these stress responses, as transcriptome analyses alone often miss broader, pathway-oriented metabolic coordination. Ultimately, these studies point towards a need to carefully re-evaluate the molecular stress response in *Chlamydia*, using more targeted approaches to answer more specific questions. We anticipate that the rapid progress of the field in recent years will continue to catalyze exciting and important discoveries regarding the fundamental biology of *Chlamydia*.

Materials and Methods

Please refer to the *SI Appendix* for a complete and detailed description of all experimental reagents and methodology. For all infections, *Chlamydia trachomatis* LGV serological variant type II was used to infect human cervical epithelial adenocarcinoma HeLa cells at a multiplicity of infection of 2 or 5, depending on experiment. Indirect immunofluorescent confocal microscopy experiments were performed on a Leica TCS SP8 laser scanning confocal microscope in the Integrative Physiology and Neuroscience Advanced Imaging Center at Washington State University. RT-qPCR and qPCR experiments were performed essentially as described (35, 37). Transcript abundance was normalized to genome copy number for all RT-qPCR analyses. RACE experiments were conducted using the SMARTer® RACE 5'/3' Kit (Takara Bio) with minor modifications as noted in *SI Materials and Methods*. RACE products were isolated by gel extraction using the Macherey-Nagel Nucleospin PCR/gel clean-up kit (Takara Bio) and sent to Eurofins Genomics, LLC for sequencing. Sequenced RACE products were mapped to the *C. trachomatis* L2 434/Bu genome (NCBI Accession:

NC_010287) by nucleotide BLAST on the NCBI server. The *E. coli* YtgR reporter assay was performed essentially as described (34) with minor modifications as indicated in *SI Materials and Methods.* Briefly, BL21 (DE3) *E. coli* were co-transformed with the indicated pCCT and pET vectors and clonal populations were selected on double-selective media. Clones were cultured in double-selective media and recombinant YtgR expression was induced by the addition of IPTG prior to induction of *lacZ* expression by the addition of L-arabinose. Cell lysates were collected and β-galactosidase activity was measured by the Miller Assay (63). All plasmids and primers used in this study are listed in Tables S1 and S2, respectively.

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Figure Legends

Fig. 1. Brief iron limitation via 2,2-bipyridyl treatment precedes the onset of characteristic chlamydial persistence. (*A*) *C. trachomatis* L2-infected HeLa cells were fixed and stained with convalescent human sera to image inclusion morphology by confocal microscopy following Bpdl treatment at the indicated times post-infection. Arrowheads indicate inclusions with visibly fewer organisms in the 12-hour Bpdl-treated condition. Figure shows representative experiment of three biological replicates. (*B*) Genomic DNA (gDNA) was harvested from infected HeLa cells at the indicated times post-infection under iron-replete (blue) and -deplete (red) conditions. Chlamydial genome copy number was quantified by qPCR. Chlamydial genome replication is stalled following 12 hours of Bpdl treatment, but not 6. N=2. (*C*) Total RNA was harvested from infected HeLa cells at the indicated times post-infection under iron-replete (teal) and -deplete (orange) conditions. The transcript abundance of hallmark persistence genes *euo* and (*D*) *omcB* were quantified by RT-qPCR and normalized against genome copy number. Only at 12 hours of Bpdl treatment is *omcB* expression significantly affected. N=3 for 12+6, N=2 for 12+12. Statistical significance was determined by One-Way ANOVA followed by post-hoc pairwise *t*-tests with Bonferroni's correction for multiple comparisons. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$.

Fig. 2. Brief iron limitation condition produces mild iron-starved transcriptional phenotype. (*A*) Total RNA and gDNA was harvested from infected HeLa cells at the indicated times post-infection under iron-replete (teal) and -deplete (orange) conditions. The transcript abundance of iron-regulated *ytgA,* (*B*) *ahpC,* (*C*) *devB* and (*D*) non-iron regulated *dnaB* were quantified by RT-qPCR and normalized against genome copy number. The transcript expression of *ytgA* and *ahpC* were significantly elevated following 6-hour Bpdl treatment, indicative or iron starvation to *C. trachomatis.* N=3. Statistical significance was determined by One-Way ANOVA followed by post-hoc pairwise *t*-tests with Bonferroni's correction for multiple comparisons. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$.

Fig. 3. Expression of the *trpRBA* operon in *C. trachomatis* is non-uniformly regulated by brief iron limitation. (*A*) Cartoon depiction of the *trpRBA* operon (drawn to scale) with the primary transcriptional start site upstream of *trpR* annotated. (*B*) Total RNA and gDNA were harvested from infected HeLa cells at the indicated times post-infection under Trp-replete (black) and -deplete (red) conditions. The transcript expression of *trpRBA* operon was quantified by RT-qPCR and normalized against genome copy number. All three ORFs are significantly induced relative to 12 hpi following Trp starvation. N=2. (*C*) Total RNA and gDNA were harvested from infected HeLa cells at the indicated times post-infection under iron-replete (blue) and -deplete (red) conditions. The transcript expression of *trpRBA* operon was quantified by RT-qPCR and normalized against genome copy number. Only *trpB* and *trpA* expression was significantly induced relative

to 12 hpi. N=3. Statistical significance was determined by One-Way ANOVA followed by post-hoc pairwise *t*-tests with Bonferroni's correction for multiple comparisons. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$.

