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Journal

Journal of Bacteriology, 204(10)

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Publication Date

2022-10-18

DOI

10.1128/jb.00178-22

Peer reviewed

AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

Chlamydia trachomatis RsbU Phosphatase Activity Is Inhibited by the Enolase Product, Phosphoenolpyruvate

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ABSTRACT The intracellular pathogen *Chlamydia* temporally regulates the expression of its genes, but the upstream signals that control transcription are not known. The best-studied regulatory pathway is a partner-switching mechanism that involves an anti-sigma factor, RsbW, which inhibits transcription by binding and sequestering the sigma subunit of RNA polymerase. RsbW is itself regulated by an anti-anti-sigma factor, RsbV, whose phosphorylation state is controlled by the phosphatase RsbU. In this study, we showed that *Chlamydia trachomatis* RsbU requires manganese or magnesium as a cofactor and dephosphorylates RsbV1 and RsbV2, which are the two chlamydial paralogs of RsbV. The gene for RsbU is adjacent to the enolase gene in a number of *Chlamydia* genomes, and we showed that *eno* and *rsbU* are cotranscribed from the same operon. In other bacteria, there is no known functional connection between the Rsb pathway and enolase, which is an enzyme in the glycolytic pathway. We found, however, that *Chlamydia* RsbU phosphatase activity was inhibited by phosphoenolpyruvate (PEP), the product of the enolase reaction, but not by 2-phosphoglycerate (2PGA), which is the substrate. These findings suggest that the enolase reaction and, more generally, glucose metabolism, may provide an upstream signal that regulates transcription in *Chlamydia* through the RsbW pathway.

IMPORTANCE The RsbW pathway is a phosphorelay that regulates gene expression in *Chlamydia*, but its upstream signal has not been identified. We showed that RsbU, a phosphatase in this pathway, is inhibited by phosphoenolpyruvate, which is the product of the enolase reaction. As enolase is an enzyme in the glycolytic pathway, these results reveal an unrecognized link between glucose metabolism and gene regulation in chlamydiae. Moreover, as these intracellular bacteria acquire glucose from the infected host cell, our findings suggest that glucose availability may be an external signal that controls chlamydial gene expression.

KEYWORDS glucose metabolism, glycolytic pathway, signaling pathway, gene regulation

Chlamydia is a pathogenic bacterium that requires a host cell for growth and replication. The intracellular infection is characterized by an unusual developmental cycle in which the bacterium converts between two forms, an infectious form called an elementary body (EB) and a noninfectious but metabolically active form called a reticulate body (RB) (1, 2). During the early stage of the infection, the EB enters the host cell, forms a membranous vacuole called an inclusion, and converts into an RB. RBs then divide repeatedly during the midcycle stage of the infection. Finally, at the late stage of the developmental cycle, RBs convert asynchronously into EBs and then exit the host cell via lysis or extrusion by 48 to 72 h postinfection (hpi) (3).

Chlamydial genes are transcribed in three temporal waves that correspond to these three developmental stages (4–7). For example, early genes are transcribed within the first few hours of EB entry into the host cell (4, 8, 9). Midcycle genes are transcribed

Editor Michael Y. Galperin, NCBI, NLM, National Institutes of Health

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The authors declare no conflict of interest.

Received 11 May 2022
Accepted 22 August 2022
Published 19 September 2022

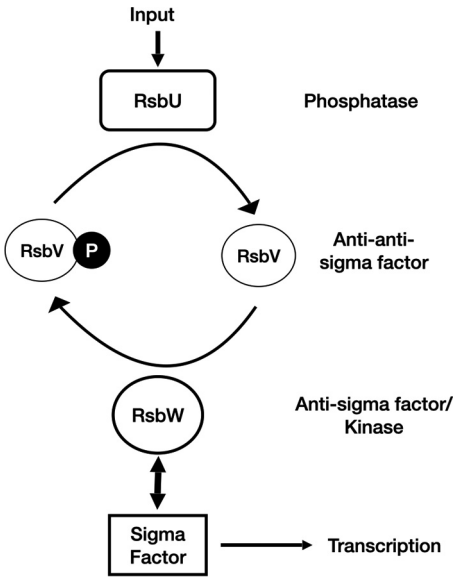

FIG 1 Diagram of the RsbW partner-switching mechanism. RsbW binds to either the sigma factor or the anti-anti-sigma factor RsbV, depending on the phosphorylation state of RsbV. RsbW binds to unphosphorylated RsbV but not phospho-RsbV. The balance between RsbV and phospho-RsbV is determined by the kinase activity of RsbW and the phosphatase activity of RsbU. RsbU is thus a positive regulator of transcription that allows RsbV to bind and sequester RsbW, which frees up the sigma factor for transcription of target genes.

during RB replication and are proposed to be regulated by changes in chlamydial DNA supercoiling (10, 11). Late genes are transcribed at the time of RB-to-EB conversion and are regulated by a transcription factor, EUO (12, 13). In addition, a subset of late genes are transcribed by an alternative form of RNA polymerase containing sigma 28 (σ^{28}) instead of the major sigma factor, sigma 66 (σ^{66}) (14–16).

In addition to these mechanisms of gene regulation, *Chlamydia* may control RNA polymerase activity through an anti-sigma factor that binds and sequesters the sigma factor (6). The sigma factor is the subunit of RNA polymerase that allows it to recognize and bind specific promoter DNA sequences and thereby transcribe its target genes. *Chlamydia* encodes an anti-sigma factor, RsbW, which binds the sigma factor sigB (σ^{B}) in *Bacillus* (17). *Chlamydia* lacks a σ^{B} ortholog but has three sigma factors. Of these, chlamydial RsbW has been proposed to bind and inhibit either σ^{66} or σ^{28}, but not σ^{54} (18–20).

Chlamydia also contains components of a signaling pathway that regulates RsbW and σ^{B} in *Bacillus* (Fig. 1) (19). The core of this pathway is a partner-switching mechanism in which RsbW binds either its sigma factor or an anti-anti-sigma factor, RsbV, depending on the phosphorylation state of RsbV. Phosphorylated RsbV cannot bind RsbW, allowing this anti-sigma factor to bind and inhibit its cognate sigma factor. However, when RsbV is unphosphorylated, it binds RsbW, which frees up the sigma factor and activates transcription.

RsbU is the phosphatase that dephosphorylates RsbV, and it thus serves as a positive regulator of transcription. *Chlamydia* has two RsbV paralogs, and *Chlamydia trachomatis* RsbU has been shown to dephosphorylate RsbV1 but has not been tested on RsbV2 (20). Soules et al. recently showed that an RsbU-null mutant lacking the C-terminal phosphatase domain had a defect in the generation of infectious progeny. Intermediates of the tricarboxylic acid (TCA) cycle bound to the periplasmic domain of *C. trachomatis* RsbU, which is separate from its phosphatase domain (21). However, it is not known if chlamydial RsbU phosphatase activity can be regulated by these TCA intermediates or other factors via direct effects on the phosphatase domain or by transmission of a regulatory signal from the N-terminal sensor domain to the phosphatase. Thus, the RsbW pathway appears to be

