RESEARCH ARTICLE

Nonspecific toxicities of *Streptococcus pyogenes* and *Staphylococcus aureus* dCas9 in *Chlamydia trachomatis*

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One sentence summary: Expression of two deactivated Cas proteins (dCas) commonly used for CRISPRi gene-silencing both cause unintended toxicity in the sexually transmitted human pathogen *Chlamydia trachomatis*.

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ABSTRACT

Chlamydiae are common, important pathogens for humans and animals alike. Despite recent advancement in genetics, scientists are still searching for efficient tools to knock out or knock down the expression of chromosomal genes. We attempted to adopt a dCas9-based CRISPR interference (CRISPRi) technology to conditionally knock down gene expression in *Chlamydia trachomatis* using an anhydrotetracycline (ATC)-inducible expression system. Surprisingly, expression of the commonly used *Streptococcus pyogenes* dCas9 in *C. trachomatis* causes strong inhibition in the absence of any guide RNA (gRNA). *Staphylococcus aureus* dCas9 also shows strong toxicity in the presence of only an empty gRNA scaffold. Toxicity of the *S. pyogenes* dCas9 is readily observed with as little as 0.2 nM ATC. Growth inhibition by *S. aureus* dCas9 is evident starting at 1.0 nM ATC. In contrast, *C. trachomatis* growth was not affected by methionine-tRNA ligase overexpression induced with 10 nM ATC. We conclude that *S. pyogenes* and *S. aureus* dCas9 proteins in their current forms have limited utility for chlamydial research and suggest strategies to overcome this problem.

Keywords: Chlamydia; CRISPR; CRISPRi; dCas9; conditional gene knock-down; essential gene knock-down; toxicity

INTRODUCTION

Chlamydiae are broadly distributed pathogenic bacteria. For example, *Chlamydia trachomatis* is one of the most prevalent sexually transmitted pathogens worldwide (WHO 2012). In the United States, cases of sexually transmitted *C. trachomatis* infection consistently account for 60% of the total infected cases reported to the Centers for Diseases and Prevention (CDC 2018). Common complications from *C. trachomatis* infection include infertility, abortion, ectopic pregnancy and pelvic inflammatory syndrome (Zhong et al. 2017). Unfortunately, an effective vaccine

has yet to be developed despite numerous efforts by the scientific community over the past 60 years (de la Maza, Zhong and Brunham 2017; Zhong et al. 2017). There is a clear need to better understand the *Chlamydia* biology to develop effective preventives.

Chlamydiae are obligate intracellular bacteria with a unique developmental cycle that is characterized by two contrasting cellular forms (Abdelrahman and Belland 2005). The small, electron-dense and non-dividing elementary body (EB) attaches to the host cell and enters a host membrane-derived vacuole through endocytosis (Hybiske and Stephens 2007). Within the

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vacuole, termed chlamydial inclusion, the EB transitions to the non-infectious reticulate body (RB), which is larger and less electron dense than the EB. RBs undergo several rounds of replication before asynchronously transitioning back into EBs, which exit the host cell (Abdelrahman and Belland 2005; Lee et al. 2018).

Progression of the chlamydial developmental cycle is dictated by transcription (Belland et al. 2003; Nicholson et al. 2003; Humphrys et al. 2013). Studies using animal models indicate that expression of specific genes is required for chlamydiae to colonize different tissues and/or organs, even though these genes may not affect chlamydial growth in cell culture (Yang et al. 2017; Koprivsek et al. 2019). However, the exact roles that many genes play in these processes have yet to be defined.

The *C. trachomatis* genome carries fewer than 1000 genes, of which nearly a third encode hypothetical proteins. Many of these encoding genes are unique to chlamydiae and require robust genetic tools to study their functions (Stephens et al. 1998). Since the first report of reproducible transformation of *C. trachomatis* with an engineered plasmid (Wang et al. 2011), methodologies for conditional gene expression and inactivation of non-essential genes have been developed (Agaisse and Derre 2013; Wickstrum et al. 2013; Bauler and Hackstadt 2014; Key and Fisher 2017; Mueller, Wolf and Fields 2017). More recently, a strategy to conditionally knock down chlamydial genes using CRISPR interference (CRISPRi) was reported. The strategy relies on a catalytically-deactivated Cas9 (dCas9) from *Staphylococcus aureus* to block the transcription of a *C. trachomatis* gene that encodes a inclusion protein termed IncA (Ouellette 2018). If proven reliable, this technology would provide great power for studying essential genes, even though IncA is non-essential. However, in our attempt to adopt this technology, we found that conditional expression of the staphylococcal dCas9 (Sa-dCas9) strongly inhibits chlamydial growth in the absence of any specific guide RNA (gRNA). We also detected serious, non-specific toxicity of a *Streptococcus pyogenes* dCas9 (Sp-dCas9) in *C. trachomatis*. These findings suggest new strategies are needed to conditionally knock down essential genes in this important pathogen.