Fig. 4. Iron-dependent induction of *trpBA* expression initiates within the *trpRBA* intergenic region from a novel alternative transcriptional start site. (*A*) Total RNA was harvested from infected HeLa cells at the indicated times post-infection to examine iron-dependent and Trp-dependent changes in the 5'-cDNA profile of the *trpRBA* operon by Rapid Amplification of 5' cDNA Ends (5'-RACE). RACE products were separated on an agarose gel, revealing three distinct and specific bands with apparent sizes of 1.5, 1.1 and 1.0 kb. Trp depletion led to the apparent enrichment of the 1.5 kb product, while Bpdl treatment produced a similarly enriched 1.0 kb RACE product. Figure shows representative experiment of three biological replicates. (*B*) To confirm that iron-dependent induction of *trpBA* could originate from alternative transcription initiation, RT-qPCR was performed on 5'-RACE cDNA to quantify the abundance of *trpB* transcripts relative to *trpR.* Only under iron-limited conditions were *trpB* transcripts enriched relative to *trpR*. N=3. Statistical significance determined by One-way ANOVA followed by post-hoc pairwise *t*-tests. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$. (*C*) The nucleotide position of the 5' cDNA ends generated from RACE were mapped to the *C. trachomatis* L2 434/Bu genome by nucleotide BLAST. Figure displays histogram (semi-continuous; bin width=20) and overlaid density plot (continuous) distribution of 5' nucleotide positions generated from each 5'-RACE product. The dotted line represents the weighted mean of the distribution, as indicated by the integer value above each line.

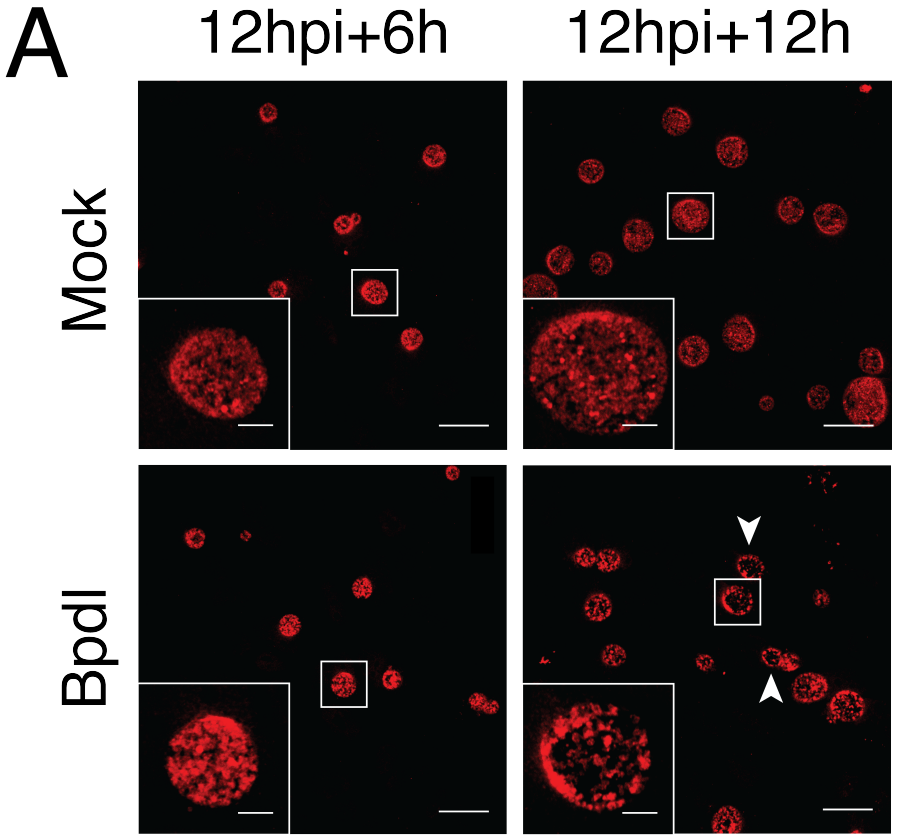
The identified alt. TSSs are depicted on the *trpRBA* operon (drawn to scale) above the plot. N=3.

Fig. 5. Ectopically expressed YtgR domain is capable of binding the putative *trpBA* promoter element in an operator-specific manner and repress transcription in a heterologous system. (*A*) Identification of putative YtgR operator sequence by local and global nucleotide sequence alignment using EMBOSS Water and Needle algorithms, respectively, to align the previously identified YtgR operator to both the *trpRBA* IGR and palindromic candidate sequence. The palindrome was then mutated in our YtgR repression assay as depicted to abolish palindromicity and AT-richness. (*B*) Ectopic expression of YtgR significantly represses β-galactosidase activity only from the promoter of its own operon, *ytgABCD*, and not from an empty vector or another iron-regulated but presumably non-YtgR targeted promoter, *lpdA.* $3 \leq N \geq 2$. (*C*) Expression of recombinant YtgR represses β-galactosidase activity from the putative *trpBA* promoter element, but not the *trpR* promoter, and this repression is dependent on the unaltered operator sequence identified in Fig. 5A. $3 \leq N \geq 2$. Statistical significance determined by two-sided unpaired Student's *t*-test with Welch's correction for unequal variance. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$.