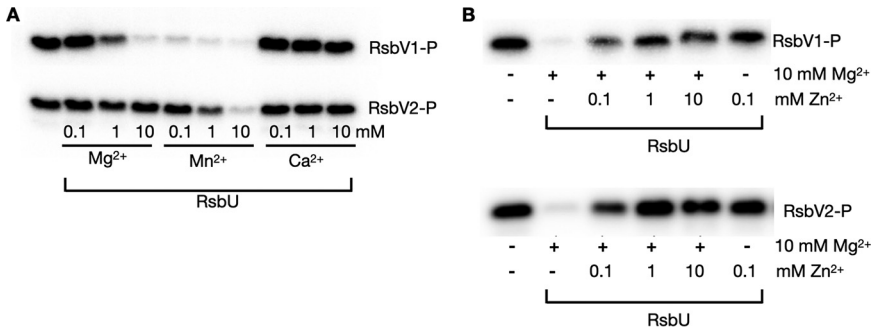

FIG 2 RsbU dephosporylates RsbV1 and RsbV2. (A) *In vitro* RsbU phosphatase assay with purified recombinant RsbU (6 μM) and ^{32}P-labeled RsbV1 or RsbV2 (5 μM each). Reactions were performed in the presence of increasing concentrations (0.1, 1, and 10 mM) of $MgCl_2$, $MnCl_2$, or $CaCl_2$. Labeled RsbV was visualized by autoradiograph. (B) Inhibition of RsbU phosphatase activity with increasing concentrations (0.1, 1, and 10 mM) of $ZnCl_2$ in the presence of cofactor ($MgCl_2$ or $MnCl_2$).

important for chlamydial growth and development, but the upstream signals that control this pathway have not been identified.

In this study, we investigated how the phosphatase activity of *C. trachomatis* RsbU is regulated. We were intrigued by the location of the enolase gene next to the RsbU gene in several *Chlamydia* spp. and explored if this glycolytic enzyme could somehow regulate RsbU. We found that phosphoenolpyruvate (PEP), which is the product of the enolase reaction, inhibited RsbU enzymatic activity. This finding suggests that the glycolytic pathway may have an unexpected role in regulating gene expression in *Chlamydia*.

RESULTS

Cofactor requirement of *C. trachomatis* RsbU. We first determined the metal cofactor that is necessary for the phosphatase activity of chlamydial RsbU. RsbU belongs to the PP2C phosphatase family that requires magnesium or manganese for activity but is inhibited by zinc (22). We were unable to purify full-length recombinant RsbU, which is a large transmembrane protein, but successfully purified a truncated form of RsbU containing the C-terminal 350 amino acids, which encompass the phosphatase domain but lack the periplasmic domain. We then tested our RsbU polypeptide in an *in vitro* phosphatase assay that utilized recombinant *C. trachomatis* RsbV1 and RsbV2 that had been phosphorylated by RsbW with ^{32}P-radiolabel.

RsbU dephosphorylated RsbV1 and RsbV2, but there were differences in its activity against these two substrates (Fig. 2). RsbV1 was dephosphorylated by RsbU in the presence of either Mn^{2+} or Mg^{2+}, but RsbV2 was only dephosphorylated at high Mn^{2+} concentration and not with Mg^{2+} (Fig. 2A). The concentration of Mn^{2+} required for comparable RsbU phosphatase activity against RsbV1 was 100-fold lower than for Mg^{2+} (Fig. 2A, lanes 4 and 5), which suggests that Mn^{2+} is a better cofactor than Mg^{2+}. There was no phosphatase activity with Ca^{2+} or Zn^{2+}, and in fact, Zn^{2+} inhibited RsbU activity against both RsbV1 and RsbV2 (Fig. 2A and B). These results demonstrate that RsbU, like other PP2C phosphatases, can use manganese or magnesium as a cofactor, while its activity is inhibited by zinc (23).

Transcriptional organization of the RsbU and enolase genes. While searching for potential regulators of RsbU, we were intrigued by enolase because the genes for these two proteins are adjacent in many *Chlamydia* genomes (Fig. 3A) (24–26).

We first used reverse transcription PCR (RT-PCR) to investigate if *eno* and *rsbU* are in the same operon. Using a 5′ primer in *eno* and a 3′ primer in *rsbU*, we successfully amplified a 1.3-kb PCR product from chlamydial RNA harvested from L929 mouse fibroblast cells infected with *C. trachomatis* serovar L2 (Fig. 3B). These data provide evidence that *eno* and *rsbU* are cotranscribed on the same polycistronic message in this bacterium.

We then used 5′ rapid amplification of cDNA ends (RACE) to examine the temporal transcription of *eno* and *rsbU* in *C. trachomatis*-infected cells from their published transcription start sites (27). At 18 hpi, we detected a 5′ RACE PCR product from the *eno*