MATERIALS AND METHODS

Vectors

DNA primers for vector construction and/or sequencing (Table S1, Supporting Information) were custom-synthesized at Millipore Sigma. Q5 high fidelity DNA polymerase (used for amplifying DNA fragments; catalog # E5520S), Q5 site-directed mutagenesis kit (New England Biolabs catalog E0554) and NEBuilder HiFi DNA Assembly Cloning Kit (used for vector construction; catalog # M0491) were purchased from New England Biolabs. Plasmid pTRL2(Δ gfp) (Fig. 1A) was derived by deleting the GFP gene from the shuttle vector pASK-GFP-L2-mKate2 (Wickstrum et al. 2013). Primers Del-gfp-pASK-GFP-F and Del-gfp-pASK-GFP-R (Table S1, Supporting Information) were used to amplify the remaining portion of pASK-GFP-L2-mKate2. The amplified fragment was circled using the Q5 site-directed mutagenesis kit.

Plasmid pTRL2-Sp-dCas9 (Fig. 1B) was constructed by assembling two DNA fragments using the NEBuilder HiFi DNA Assembly Cloning Kit. Fragment 1, essentially a linearized shuttle vector pTRL2(Δ gfp), was amplified using pASK-GFP-L2-mKate2 as the template, primer PTRL2-Frag-2-F and primer PTRL2-Frag-1-R (Table S1, Supporting Information). Fragment 2, which contained the open reading frame of Sp-dCas9, was amplified using pTDH3-dCas9 as the template and primers PTRL2-

dCas9-F and PTRL2-dCas9-R (Table S1, Supporting Information). pTDH3-dCas9 was a gift from Stanley Qi & Jonathan Weissman (Addgene plasmid # 46920; <http://n2t.net/addgene:46920>; RRID:Addgene_46920).

Plasmid pTRL2-Sa-dCas9 (Fig. 1C) was constructed by assembling four DNA fragments using the NEBuilder HiFi DNA Assembly Cloning Kit. Fragment 1 contained the pASK-GFP-L2-mKate2-originated mKate gene, ampicillin-resistance gene, tet-regulated transcription repressor (tetR), *E. coli* replication origin ColE1 and the tet-inducible promoter (P_{tetA}). The fragment was amplified using pASK-GFP-L2-mKate2 as the template, and primers PTRL2-Frag1-F and PTRL2-Sa-dCas9-Frag1-R (Table S1, Supporting Information). Fragment 2, which contained all components of the Ctl2 plasmid and a mutated *dnaK* promoter (Wickstrum et al. 2013), was amplified using pTRL2 as the template, and primers PTRL2-Sa-dCas9-Frag2-F and PTRL2-Sa-dCas9-Frag2-R (Table S1, Supporting Information). Fragment 3, which contained a gRNA scaffold sequence followed by the transcription termination signal of *ctl0608* and the *defA* (*ctl0607*) promoter (P_{defA}), was amplified using pUC57-Kan-GrgA gRNA-scaffold-TTS- P_{defA} as the template, and primers PTRL2-gRNA-C-F and PTRL2-gRNA-R (Table S1, Supporting Information). Fragment 4, which contained the Sa-dCas9 open reading frame, was amplified using pX603-AAV-CMV::NLS-dSaCas9 (D10A, N580A)-NLS-3xHA-bGHpA as the template, and primers Sa-dCas9-pTRL2-F and Sa-dCas9-pTRL2-R (Table S1, Supporting Information). pX603-AAV-CMV::NLS-dSaCas9 (D10A, N580A)-NLS-3xHA-bGHpA was a gift from Feng Zhang (Addgene plasmid # 61594; <http://n2t.net/addgene:61594>; RRID:Addgene_61594).