Fig. 6. Transcription termination at *trpRBA* YtgR operator site coincides with iron-dependent transcription read-through. (*A*) Total RNA was harvested from *C. trachomatis*-infected HeLa cells to analyze 3'-cDNA landscape downstream of *trpR* promoter. The top panel displays representative RT-PCR of full-length *trpRBA* message

across experimental conditions (NTC = No Template Control). Bottom panel depicts electrophoresed 3'-RACE products and estimated sizes. N=3. (*B*) 3'-RACE products were sequenced and mapped to *C. trachomatis* L2 434/Bu genome by nucleotide BLAST. Figure displays histogram (semi-continuous; bin width=20) and overlaid density plot (continuous) distribution of 3' nucleotide positions generated from each 3'-RACE product. The dotted line represents the weighted mean of the distribution, as indicated by the integer value above each line. The identified alt. TTSs are depicted on the *trpRBA* operon (drawn to scale) above the plot. The 0.40 kb RACE product mapped to a region overlapping the predicted YtgR operator site. N=3. (*C*) Analysis of transcription read-through by RT-qPCR was performed on 3'-RACE cDNAs at three distinct loci across the *trpRBA* operon representing upstream transcription initiation (511,416-531), YtgR operator site termination (511,639-764) and terminal *trpBA* transcription (513,856-968). Abundance of each amplicon was normalized to a region (Read-through) predicted to be transcribed only as a part of the full-length product based on 5' and 3'-RACE data (511,792-512,080). Thus, the ratio of each amplicon to the normalization amplicon represents the proportion of that amplicon encoded as part of the full-length transcript, approaching one as the two more closely coincide. At the YtgR operator termination site, iron limitation reduces the ratio relative to 12 hpi, suggesting that transcription read-through increases at this site under this condition. The transcription initiation ratio is unaffected by iron limitation, while the terminal *trpBA* amplicon is increased, consistent with alternative transcription from the alt. TSS. Statistical significance determined by One-way ANOVA followed by post-hoc pairwise *t*-tests. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$.