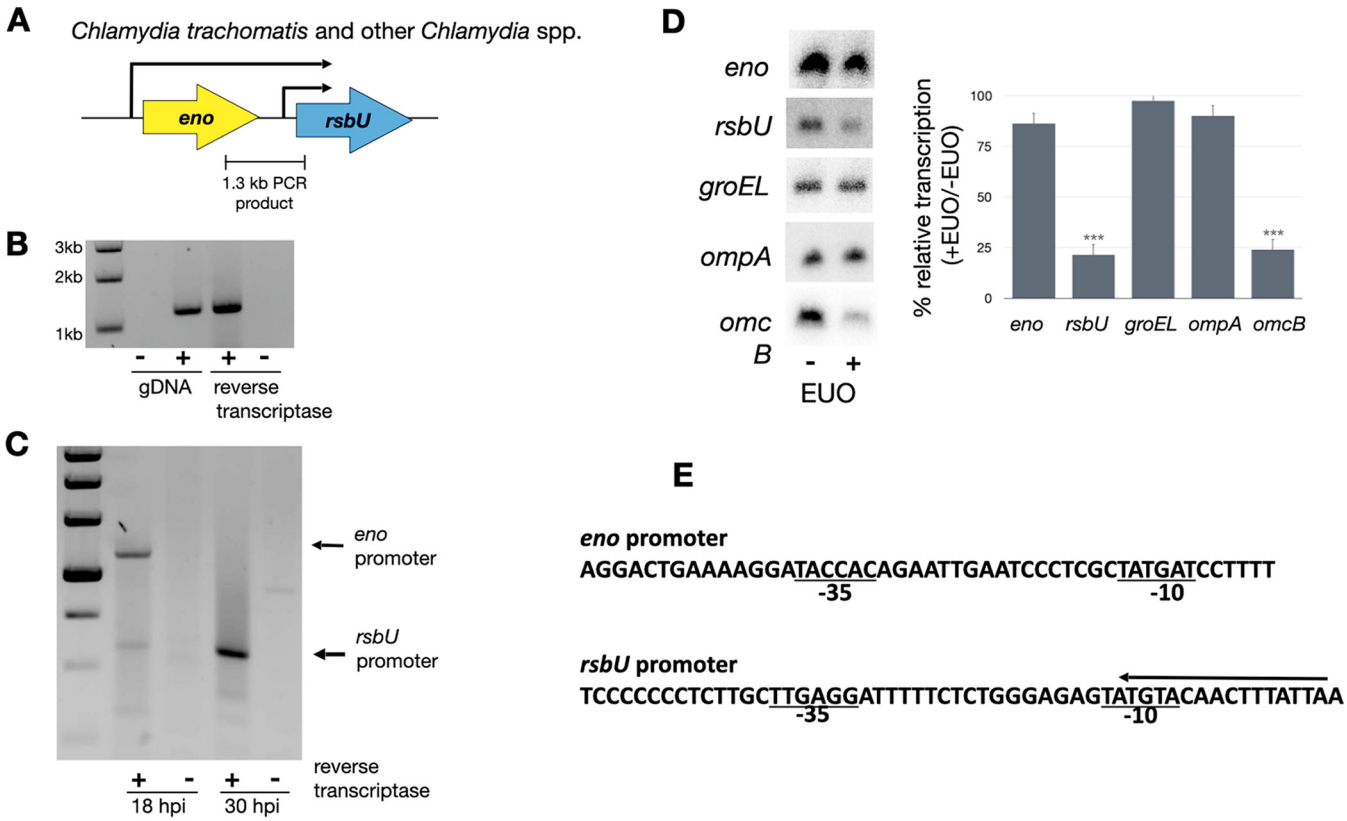

FIG 3 The *eno* and *rsbU* genes are in an operon. (A) Gene organization of *eno* and *rsbU* genes in *C. trachomatis* and other *Chlamydia* spp. Arrows above the genes represent transcriptional start sites identified by transcriptome sequencing (RNA-seq) (27). The 1.3-kb PCR product used for RT-PCR is indicated below the *C. trachomatis* gene diagram. (B) The 1.3-kb PCR product was detected by reverse transcription of *C. trachomatis* RNA collected at 24 hpi. Primers used in the PCR amplified a segment that annealed to the 3′ end of *eno* and 5′ end of *rsbU*. *C. trachomatis* genomic DNA (gDNA) was used as a control. (C) 5′ RACE showing differential expression of the *eno* and *rsbU* promoters. 5′ RACE was performed with a primer that annealed to the 3′ end of *rsbU* and specific 5′ primers for the *eno* and *rsbU* promoters. *C. trachomatis* RNA was collected at 18 and 30 hpi. PCR products were visualized on a 1% agarose gel. Predicted PCR products corresponding to transcripts from the *eno* and *rsbU* promoters are indicated by the arrows. (D) EUO-mediated repression of the *rsbU*, but not *eno*, promoter. *In vitro* transcription assays of the promoters of *eno*, *rsbU*, *omcB* (positive control), *groEL*, and *ompA* (negative controls) were transcribed with *E. coli* RNA polymerase in the presence or absence of 5 μM EUO. Transcripts were quantified with Quantity One software. For each promoter, relative transcription was calculated as the percentage of transcripts in the presence of EUO compared to transcripts in the absence of EUO. Values are from the average of at least three independent experiments with standard deviation indicated by the error bar, and statistically significant differences (unpaired two-sided *t* tests, $P < 0.0001$) are indicated by asterisks. (E) DNA sequences of the *eno* and *rsbU* promoters, with putative −10 and −35 promoter elements underlined. The arrow above the *rsbU* promoter sequence is the putative EUO binding site.

promoter and a weak product from the *rsbU* promoter (Fig. 3C). In contrast, at 30 hpi, the 5′ RACE PCR product was primarily from the *rsbU* promoter, with no product from the *eno* promoter. Together, these findings suggest that *eno* and *rsbU* are cotranscribed from a promoter that is active at 18 hpi, which is midcycle in the chlamydial developmental cycle but downregulated at 30 hpi, which is a late time. In addition, detection of *rsbU* transcripts at 30 hpi suggests that *rsbU* may have its own promoter that is preferentially transcribed at late times.

We performed *in vitro* transcription assays to study the promoters for *eno* and *rsbU*. Both promoters were transcribed by *Escherichia coli* σ^{70} RNA polymerase (which is equivalent to *C. trachomatis* σ^{66} RNA polymerase in transcribing *Chlamydia* σ^{66} promoters) (Fig. 3D) (12). As *rsbU* appears to be a late gene, we investigated if it is regulated by the late regulator EUO (12, 13). EUO repressed the *rsbU* promoter and a positive-control late promoter *omcB*, but not the *eno* promoter or two negative-control midcycle promoters, *groEL* and *ompA* (Fig. 3D). These findings provide evidence that *rsbU* can be expressed from two promoters, a midcycle promoter for the *eno-rsbU* operon and an internal promoter that transcribes *rsbU* at late times.

RsbU phosphatase activity is regulated by the product of the enolase reaction. To study chlamydial enolase, we first checked if *C. trachomatis* enolase is enzymatically

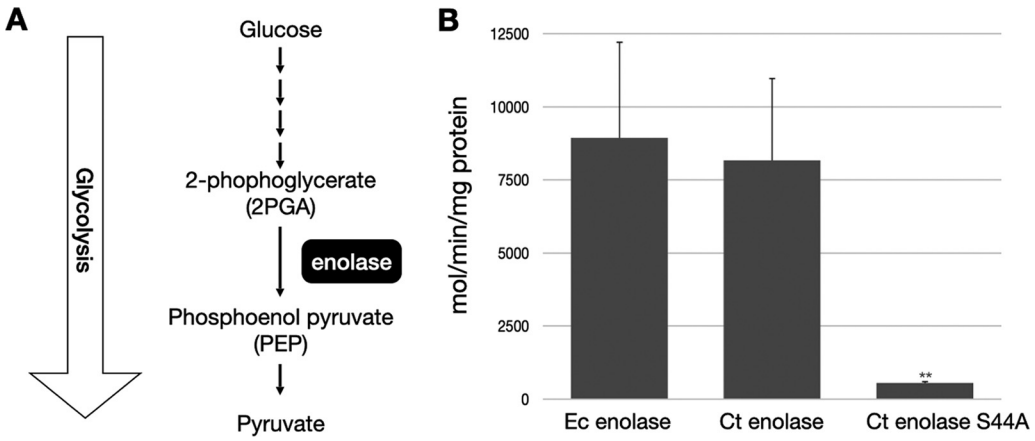

FIG 4 *C. trachomatis* enolase can convert 2PGA to PEP. (A) Diagram of the glycolytic pathway and the enolase reaction step. (B) *In vitro* enolase assay using purified recombinant *E. coli* enolase, *C. trachomatis* enolase, or the S44A mutant of *C. trachomatis* enolase. Enzymatic activity was calculated as the amount of PEP produced per minute per mg protein. Ec enolase is the *E. coli* recombinant protein; Ct enolase and its mutant are *C. trachomatis* recombinant protein. Statistically significant difference between the *C. trachomatis* wild type and mutant enolase is indicated by asterisks (unpaired two-sided *t* test, $P < 0.05$).

active. Enolase is an enzyme in the glycolytic pathway that catalyzes the conversion of 2PGA to PEP in bacteria and many other organisms (Fig. 4A) (24–26). Using an *in vitro* assay, we showed that purified recombinant *C. trachomatis* enolase converted 2PGA to PEP in a similar manner as *E. coli* enolase (Fig. 4B). In contrast, a mutant *C. trachomatis* enolase containing point substitutions at conserved residues at S44A lacked enzymatic activity (Fig. 4B) (28). These data demonstrate that *C. trachomatis* enolase is functional and verify that the activity that we measured was not due to contaminating *E. coli* enolase in our purified recombinant *C. trachomatis* enolase preparation.