Plasmid pTRL2-NH-metG (Fig. 1D) was constructed by assembling two DNA fragments using the NEBuilder HiFi DNA Assembly Cloning Kit. Fragment 1, which contained all Ctl2 plasmid encoding genes, mKate gene, ampicillin-resistance, tetR, ColE1, P_{tetA} and a polyhistidine tag, was amplified using pTRL2-NH-GrgA (unpublished work) as the template, primers metG-NH-pTRL-F and metG-NH-pTRL-R (Table S1, Supporting Information). Fragment 2, which contained the open reading frame of metG gene, was amplified using Ctl2 genomic DNA as the template and primers NH-pTRL-metG-F and NH-pTRL-metG-R (Table S1, Supporting Information).

All four plasmids constructed for this study were sequenced almost entirely using services provided by MacrogenUSA or GenScript. The only portions that were not sequenced were *ampR* and ColE1. Sequences of the constructed plasmids were aligned against source sequences. None of the final plasmid used for experiments contained any nucleotide changes.

Chlamydiae

Chlamydia trachomatis L2 (Ctl2) strain 434/BU was purchased from American Type Culture Collection (cat. # VR-902B) and maintained with L929 host cells (Bao et al. 2014). Ctl2 transformation and selection of transformants were carried out as previously described (Xu et al. 2013).

Chlamydia trachomatis growth determination

L929 cells grown on 6-well plates at about 90% confluency were inoculated with Ctl2 transformants and cultured at 37°C with or without ATC (Millipore Sigma, catalog # 37919). Bright field and mKate images were acquired on an Olympus IX51 fluorescence microscope using constant exposure time for each channel 30 hours post-inoculation. Image overlay was performed

Figure 1. Structures of pTRL2(Δ gfp) (A), pTRL2-Sp-dCas9 (B), pTRL2-Sa-dCas9 (C) and pTRL2-metG (D). Abbreviations: Sp, *S. pyogenes*; Sa, *S. aureus*; dCas9, deactivated Cas9; MetG, methionine-tRNA ligase; Pgp, chlamydial plasmid-encoded protein; P_{dnaK} , *dnaK* promoter; mKate, a monomeric basic far-red fluorescence protein; gRNA, guide RNA; P_{defA} , *defA* promoter; AmpR, penicillin/ampicillin-resistance gene; tetR, tet repressor; ColE1, *E. coli* replication origin E1; P_{tetA} , tetracycline/anhidrotetracycline (ATC)-activated promoter. pL2 ori, Ctl2 plasmid replication origin.

using the PictureFrame software. To quantify progeny EB formation, cultures were terminated 30 hours post-inoculation. Cells were disrupted by sonication to release chlamydiae. 1:10-serially diluted cell lysates were inoculated onto L929 cells grown on 96-well plates. Recoverable inclusion-forming units were scored 30 hours later (Gong et al. 2014).

Immunostaining

L929 cells grown on 24-well plates were infected with pTRL2-Sa-dCas9 transformants and cultured at 37°C for 17 hours. Sa-dCas9 expression was induced for 4 hours following addition of ATC to the media. Cells were fixed sequentially with paraformaldehyde and cold methanol as previously described. The double-fixation protocol preserves mKate signal (Zhang et al. 2017).

The fixed cells were sequentially reacted with a mouse monoclonal anti-Sa-Cas9 primary antibody (Boster Biological Technology, catalog # M30929–3) and an FITC-conjugated anti-mouse IgG secondary antibody (Millipore Sigma, catalog # F9137). Evan Blue and Hoechst dye 33342 were used to counter stain non-nuclear proteins and DNA, respectively, before microscopy. Images were acquired using constant exposure time for each channel.

RESULTS

Toxicity of Sp-dCas9 in *C. trachomatis*

We initially chose Sp-dCas9 for CRISPRi in *C. trachomatis* because (i) it is the most extensively used dCas in the literature (Jiang

Figure 2. *Chlamydia trachomatis* L2 (CtL2) growth inhibition by ATC-induced Sp-dCas9 expression. (A) Microscopic images showing reduced mKate expression in inclusions formed by pTRL2-Sp-dCas9 transformants of CtL2 cultured with indicated ATC concentrations. (B) ATC concentration-dependent inhibition of EB production by the transformants. Data are averages $\pm$ standard deviations of triplicate experiments. P values are of two-tailed t tests. (A, B) ATC was added at the time of inoculation. Microscopy and determination of recoverable inclusion-forming units were performed at 30 hours post-inoculation.