Author Contributions: N.D.P. and R.A.C. wrote the manuscript; N.D.P. and R.A.C. designed the experiments; N.D.P. performed the experiments; N.D.P. and R.A.C. analyzed and interpreted the data.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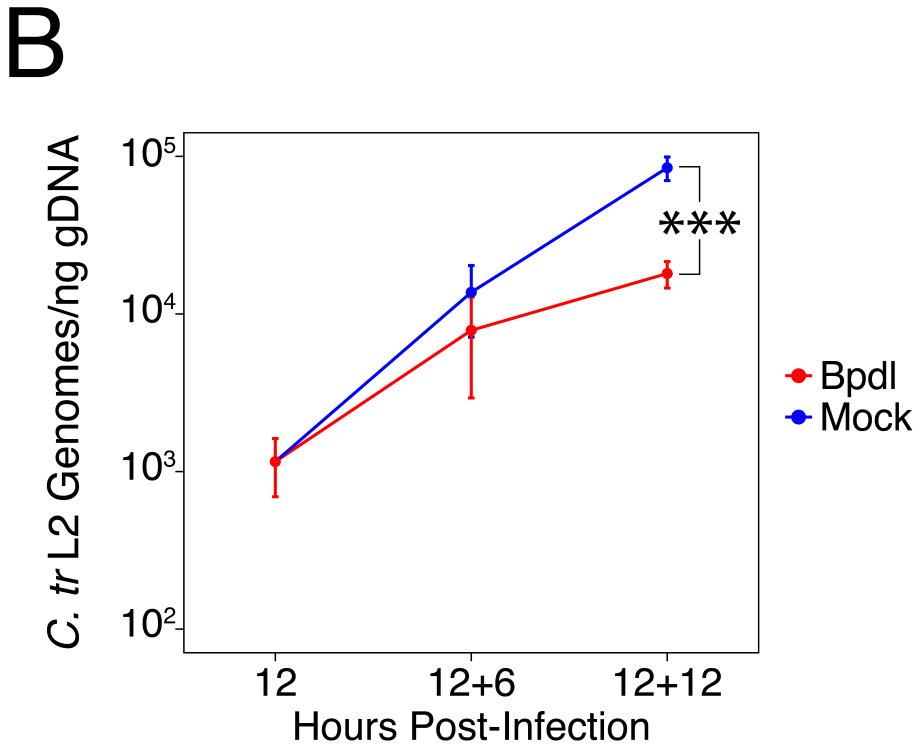
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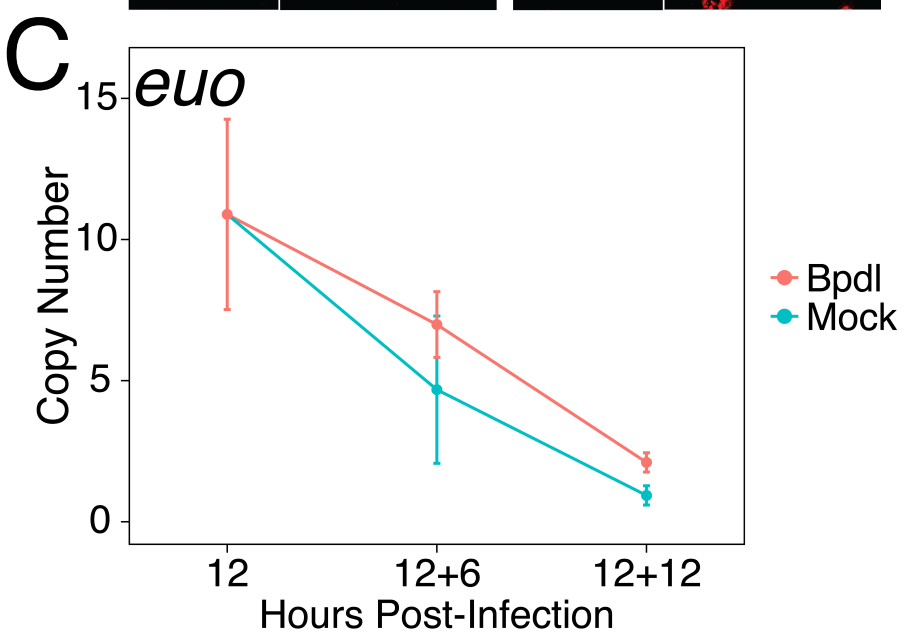
Hours Post-Infection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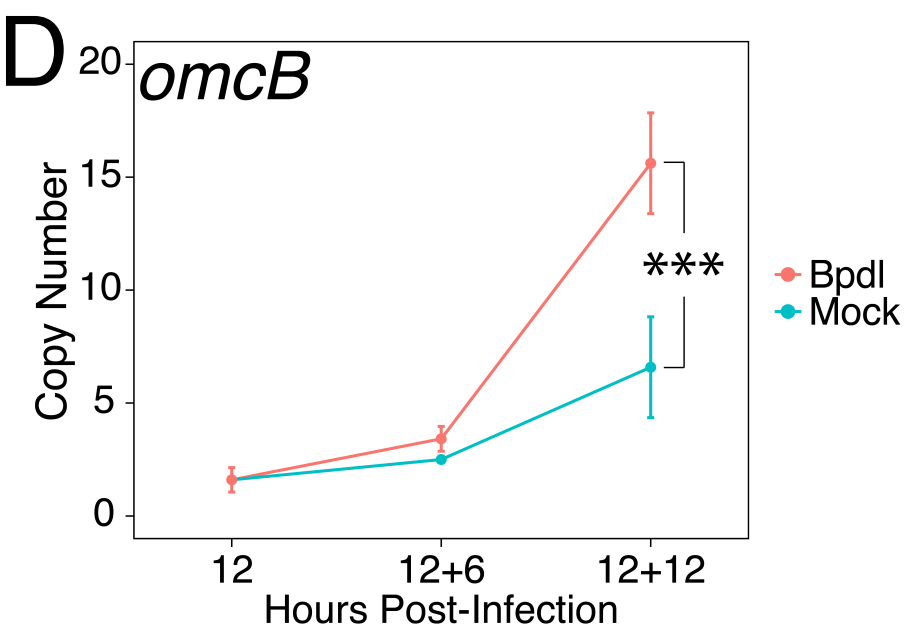