We next used our *in vitro* phosphatase assay to examine if enolase could be involved in regulating RsbU activity. In the presence of Mg^{2+} or high Mn^{2+}, PEP inhibited the ability of RsbU to dephosphorylate RsbV1 and RsbV2 (Fig. 5), while 2PGA did not. (Fig. 5). We also tested TCA intermediates that have been shown to interact with the periplasmic domain of RsbU (21). 2-Ketoglutarate and malic acid each had minimal effects on RsbU activity against RsbV1 and RsbV2, equivalent to the effect of the succinic acid negative control (Fig. 6). Oxaloacetate (OAA) inhibited RsbU but only under

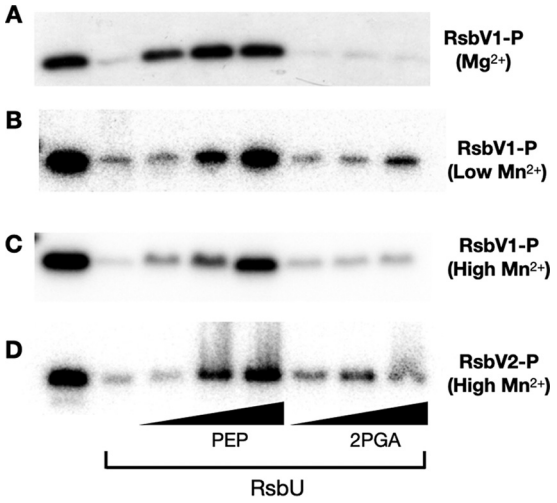

FIG 5 Inhibition of RsbU phosphatase activity by PEP. Autoradiographs showing *in vitro* phosphatase assay with purified recombinant RsbU (6 μM) and ^{32}P-labeled RsbV1 and RsbV2 (5 μM) were performed with increasing concentrations (2.5, 5, and 10 mM) of PEP or 2PGA in the presence of 10 mM $MgCl_2$ (A), 0.1 mM $MnCl_2$ (B), 10 mM $MnCl_2$ (C), and 10 mM $MnCl_2$ (D) as the cofactor.

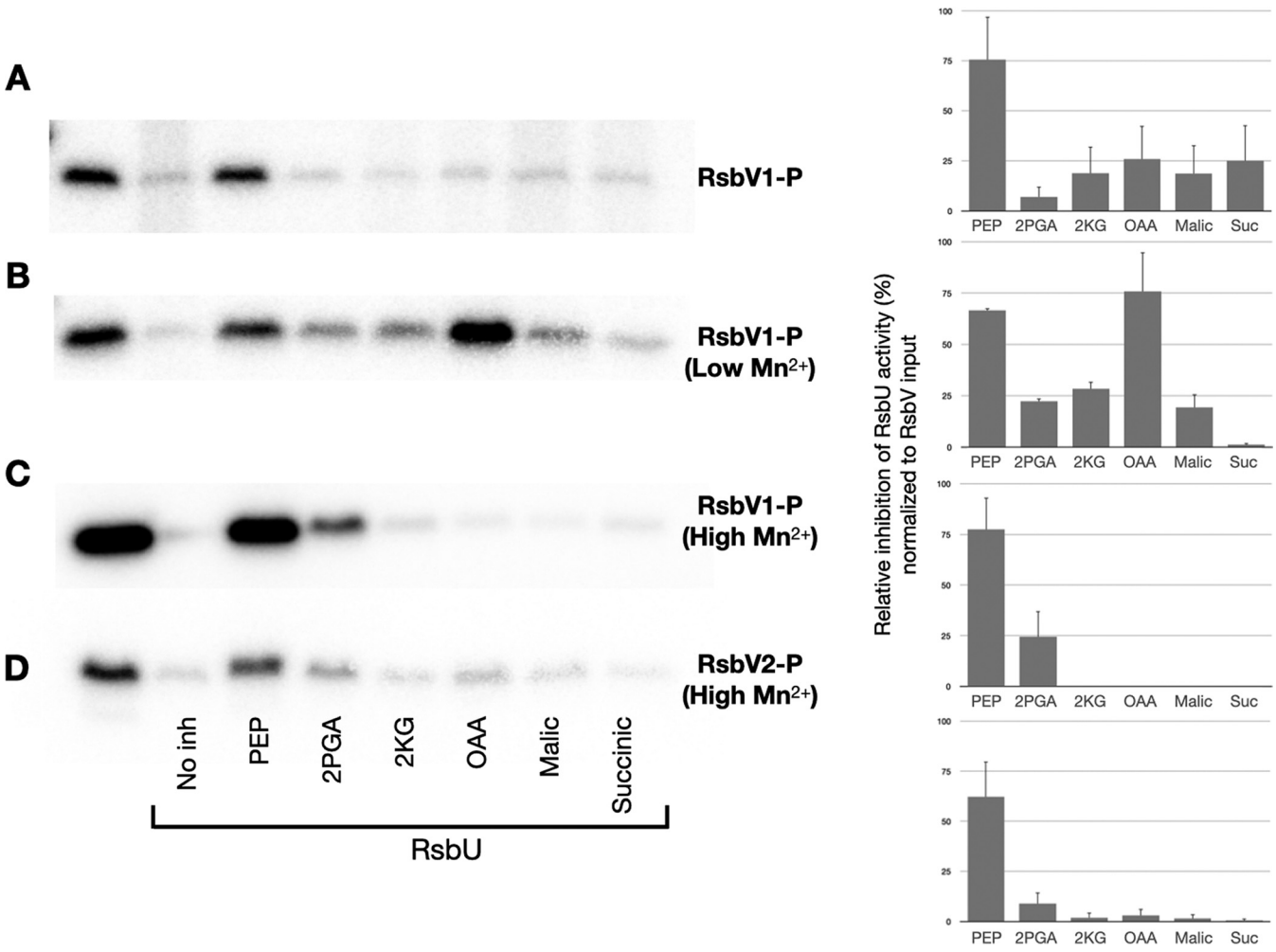

FIG 6 Effect of TCA intermediates on RsbU phosphatase activity. Autoradiographs showing *in vitro* phosphatase assay with purified recombinant RsbU (6 μM) and ^{32}P-labeled RsbV1 and RsbV2 (5 μM) were performed with 10 mM PEP, 2PGA, 2-ketoglutarate (2KG), oxaloacetate (OAA), malic acid, or succinic acid in the presence of 10 mM $MgCl_2$ (A), 0.1 mM $MnCl_2$ (B), 10 mM $MnCl_2$ (C), and 10 mM $MnCl_2$ (D) as the cofactor. Quantification of each autoradiograph shown to the right. Relative inhibition is expressed as the percentage of inhibition, calculated as the amount of labeled RsbV divided by the RsbV input (these values were adjusted by subtracting the background of the no-inhibitor control). Values represent the mean from at least three experiments, with standard deviation indicated by the error bar.

specific conditions, i.e., against RsbV1 but not RsbV2, and only at low Mn^{2+} concentration and not in the presence of high Mn^{2+} or Mg^{2+} concentration (Fig. 6).

DISCUSSION

In this study, we characterized the phosphatase activity of *C. trachomatis* RsbU, including its cofactor requirement and putative substrates. We showed that manganese is a better cofactor than magnesium and that RsbU was able to dephosphorylate both RsbV1 and RsbV2. *C. trachomatis* RsbU has been previously shown to dephosphorylate RsbV1 (20), but prior to this study, it was not known if it could also dephosphorylate RsbV2. However, we found that RsbV2 was not as good a substrate as RsbV1, leaving open the question of whether RsbU or another enzyme is the cognate phosphatase for RsbV2 in *Chlamydia*.