Figure 3. CtL2 growth inhibition by ATC-induced Sa-dCas9 expression. (A) Microscopic images showing reduced mKate expression in inclusions formed by pTRL2-Sa-dCas9 transformants cultured with indicated ATC concentrations. (B) ATC concentration-dependent inhibition of EB production by the transformants. Data are averages $\pm$ standard deviations of triplicate experiments. P values are of two-tailed t tests. (A, B) ATC was added at the time of inoculation. Microscopy and determination of recoverable inclusion-forming units were performed at 32 hours post-inoculation.

Figure 4. ATC concentration-dependent induction of Sa-dCas9 expression in pTRL2-Sa-dCas9 transformants. ATC was added at 17 hours post-noculation. Cultures were fixed at 21 hours for Sa-dCas9 expression analysis using immunofluorescence.

and Doudna 2017) and (ii) it recognizes a short protospacer adjacent motif (PAM) sequence of only three nucleotides (NGG where N is any nucleotide). This notably short PAM sequence consequently allows for numerous targeting sites. We constructed a *C. trachomatis* expression vector for Sp-dCas9 termed pTRL2-Sp-dCas9 (Fig. 1A), in which the Sp-dCas9 sequence is placed downstream of an ATC-inducible promoter (Wickstrum et al. 2013). This vector also carries an mKate gene, which encodes a far-red fluorescence protein under the control of a mutated *dnaK* promoter (P_{dnaK}) (Wickstrum et al. 2013). After a single-time transformation with pTRL2-Sp-dCas9, mKate-expressing inclusions were observed in 2 wells of a 6-well plate of L929 cells infected with transformed EBs at passage two of selection with penicillin. Both populations demonstrated severely reduced mKate signals in their inclusions 30 hours after inducing Sp-dCas9 expression with 10 nM ATC. One population was further analyzed using various concentrations of ATC. Reduced mKate inclusion signals were readily observed with 0.5 nM ATC, with progressively decreased signal intensity observed at higher concentrations (Fig. 2A). An 80% inhibition of progeny EB production was observed with even 0.2 nM ATC (Fig. 2B). The inhibition increased to 96 and 98%, with 0.5 nM and 1.0 nM ATC, respectively (Fig. 2B).

Toxicity of Sa-dCas9 in *C. trachomatis*

In light of a recent publication showing Sa-dCas9-mediated IncA expression inhibition (Ouellette 2018), we decided to adopt the same system by constructing the pTRL2-Sa-dCas9 vector with the addition of an mKate gene. In pTRL2-Sa-dCas9, P_{dnaK} controls expression of a gRNA scaffold carrying no specific targeting sequence; a peptide deformylase gene promoter (P_{defA}) drives mKate expression (Fig. 2B). After a single-time transformation with pTRL2-Sp-dCas9, mKate-expressing inclusions emerged in 5 wells of a 6-well plate of L929 cells infected with transformed EBs at passage two of selection with penicillin. Disappointingly, the transformants also showed growth inhibition following Sa-dCas9 induction as evidenced by a dose-dependent decrease in both mKate expression (Fig. 3A) and progeny EB production (Fig. 3B). Although 0.5 nM ATC did not exhibit an inhibitory effect, 1, 2, 5 and 10 nM ATC inhibited EB production by 55, 82, 93 and 93%, respectively.