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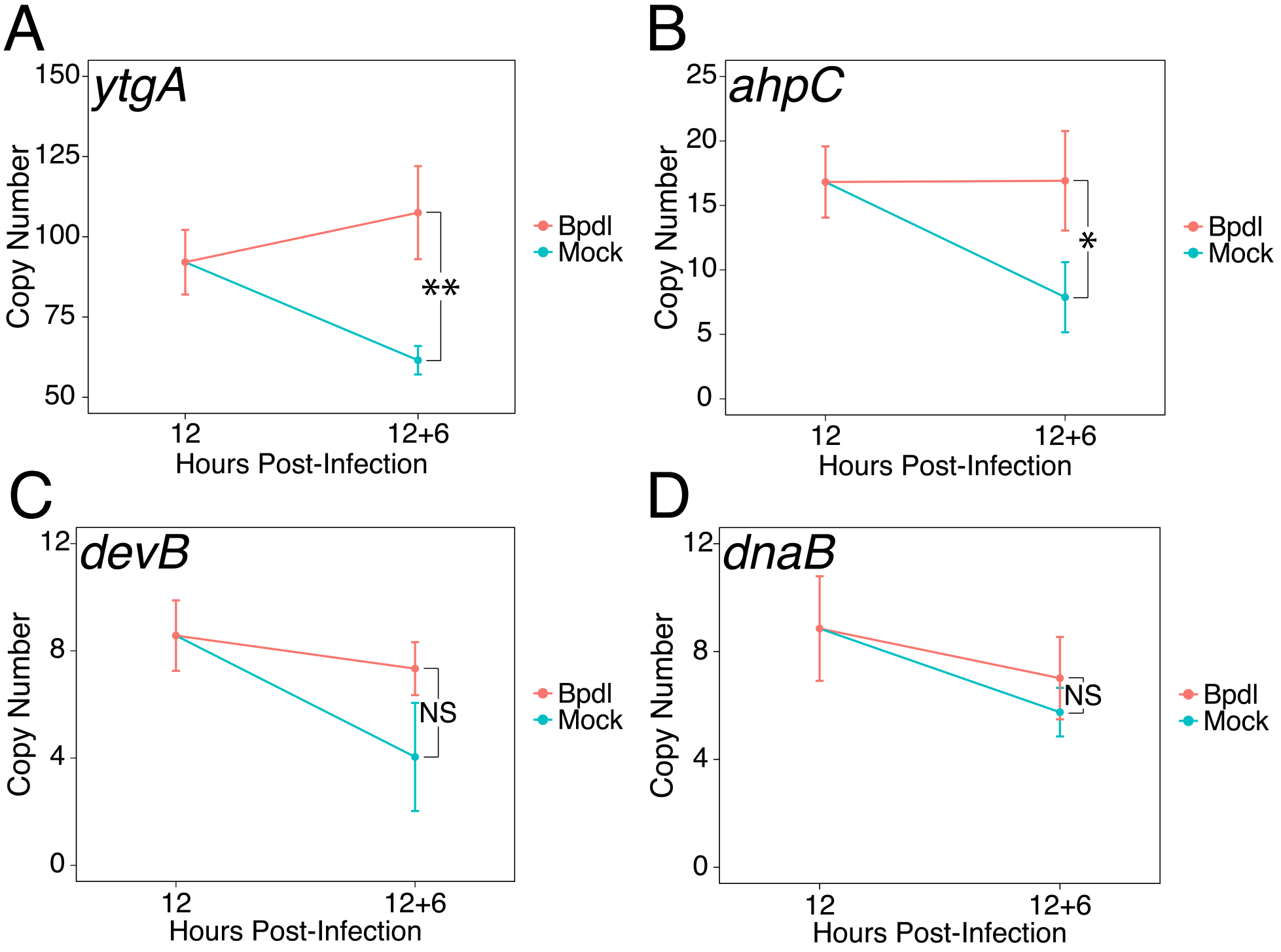
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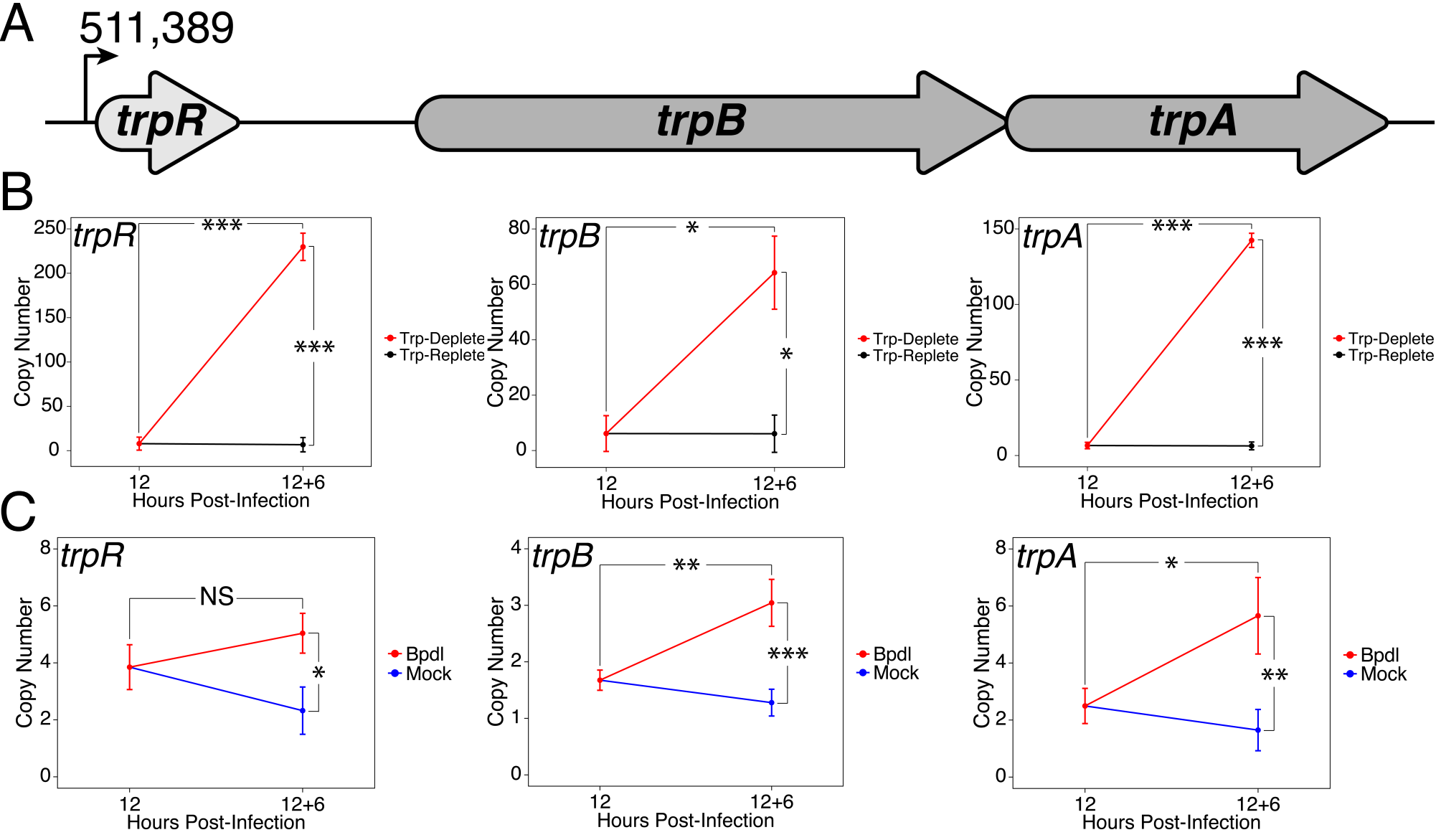
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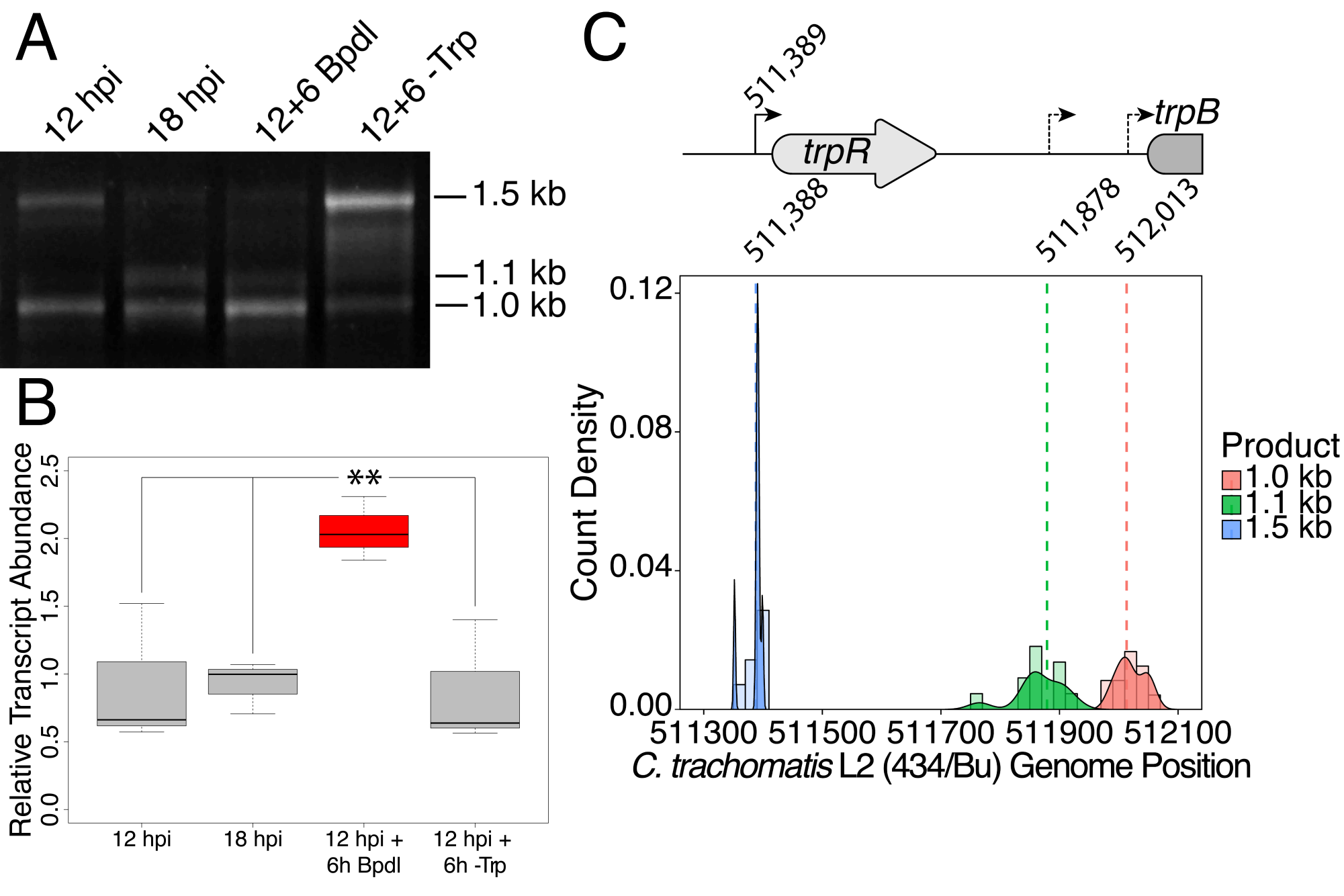
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	Local Pairwise Alignment	Identity
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	Global Pairwise Alignment	Identity
YtgR Operator Palindrome	1 - - - - -TCTCTTCGAGATGAGAAA 18 • | | • | | | • | | •| | | • • 1 AATCAGCTTTTCAAGCTGAT T- - 21	43.5%