The transcriptional organization of the *rsbU* gene in *C. trachomatis* provided a clue that enolase and RsbU might have a functional linkage in *Chlamydia*. The presence of *eno* and *rsbU* in the same operon was at first puzzling because there was no known relationship between glycolysis and the Rsb pathway of transcriptional regulation in other bacteria. The location of *eno* as the first gene in the *eno-rsbU* operon was particularly intriguing because the regulator of an operon is often its first gene, as is the case

for the chlamydial transcription factors TrpR and HrcA (29, 30). This gene organization appears to be conserved, as *eno* is immediately upstream of *rsbU* in other *Chlamydia* spp., with the exception of *Chlamydia pneumoniae*, which has 5 genes between them. Another feature of this operon is that *rsbU* expression is controlled by two promoters. We propose that *rsbU* is transcribed from the *eno* promoter prior to late times. In addition, the EUO-dependent *rsbU* promoter provides a means to independently upregulate RsbU expression at late times.

Our data provide support for a novel functional connection between enolase and RsbU in *Chlamydia* through PEP, the product of the enolase reaction. We demonstrated that RsbU phosphatase activity is inhibited by PEP, but not by 2PGA, which is the substrate that is converted into PEP by enolase. These findings support a model in which enolase regulates RsbU by controlling the production of an inhibitor of this phosphatase.

Interestingly, RsbU may also be regulated by the TCA cycle. TCA intermediates, including 2-ketoglutarate, malic acid, and oxaloacetate, have been reported to bind the C-terminal periplasmic domain of RsbU (21), but binding to the phosphatase domain was not examined. In our studies, 2-ketoglutarate and malic acid did not inhibit RsbU phosphatase activity, and oxaloacetate only inhibited RsbU activity under specific cofactor conditions and only against RsbV1 (Fig. 6). It is possible that the periplasmic domain of RsbU is a regulatory domain, and binding of TCA intermediates could inhibit RsbU phosphatase activity through allosteric mechanisms. We were unable to test this model because we could not purify full-length RsbU and thus only performed experiments with the phosphatase domain of RsbU.

The ability of PEP to inhibit RsbU suggests several potential mechanisms for controlling the RsbW pathway and chlamydial gene expression. First, PEP production could be decreased late in the developmental cycle when there is decreased expression of chlamydial glycolytic enzymes (31). PEP production could also be controlled by chlamydial glucose levels since PEP is a product of glucose metabolism through the glycolytic pathway. *Chlamydia*, as an intracellular bacterium, obtains glucose from the host cell in the form of glucose-6-phosphate as a carbon source (32–35). Thus, enolase and RsbU could act as a sensor that activates transcription of Rsb-regulated genes when host glucose is limited. Alternatively, PEP production could be controlled by enolase activity. In *E. coli*, enolase enzymatic activity is regulated by its phosphorylation state (36). Enolase may be developmentally regulated in *Chlamydia* because phosphorylated enolase has been detected in EBs but not in RBs in *Chlamydia caviae* (37). Other potential regulators of enolase activity include inhibitors, such as fluoride, SF2312 phosphonate, and tropolone derivatives (38–40), or posttranslational modification by lysine acetylation (41). In this regard, lysine-acetylated enolase has been detected in *Chlamydia* EBs, although its significance has not been investigated (42).

We propose the following model for how enolase and RsbU could regulate gene expression in *Chlamydia* (Fig. 7). When the host cell supplies sufficient glucose in the form of glucose-6-phosphate to RBs, the chlamydial glycolytic pathway produces 2PGA, which is converted into PEP by chlamydial enolase. PEP inhibits the phosphatase activity of RsbU, leaving its substrate, RsbV1, in a phosphorylated form that is unable to bind RsbW. RsbW is thus free to bind its cognate sigma factor, inhibiting transcription by the form of RNA polymerase containing this sigma factor, be it σ^{66} or σ^{28}. However, when host glucose is limited, chlamydiae are unable to produce PEP, and RsbU becomes enzymatically active and dephosphorylates RsbV1. Unphosphorylated RsbV1 binds RsbW, which frees up its cognate sigma factor so that it can direct the transcription of its target genes. We have illustrated this model with RsbV1 because it is the best studied of the two chlamydial RsbV paralogs, but RsbV2 may have a parallel or redundant role in this pathway. This model provides a novel mechanism by which chlamydial gene expression could be regulated by nutrient availability. It does not, however, exclude the possibility of an additional regulatory mechanism mediated through interactions between the N-terminal periplasmic domain of RsbU and TCA intermediates (21).

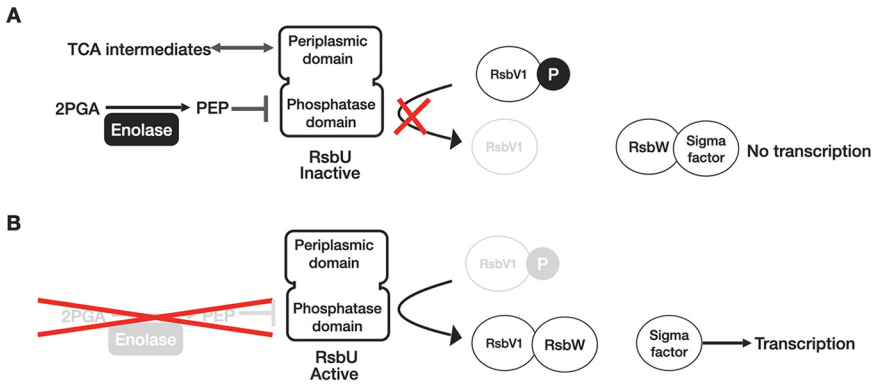

FIG 7 Model for regulation of RsbU phosphatase activity by enolase. (A) PEP production by enolase inhibits transcription controlled by the Rsb pathway. PEP inhibits RsbU phosphatase activity, which results in accumulation of phosphorylated RsbV1 that is unable to bind RsbW. As a result, RsbW binds to its cognate sigma factor and inhibits transcription of target genes. RsbU may also be regulated through physical interactions between its N-terminal periplasmic domain and TCA intermediates, although the effect of these interactions on RsbU phosphatase activity have not been determined (21). (B) Active RsbU positively regulates transcription controlled by the Rsb pathway. In the absence of PEP, either because of decreased enolase activity or the lack of substrate (e.g., 2PGA or glucose), RsbU phosphatase is active and dephosphorylates RsbV. Unphosphorylated RsbV binds to RsbW, which frees up the cognate sigma factor to associate with other RNA polymerase subunits and transcribe target genes.

In summary, we have uncovered evidence of a functional connection between the glycolytic pathway and the Rsb pathway of gene regulation in *Chlamydia*. The linkage appears to be through enolase, which catalyzes the production of PEP, a novel inhibitor of chlamydial RsbU phosphatase activity. There has long been speculation about external stimuli that could regulate chlamydial gene expression and development, but upstream signals have not been identified (43, 44). Our findings provide a potential mechanism in which host glucose availability could be an upstream signal that regulates chlamydial gene expression.

MATERIALS AND METHODS

Plasmids and strains. Plasmids used in this assay are listed in Table 1. Primer sequences used for plasmid construction are listed in Table 2. *C. trachomatis* LGV serovar L2 434/Bu was used as a source of genomic DNA for cloning and RNA extraction. L929 mouse fibroblast cells were used for *C. trachomatis* infections. Cells and infections were grown in RPMI 1640 supplemented with 25 mM HEPES and 5% fetal bovine serum (FBS). *E. coli* BL21 was used as a source of genomic DNA for cloning and protein purification.