Compared to pTRL2-Sp-dCas9 transformants (Fig. 2), pTRL2-Sa-dCas9 transformants were inhibited by ATC somewhat less efficiently (Fig. 3). We hoped that there would be a Sa-dCas9 expression range induced by ATC concentrations that allowed

Figure 5. Growth of CtL2 transformed with control pTRL2(Δ gfp) plasmid unaffected by ATC. (A) Microscopic images showing mKate-expressing inclusions formed in the presence of 0 or 10 nM ATC. (B) Recoverable IFUs formed in the presence of 0 or 10 nM ATC. Data are averages $\pm$ standard deviations of triplicate experiments. P values are of two-tailed t tests. (A, B) ATC was added at the time of inoculation. Microscopy and determination of recoverable inclusion-forming units were performed at 35 hours post-inoculation.

for CRISPRi without nonspecific toxicity. To assess this possibility, we performed immunofluorescence assays using a monoclonal anti-Sa-dCas9 antibody to analyze Sa-dCas9 expression following culture with different concentrations of ATC for 4 hours. pTRL2-Sa-dCas9 transformants induced with 0.5 and 1.0 nM ATC had indistinguishable staining profiles as the non-induced control. Specific dCas9 signals became definitively evident only with ≥ 2 nM ATC (Fig. 4), even though toxicity was readily detectable in cultures treated with 1 nM ATC (Fig. 3). Together, these findings do not support the existence of a practical ATC concentration range within which efficient gene transcription interference can be induced without nonspecific toxicity.

Lack of toxicity by ATC

ATC is derived from tetracycline, a potent and known antichlamydial. To eliminate the possibility that residual antibacterial ATC activity was responsible for the observed toxicities following dCas9-overexpression, we determined the effects of ATC on the growth of CtL2 transformed with control plasmid pTRL2(Δ gfp) (Fig. 1A). When compared to a non-treated control, mock-induction with 10 nM ATC did not demonstrate any inhibition of inclusion size or the mKate intensity (Fig. 5A). Furthermore, subsequent EB formation was also un-impaired (Fig. 5B). These data are consistent with findings by Wickstrum et al., that show a lack of inhibition by 10 nM ATC in wild-type CtL2 (Wickstrum et al. 2013).

Lack of toxicity by methionine-tRNA ligase (MetG) overexpression

We considered the possibility that overexpression of any protein in CtL2 may induce toxicity, for which we constructed the plasmid pTRL2-NH-metG to overexpress N-terminally (His)₆-tagged

Figure 6. CtL2 growth unaffected by ATC-induced NH-MetG expression. (A) Western blot detecting the 65 kDa NH-MetG in pTRL2-NH-metG transformants of CtL2 cultured in the presence of 0 or 10 nM ATC. (B) mKate-expressing inclusions formed in the presence of 0 or 10 nM ATC. (C) Recoverable IFUs formed in the presence of 0 or 10 nM ATC. Data are averages $\pm$ standard deviations of triplicate experiments. P values are of two-tailed t tests. (A–C) ATC was added at the time of inoculation. Microscopy and determination of recoverable inclusion-forming units were performed at 30 hours post-inoculation.

MetG (NH-MetG) under the control of P_{tetA} (Fig. 1D). For transformants cultured in the absence of ATC, western blot using anti-(His)₆ tag antibody detected only a low level of NH-MetG. However, when cultured in the presence of 10 nM ATC, a markedly increased level of NH-MetG was detected (Fig. 6A). Further analysis of inclusion size, mKate intensity (Fig. 6B) and EB production (Fig. 6C) corroborate the absence of growth inhibition following ATC-induced NH-MetG overexpression. Taken together, these data indicate Sp-dCas9 and Sa-dCas9 are themselves responsible for observed growth inhibition rather than the ATC used to induce protein expression.

DISCUSSION

CRISPR-Cas9 technologies have armed scientists with unprecedented genome editing powers. As a result, genes can now be efficiently inactivated or modified in a wide range of organisms, whereas inducible dCas9 expression has been used to conditionally knock down essential genes (Jiang and Doudna 2017). Although there has been no report of successful *Chlamydia* genome editing with wild-type Cas9 (or any other Cas proteins),

a recent study documented knock down of *C. trachomatis* IncA expression with Sa-dCas9 (Ouellette 2018). It showed decreased IncA expression in select cells following induction of the Sa-dCas9 with 10 nM ATC. However, the effect of Sa-dCas9 expression on chlamydial growth was not quantified (Ouellette 2018). Our data presented here clearly demonstrate that *C. trachomatis* is highly sensitive to both Sp-dCas9 and Sa-dCas9 in the absence of any gRNA. Accordingly, significant reduction in EB formation was detected with only 0.2 and 1.0 nM ATC to induce expression of Sp-dCas9 and Sa-dCas9, respectively.