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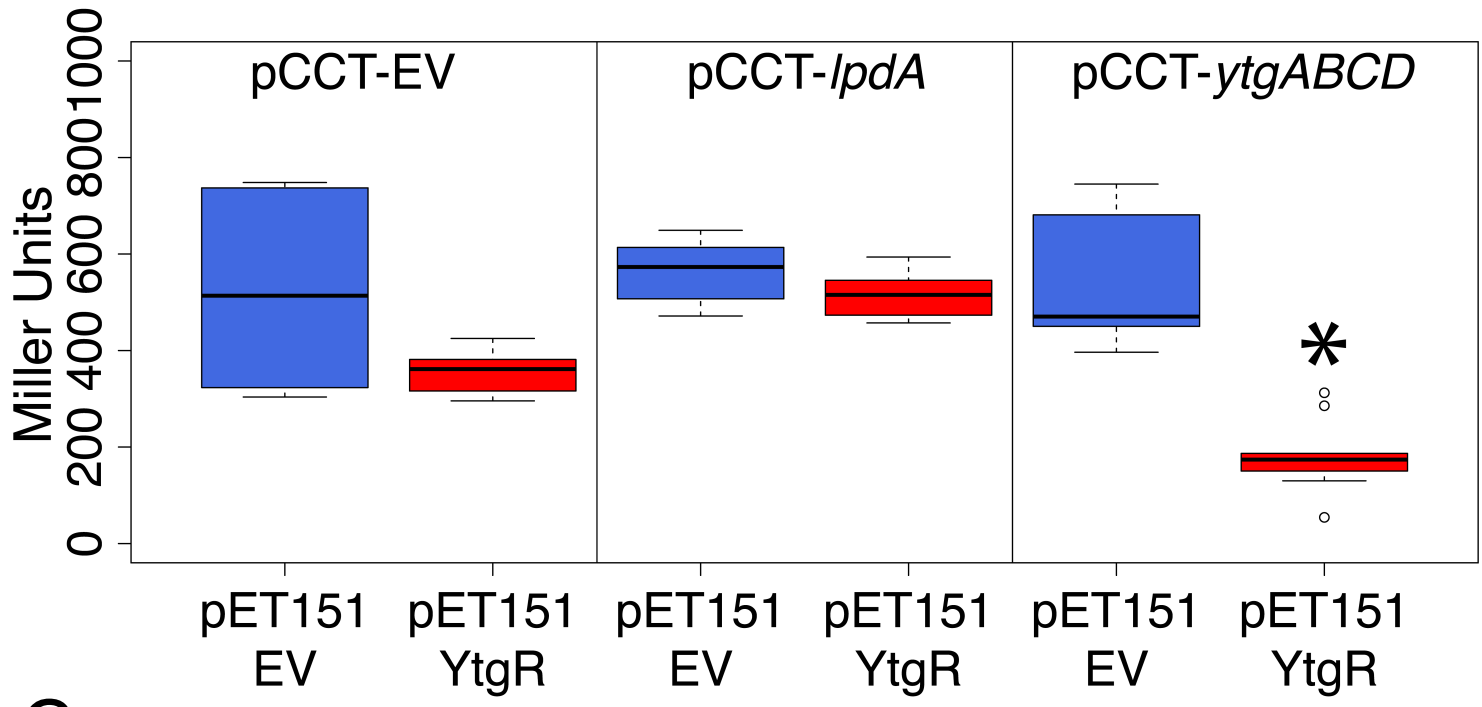