Protein purification. For protein expression, *C. trachomatis* or *E. coli* genes were cloned into pRSET C (Thermo Fisher) or pQE30 (Qiagen), which adds a 6× His moiety to the N terminus of the recombinant protein. Plasmids were used to transform *E. coli* strain BL21, and transformed cells were grown in LB

TABLE 1 List of plasmids used in this study

Plasmid	Description	Source or reference
pRSET-C	Expression plasmid	Thermo Fisher
pEpQE30	Expression plasmid	Qiagen
pMT1125	G-less transcription template	45
pMT1149	*C. trachomatis ompA* promoter in pMT1125	A. Wilson and M. Tan, unpublished data
pMT1178	*C. trachomatis groESL* promoter in pMT1125	46
pMT1636	*C. trachomatis omcB* promoter in pMT1125	12
pMT1669	*C. trachomatis rsbU* promoter in pMT1125	This work
pMT1781	*E. coli* enolase in pRSET-C	This work
pMT1673	*C. trachomatis* enolase in pRSET-C	This work
pMT1972	*C. trachomatis* enolase containing a serine-to-alanine substitution at amino acid 44 in pRSET-C	This work
pMT1135	*C. trachomatis* RsbW in pRSET-C	C. Schaumburg and M. Tan, unpublished data
pMT1136	*C. trachomatis* RsbV2 in pRSET-C	C. Schaumburg and M. Tan, unpublished data
pMT1754	*C. trachomatis* RsbV1 in pEpQE30	This work
pMT1676	*C. trachomatis* RsbU from amino acids3 01–650 in pSET-C	This work
pMT1912	*C. trachomatis eno* promoter in pMT1125	This work

TABLE 2 List of primers used in this study

Description	Sequence[a]
Protein expression primers	
E. coli enolase	5′-GCCGGATCAAGCTTCGAATTTTATGCCTGGCCTTTGAT
	5′-GCCGGATCAAGCTTCGAATTTTATGCCTGGCCTTTGATCT
C. trachomatis enolase	5′-ATCTGCAGCTGGTACCATGGATGTTTGATGTCGTCATC
	5′-GCCGGATCAAGCTTCGAATTCTATGCTTTAGAAAAGGG
C. trachomatis internal primer for S44A enolase mutant	5′-CCTTCTGGAGCAGCAACAGGCATCAAG
	5′-CTTGATGCCTGTTGCTGCTCCAGAAGG
C. trachomatis RsbU	5′-ATGGAAGAAAAATCTATAATTTTTGCT
	5′-ATAAGCGGAAGGTTCCTTAGGTATTTTC
C. trachomatis RsbV1	5′-GCTCGGTACCCCGGGTCGAATGAGTAACTTTCAGAAA
	5′-AGCTCAGGTAATTAAGCTTTAACTGTTTTCTTTTGC
Transcription primers	
eno promoter	5′-AAGAATTCACTGAAAAGGATACCACAGAATTGAATCCC
	5′-TAGTAGAAAAGGATCATAGCGAGGGATTCAATTCTGTG
rsbU promoter	5′-TCCCTTCCCCCCTCTTGCTTGAGGATTTTTCTCTGGGA
	5′-TTAATAAAGTTGTACATACTCTCCCAGAGAAAAATCC

[a]Each primer pair represents the upstream and downstream primer sequence used to amplify the PCR insert for construction of the respective plasmid.

broth. Protein expression was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h. Cells were pelleted and resuspended in buffer N (10 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM $MgCl_2$, and 10 mM 2-mercapoethanol) containing 25 mM imidazole. Cells were lysed by sonication in a Branson digital sonifier 250D for 2 min at 25% output. Cell debris was pelleted, and the supernatant was incubated with a 2-mL slurry of Ni-nitrilotriacetic acid (Ni-NTA; Sigma-Aldrich) for 1 h at 4°C with agitation. Bound protein was washed with 50 mL buffer N containing 25 mM imidazole. Protein was eluted with buffer N containing 250 mM imidazole. Eluted protein was dialyzed against storage buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 10 mM 2-mercaptoethanol, and 30% [vol/vol] glycerol). Dialyzed protein was aliquoted and stored at −70°C.

RsbU phosphatase assay. Purified recombinant RsbV1 or RsbV2 (20 μg) was radiolabeled by incubating with purified recombinant RsbW (5 μg) and approximately 30 μCi [g-^{32}P]ATP (10 mCi mmol^{-1}; PerkinElmer) in CutSmart buffer (NEB) at 37°C for 30 min. Radiolabeled protein was separated from free ^{32}P-ATP by centrifugation in Mini Quick Spin DNA column (Roche). Radiolabeled protein was diluted in glycerol (20% [vol/vol] final concentration) and stored at −20°C.

For the phosphatase assay, radiolabeled RsbV protein (approximately 0.02 μg; 5 μM) was incubated with purified recombinant RsbU (0.5 μg; 6 μM) in buffer P (10 mM Tris, pH 7.5) with magnesium chloride or manganese chloride (as indicated in text) for 30 min at 37°C. For assays with other ions, Mg^{2+} was not present in buffer P. Phosphoenolpyruvate (Fisher; catalog no. 50-505-440), L-2-phosphoglyceric acid (Sigma-Aldrich; catalog no. 19710), alpha-ketoglutaric acid (Spectrum Labs; catalog no. KE110), oxaloacetic acid (Sigma-Aldrich; catalog no. O4126), L-(−)-malic acid (Spectrum Labs; catalog no. M2007), and succinic acid (Fisher Scientific; catalog no. S3674) were used as indicated. Reactions were stopped by addition of SDS-PAGE loading buffer. Samples were analyzed by SDS-PAGE electrophoresis on an 18% SDS-PAGE gel. Gels were exposed to a phosphoimager screen, which was then scanned on an Amersham Typhoon biomolecular imager.

RT-PCR. L929 mouse fibroblast cells were infected with *C. trachomatis* L2 at a multiplicity of infection (MOI) of 3. At indicated times, infected cells were collected with glass beads in phosphate-buffered saline (PBS) buffer. Cells were pelleted and used for RNA extraction using a Macherey-Nagel RNA extraction kit. RNA was treated with RQ1 DNase (Promega) to remove any DNA. Approximately 5 μg total RNA was used for reverse transcription with Moloney murine leukemia virus (MMLV) reverse transcriptase, with a primer located 1,697 bp downstream of the ATG translational start site of the *rsbU* gene. cDNA was used for PCR using primers amplifying a 1,309-bp fragment located 1,162 bp downstream (5′-GGG TCTCTTTCCCGTTCTGA) of the ATG translational start site of the *eno* gene to 1,057 bp downstream (5′-GGATGGTGTTGATTGCGACG) of the ATG translational start site of the *rsbU* gene. PCR was performed with Bioneer AccuPower *Pfu* PCR premix with the following amplification conditions: 30 s at 95°C, 45 s at 57°C, and 90 s at 71°C for 40 cycles. PCR products were analyzed by electrophoresis on a 1% agarose gel in Tris-borate-EDTA (TBE). Gels were stained with ethidium bromide, and bands were visualized by UV light with an Amersham Imager 680.