Nonspecific dCas9 toxicities in *Escherichia coli* and other bacteria have also been reported, but only when very high concentrations of ATC or doxycycline were used to induce expression (Rock et al. 2017; Cho et al. 2018). For example, 1 μ M ATC is needed to demonstrate toxicity of Sp-dCas9 in *E. coli* K-12 1655 (Cho et al. 2018). *Chlamydia trachomatis* seems particularly susceptible to the toxicity of Sp-dCas9, where 0.2 nM ATC is sufficient for growth inhibition.

While the toxicity mechanism remains unclear, nonspecific binding of Sp-dCas9 at high concentrations to the PAM site in the absence of any gRNA is a potential mechanism (Jones et al. 2017). The PAM sequences of Sp-dCas9 and Sa-dCas9 are NGG and NNGRRT, respectively. The *C. trachomatis* genome (or any other genome) thereby has a higher number of Sp-dCas9 PAM sites than Sa-dCas9 PAM sites. This may explain the need for lower ATC concentrations to induce the toxicity of Sp-dCas9. However, we think that the differential ATC susceptibility is more likely due to a lower expression efficiency of Sa-dCas9 than Sp-dCas9 although we have only performed Sa-dCas9 expression analysis. Even though expression of both dCas is driven by P_{tetA} , the Sp-dCas9 gene may have a higher transcription efficiency if the Sa-dCas gene has a higher tendency for abortive initiation (Duchi et al. 2016). The Sa-dCas9 mRNA may also have a lower translation efficiency if its codons are biased against by Ctl2 (Heizer et al. 2006).

In conclusion, nonspecific toxicities of Sp-dCas9 and Sa-dCas9 as documented in this report limit their utilities in *Chlamydia* biology. There are potential solutions to the problem. First, one might be able to select *Chlamydia* repressors that are no longer inhibited by either Sp-dCas9 or Sa-dCas9. Second, some of the strategies used to produce enzymatically active Sp-Cas9 variants with reduced toxicity could be used to generate dCas variants with less chlamydial toxicity (Bolukbasi et al. 2015; Wright et al. 2015). Finally, since a large number of Cas with dissimilar properties have been identified and characterized (Makarova, Wolf and Koonin 2018), the deactivated form of some of the alternative Cas may be tolerated by *Chlamydia*.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSPD](#) online.

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Conflict of interest. None declared.

REFERENCES

- Abdelrahman YM, Belland RJ. The chlamydial developmental cycle. *FEMS Microbiol Rev* 2005;29:949–59.
- Agaisse H, Derre I. A *C. trachomatis* cloning vector and the generation of *C. trachomatis* strains expressing fluorescent proteins under the control of a *C. trachomatis* promoter. *PLoS One* 2013;8:e57090.
- Bao X, Gylfe A, Sturdevant GL et al. Benzylidene acylhydrazides inhibit chlamydial growth in a type III secretion- and iron chelation-independent manner. *J Bacteriol* 2014;196:2989–3001.
- Bauler LD, Hackstadt T. Expression and targeting of secreted proteins from *Chlamydia trachomatis*. *J Bacteriol* 2014;196:1325–34.
- Belland RJ, Zhong G, Crane DD et al. Genomic transcriptional profiling of the developmental cycle of *Chlamydia trachomatis*. *Proc Natl Acad Sci USA* 2003;100:8478–83.
- Bolukbasi MF, Gupta A, Oikemus S et al. DNA-binding-domain fusions enhance the targeting range and precision of Cas9. *Nat Methods* 2015;12:1150.
- CDC. *Sexually Transmitted Disease Surveillance* 2016. Atlanta: U.S. Department of Health and Human Services, 2018.
- Cho S, Choe D, Lee E et al. High-level dCas9 expression induces abnormal cell morphology in *Escherichia coli*. *ACS Synthetic Biology* 2018;7:1085–94.
- de la Maza LM, Zhong G, Brunham RC. Update on *Chlamydia trachomatis* vaccinology. *Clin Vaccine Immunol* 2017;24:e00543–16.
- Duchi D, Bauer DLV, Fernandez L et al. RNA polymerase pausing during initial transcription. *Mol Cell* 2016;63:939–50.
- Gong Z, Luna Y, Yu P et al. Lactobacilli inactivate *Chlamydia trachomatis* through lactic acid but not H₂O₂. *PLoS One* 2014;9:e107758.
- Heizer EM, Jr., Raiford DW, Raymer ML et al. Amino acid cost and codon-usage biases in 6 prokaryotic genomes: a whole-genome analysis. *Mol Biol Evol* 2006;23:1670–80.
- Humphrys MS, Creasy T, Sun Y et al. Simultaneous transcriptional profiling of bacteria and their host cells. *PLoS One* 2013;8:e80597.
- Hybiske K, Stephens RS. Mechanisms of *Chlamydia trachomatis* entry into nonphagocytic cells. *Infect Immun* 2007;75:3925–34.
- Jiang F, Doudna JA. CRISPR–Cas9 structures and mechanisms. *Annu Rev Biophys* 2017;46:505–29.
- Jones DL, Leroy P, Unoson C et al. Kinetics of dCas9 target search in *Escherichia coli*. *Science* 2017;357:1420–4.
- Key CE, Fisher DJ. Use of group II Intron technology for targeted mutagenesis in *Chlamydia trachomatis*. *Methods Mol Biol* 2017;1498:163–77.
- Koprivsek J, Zhang T, Tian Q et al. Distinct roles of chromosome-versus plasmid-encoded genital tract virulence factors in promoting *Chlamydia muridarum* colonization in the gastrointestinal tract. *Infect Immun* 2019, DOI 10.1128/iai.00265-19:IAI.00265-19.
- Lee JK, Enciso GA, Boassa D et al. Replication-dependent size reduction precedes differentiation in *Chlamydia trachomatis*. *Nat Commun* 2018;9:45.