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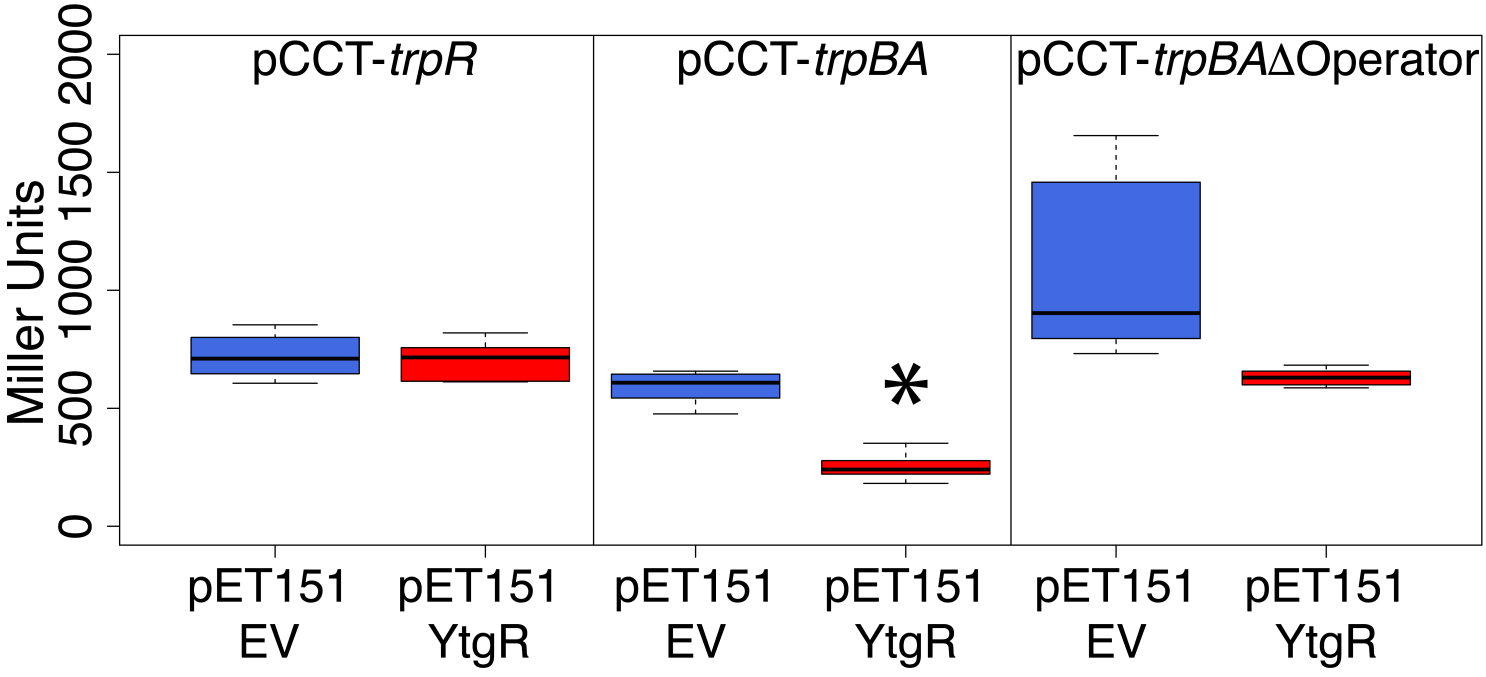