5′ RACE. First Choice RLM-RACE reactions (Thermo Fisher) were performed with RNA extracted from infected L929 cells at 18 and 30 hpi. 5′ RACE assays were performed per the manufacturer's instructions. A primer (5′-GCAGGGTGAGGAGGAACTTC) located 1,697 bp downstream of the ATG translation start site of *rsbU* was used for cDNA synthesis. The same primer was used to amplify a PCR product to the modified 5′ end of the mRNA. Based on published transcription start sites (27), these primers generate expected PCR products of ~3.1 kB and ~1.6 kB for the *eno* and *rsbU* promoters, respectively. PCR

products were analyzed by electrophoresis on a 1% agarose gel in TBE. Gels were stained with ethidium bromide, and bands were visualized by UV light with an Amersham Imager 680.

Transcription assays. Transcription assays were performed as previously described (14). Essentially, 2.5 μM recombinant EUO was incubated with 13 nM plasmid DNA at room temperature for 15 min. Transcription was initiated with 0.4 U *E. coli* holoenzyme (Epicentre) in the presence of ^{32}P-CTP at 37°C for 15 min. Transcripts were resolved on an 8 M urea-6% polyacrylamide gel. Gels were placed on Whatman paper and exposed to a phosphorimager screen. The phosphoimager screen was scanned on an Amersham Typhoon biomolecular imager. Band intensities were quantified using Quantity One software (Bio-Rad). For each promoter, the relative transcript levels were calculated by measuring the transcript levels in the presence of EUO and normalizing them to levels in the absence of EUO. Values are reported as the mean of the relative transcript levels with standard deviations from at least three individual experiments. Statistical significance was calculated by unpaired *t* test using GraphPad Prism.

Enolase assay. Enolase assays were performed using an enolase activity kit (Sigma-Aldrich; catalog no. MAK178). Essentially, purified recombinant His-tagged enolase was incubated in 2-phosphoglycerate in a Costar 3603 clear, flat-bottom, 96-well assay plate. The production of phosphoenolpyruvate is proportional to the amount of hydrogen peroxide produced. The production of hydrogen peroxidase was measured fluorometrically (excitation wavelength [λ_{ex}], 535 nm; emission wavelength [λ_{em}], 587 nm) on a SpectraMax i3x, with readings taken every 5 min for 30 min at 25°C. Activity was calculated as the rate of nanomole of hydrogen peroxide produced per time (minutes) per milliliter of sample. Statistical significance was calculated by unpaired *t* test using GraphPad Prism.

ACKNOWLEDGMENT

This work was supported by a grant from the NIH (AI044198).

REFERENCES

1. Abdelrahman YM, Belland RJ. 2005. The chlamydial developmental cycle. FEMS Microbiol Rev 29:949–959. https://doi.org/10.1016/j.femsre.2005.03.002.
2. Moulder JW. 1991. Interaction of chlamydiae and host cells *in vitro*. Microbiol Rev 55:143–190. https://doi.org/10.1128/mr.55.1.143-190.1991.
3. Hybiske K, Stephens RS. 2007. Mechanisms of host cell exit by the intracellular bacterium *Chlamydia*. Proc Natl Acad Sci U S A 104:11430–11435. https://doi.org/10.1073/pnas.0703218104.
4. Belland RJ, Zhong G, Crane DD, Hogan D, Sturdevant D, Sharma J, Beatty WL, Caldwell HD. 2003. Genomic transcriptional profiling of the developmental cycle of *Chlamydia trachomatis*. Proc Natl Acad Sci U S A 100:8478–8483. https://doi.org/10.1073/pnas.1331135100.
5. Nicholson TL, Olinger L, Chong K, Schoolnik G, Stephens RS. 2003. Global stage-specific gene regulation during the developmental cycle of *Chlamydia trachomatis*. J Bacteriol 185:3179–3189. https://doi.org/10.1128/JB.185.10.3179-3189.2003.
6. Rosario CJ, Soules K, Hefty PS, Tan M. 2020. Chlamydia gene regulation, p 219–240. *In* Tan M, Hegemann JH, Sütterlin C (ed), Chlamydia biology: from genome to disease. Caister Academic Press, Norfolk, UK.
7. Shaw EI, Dooley CA, Fischer ER, Scidmore MA, Fields KA, Hackstadt T. 2000. Three temporal classes of gene expression during the *Chlamydia trachomatis* developmental cycle. Mol Microbiol 37:913–925. https://doi.org/10.1046/j.1365-2958.2000.02057.x.
8. Hayward RJ, Humphrys MS, Huston WM, Myers GSA. 2021. Dual RNA-seq analysis of in vitro infection multiplicity and RNA depletion methods in *Chlamydia*-infected epithelial cells. Sci Rep 11:10399. https://doi.org/10.1038/s41598-021-89921-x.
9. Humphrys MS, Creasy T, Sun Y, Shetty AC, Chibucos MC, Drabek EF, Fraser CM, Farooq U, Sengamalay N, Ott S, Shou H, Bavoil PM, Mahurkar A, Myers GS. 2013. Simultaneous transcriptional profiling of bacteria and their host cells. PLoS One 8:e80597. https://doi.org/10.1371/journal.pone.0080597.
10. Niehus E, Cheng E, Tan M. 2008. DNA supercoiling-dependent gene regulation in *Chlamydia*. J Bacteriol 190:6419–6427. https://doi.org/10.1128/JB.00431-08.
11. Orillard E, Tan M. 2016. Functional analysis of three topoisomerases that regulate DNA supercoiling levels in *Chlamydia*. Mol Microbiol 99:484–496. https://doi.org/10.1111/mmi.13241.
12. Rosario CJ, Tan M. 2012. The early gene product EUO is a transcriptional repressor that selectively regulates promoters of *Chlamydia* late genes. Mol Microbiol 84:1097–1107. https://doi.org/10.1111/j.1365-2958.2012.08077.x.
13. Rosario CJ, Hanson BR, Tan M. 2014. The transcriptional repressor EUO regulates both subsets of *Chlamydia* late genes. Mol Microbiol 94:888–897. https://doi.org/10.1111/mmi.12804.
14. Yu HHY, Tan M. 2003. Sigma 28 RNA polymerase regulates *hctB*, a late developmental gene in *Chlamydia*. Mol Microbiol 50:577–584. https://doi.org/10.1046/j.1365-2958.2003.03708.x.
15. Yu HHY, Kibler D, Tan M. 2006. *In silico* prediction and functional validation of sigma 28-regulated genes in *Chlamydia* and *Escherichia coli*. J Bacteriol 188:8206–8212. https://doi.org/10.1128/JB.01082-06.
16. Yu HHY, Di Russo EG, Rounds MA, Tan M. 2006. Mutational analysis of the promoter recognized by *Chlamydia* and *Escherichia coli* sigma 28 RNA polymerase. J Bacteriol 188:5524–5531. https://doi.org/10.1128/JB.00480-06.
17. Helmann JD. 1999. Anti-sigma factors. Curr Opin Microbiol 2:135–141. https://doi.org/10.1016/S1369-5274(99)80024-1.
18. Douglas A, Hatch TP. 2006. The phosphorelay system in *Chlamydia trachomatis*, p 177–180. *In* Chernesky M (ed), Chlamydial infections: proceedings of the 11th international symposium on human chlamydial infections. International Chlamydia Symposium, Niagara-on-the-Lake, Canada.
19. Hua L, Hefty PS, Lee YJ, Lee YM, Stephens RS, Price CW. 2006. Core of the partner switching signalling mechanism is conserved in the obligate intracellular pathogen *Chlamydia trachomatis*. Mol Microbiol 59:623–636. https://doi.org/10.1111/j.1365-2958.2005.04962.x.
20. Thompson CC, Griffiths C, Nicod SS, Lowden NM, Wigneshweraraj S, Fisher DJ, McClure MO. 2015. The Rsb phosphoregulatory network controls availability of the primary sigma factor in *Chlamydia trachomatis* and influences the kinetics of growth and development. PLoS Pathog 11:e1005125. https://doi.org/10.1371/journal.ppat.1005125.
21. Soules KR, Dmitriev A, LaBrie SD, Dimond ZE, May BH, Johnson DK, Zhang Y, Battaile KP, Lovell S, Hefty PS. 2020. Structural and ligand binding analyses of the periplasmic sensor domain of RsbU in *Chlamydia trachomatis* support a role in TCA cycle regulation. Mol Microbiol 113:68–88. https://doi.org/10.1111/mmi.14401.
22. Das AK, Helps NR, Cohen PT, Barford D. 1996. Crystal structure of the protein serine/threonine phosphatase 2C at 2.0 A resolution. EMBO J 15:6798–6809. https://doi.org/10.1002/j.1460-2075.1996.tb01071.x.
23. Sajid A, Arora G, Singhal A, Kalia VC, Singh Y. 2015. Protein phosphatases of pathogenic bacteria: role in physiology and virulence. Annu Rev Microbiol 69:527–547. https://doi.org/10.1146/annurev-micro-020415-111342.
24. Kanehisa M, Goto S. 2000. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res 28:27–30. https://doi.org/10.1093/nar/28.1.27.
25. Kanehisa M. 2019. Toward understanding the origin and evolution of cellular organisms. Protein Sci 28:1947–1951. https://doi.org/10.1002/pro.3715.
26. Kanehisa M, Furumichi M, Sato Y, Ishiguro-Watanabe M, Tanabe M. 2021. KEGG: integrating viruses and cellular organisms. Nucleic Acids Res 49:D545–D551. https://doi.org/10.1093/nar/gkaa970.