- Makarova KS, Wolf YI, Koonin EV. Classification and nomenclature of CRISPR-Cas systems: where from here? *The CRISPR Journal* 2018;1:325–36.
- Mueller KE, Wolf K, Fields KA. *Chlamydia trachomatis* transformation and allelic exchange mutagenesis. *Curr Protoc Microbiol* 2017;45:11A31–5.
- Nicholson TL, Olinger L, Chong K et al. Global stage-specific gene regulation during the developmental cycle of *Chlamydia trachomatis*. *J Bacteriol* 2003;185:3179–89.
- Ouellette SP. Feasibility of a conditional knockout system for *Chlamydia* based on CRISPR interference. *Front Cell Infect Mi* 2018;8:59.
- Rock JM, Hopkins FF, Chavez A et al. Programmable transcriptional repression in mycobacteria using an orthogonal CRISPR interference platform. *Nature Microbiology* 2017;2:16274.
- Stephens RS, Kalman S, Lammel C et al. Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. *Science* 1998;282:754–9.
- Wang Y, Kahane S, Cutcliffe LT et al. Development of a transformation system for *Chlamydia trachomatis*: restoration of glycogen biosynthesis by acquisition of a plasmid shuttle vector. *PLoS Pathog* 2011;7:e1002258.
- WHO. Global incidence and prevalence of selected curable sexually transmitted infections: 2008. *Reprod Health Matters* 2012;20.
- Wickstrum J, Sammons LR, Restivo KN et al. Conditional gene expression in *Chlamydia trachomatis* using the tet system. *PLoS One* 2013;8:e76743.
- Wright AV, Sternberg SH, Taylor DW et al. Rational design of a split-Cas9 enzyme complex. *Proc Natl Acad Sci* 2015;112:2984–9.
- Xu S, Battaglia L, Bao X et al. Chloramphenicol acetyltransferase as a selection marker for chlamydial transformation. *BMC Res Notes* 2013;6:377.
- Yang C, Kari L, Sturdevant GL et al. *Chlamydia trachomatis* ChxR is a transcriptional regulator of virulence factors that function in in vivo host-pathogen interactions. *Pathog Dis* 2017;75:ftx035.
- Zhang H, Kunadia A, Lin Y et al. Identification of a strong and specific antichlamydial N-acylhydrazine. *PLoS One* 2017;12:e0185783.
- Zhong G, Brunham RC, de la Maza LM et al. National Institute of Allergy and Infectious Diseases workshop report: “*Chlamydia* vaccines: the way forward”. *Vaccine* 2017, DOI <https://doi.org/10.1016/j.vaccine.2017.10.075>.