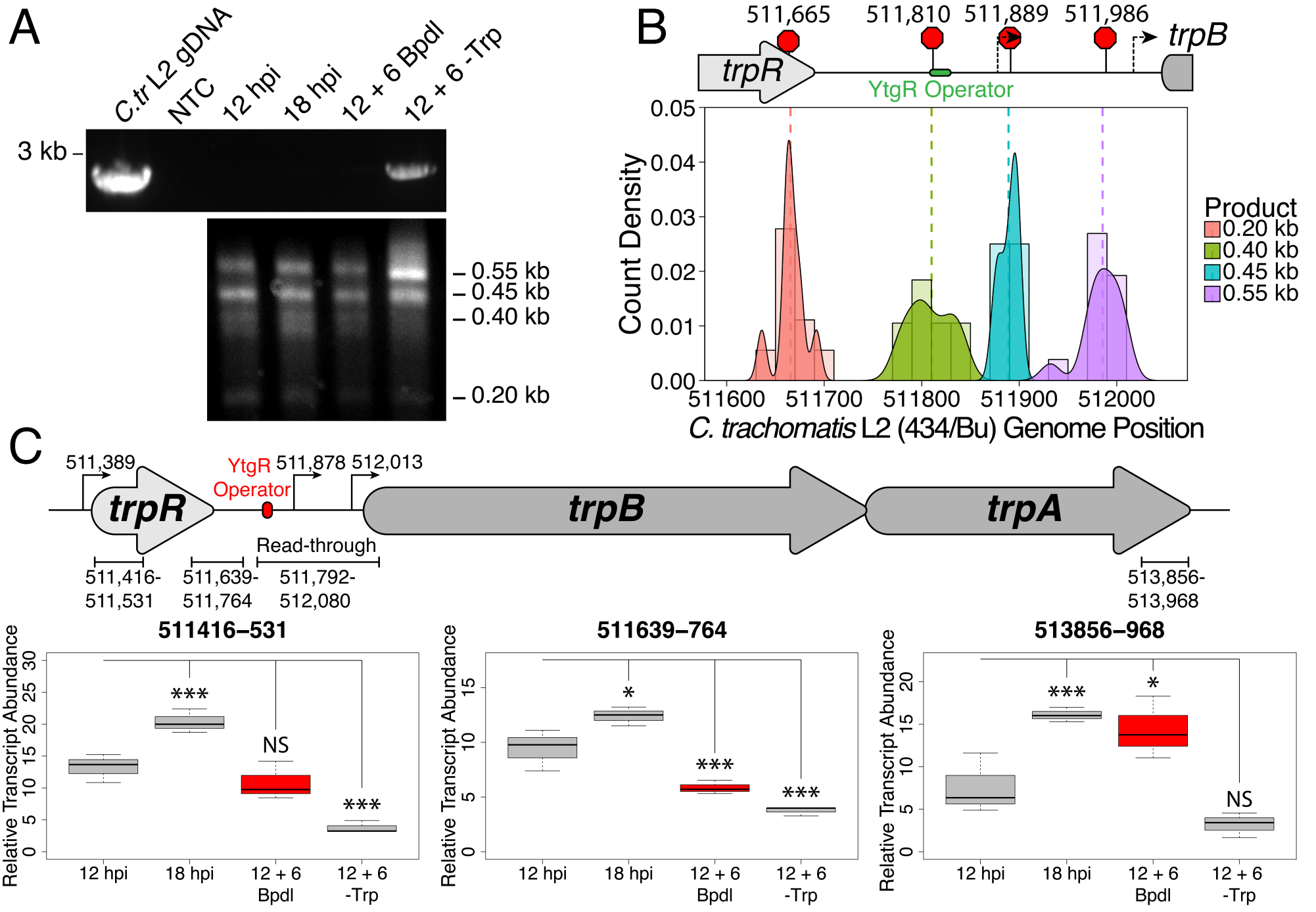
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