27. Albrecht M, Sharma CM, Reinhardt R, Vogel J, Rudel T. 2010. Deep sequencing-based discovery of the *Chlamydia trachomatis* transcriptome. Nucleic Acids Res 38:868–877. https://doi.org/10.1093/nar/gkp1032.
28. Brewer JM, Glover CV, Holland MJ, Lebioda L. 1998. Significance of the enzymatic properties of yeast S39A enolase to the catalytic mechanism. Biochim Biophys Acta 1383:351–355. https://doi.org/10.1016/S0167-4838(98)00004-1.
29. Akers JC, Tan M. 2006. Molecular mechanism of tryptophan-dependent transcriptional regulation in *Chlamydia trachomatis*. J Bacteriol 188:4236–4243. https://doi.org/10.1128/JB.01660-05.
30. Hanson BR, Tan M. 2015. Transcriptional regulation of the *Chlamydia* heat shock stress response in an intracellular infection. Mol Microbiol 97:1158–1167. https://doi.org/10.1111/mmi.13093.
31. Ende RJ, Derre I. 2020. Host and bacterial glycolysis during *Chlamydia trachomatis* infection. Infect Immun 88:e00545-20. https://doi.org/10.1128/IAI.00545-20.
32. Schwoppe C, Winkler HH, Neuhaus HE. 2002. Properties of the glucose-6-phosphate transporter from *Chlamydia pneumoniae* (HPTcp) and the glucose-6-phosphate sensor from *Escherichia coli* (UhpC). J Bacteriol 184:2108–2115. https://doi.org/10.1128/JB.184.8.2108-2115.2002.
33. Vender J, Moulder JW. 1967. Initial step in catabolism of glucose by the meningopneumonitis agent. J Bacteriol 94:867–869. https://doi.org/10.1128/jb.94.4.867-869.1967.
34. Weiss E, Wilson N. 1969. Role of exogenous adenosine triphosphate in catabolic and synthetic activities of *Chlamydia psittaci*. J Bacteriol 97:719–724. https://doi.org/10.1128/jb.97.2.719-724.1969.
35. Weiss E. 1965. Adenosine triphosphate and other requirements for the utilization of glucose by agents of the psittacosis-trachoma group. J Bacteriol 90:243–253. https://doi.org/10.1128/jb.90.1.243-253.1965.
36. Dannelly HK, Duclos B, Cozzone AJ, Reeves HC. 1989. Phosphorylation of *Escherichia coli* enolase. Biochimie 71:1095–1100. https://doi.org/10.1016/0300-9084(89)90116-8.
37. Fisher DJ, Adams NE, Maurelli AT. 2015. Phosphoproteomic analysis of the *Chlamydia caviae* elementary body and reticulate body forms. Microbiology (Reading) 161:1648–1658. https://doi.org/10.1099/mic.0.000116.
38. Krucinska J, Lombardo MN, Erlandsen H, Hazeen A, Duay SS, Pattis JG, Robinson VL, May ER, Wright DL. 2019. Functional and structural basis of *E. coli* enolase inhibition by SF2312: a mimic of the carbanion intermediate. Sci Rep 9:17106. https://doi.org/10.1038/s41598-019-53301-3.
39. Krucinska J, Falcone E, Erlandsen H, Hazeen A, Lombardo MN, Estrada A, Robinson VL, Anderson AC, Wright DL. 2019. Structural and functional studies of bacterial enolase, a potential target against Gram-negative pathogens. Biochemistry 58:1188–1197. https://doi.org/10.1021/acs.biochem.8b01298.
40. Spring TG, Wold F. 1971. The purification and characterization of *Escherichia coli* enolase. J Biol Chem 246:6797–6802. https://doi.org/10.1016/S0021-9258(19)45916-4.
41. Nakayasu ES, Burnet MC, Walukiewicz HE, Wilkins CS, Shukla AK, Brooks S, Plutz MJ, Lee BD, Schilling B, Wolfe AJ, Muller S, Kirby JR, Rao CV, Cort JR, Payne SH. 2017. Ancient regulatory role of lysine acetylation in central metabolism. mBio 8:e01894-17. https://doi.org/10.1128/mBio.01894-17.
42. Mihailovic J, Inic-Kanada A, Smiljanic K, Stein E, Barisani-Asenbauer T, Cirkovic Velickovic T. 2016. Lysine acetylation of major *Chlamydia trachomatis* antigens. EuPA Open Proteom 10:63–69. https://doi.org/10.1016/j.euprot.2016.01.007.
43. Chiarelli TJ, Grieshaber NA, Omsland A, Remien CH, Grieshaber SS. 2020. Single-Inclusion kinetics of *Chlamydia trachomatis* development. mSystems 5:e00689-20. https://doi.org/10.1128/mSystems.00689-20.
44. Lee JK, Enciso GA, Boassa D, Chander CN, Lou TH, Pairawan SS, Guo MC, Wan FYM, Ellisman MH, Sutterlin C, Tan M. 2018. Replication-dependent size reduction precedes differentiation in *Chlamydia trachomatis*. Nat Commun 9:45. https://doi.org/10.1038/s41467-017-02432-0.
45. Wilson AC, Tan M. 2002. Functional analysis of the heat shock regulator HrcA of *Chlamydia trachomatis*. J Bacteriol 184:6566–6571. https://doi.org/10.1128/JB.184.23.6566-6571.2002.
46. Wilson AC, Tan M. 2004. Stress response gene regulation in *Chlamydia* is dependent on HrcA-CIRCE interactions. J Bacteriol 186:3384–3391. https://doi.org/10.1128/JB.186.11.3384-3391.2004.