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Intra-ChIP: studying gene regulation in an intracellular pathogen

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Abstract Intracellular bacteria that reside within a host cell use a variety of strategies to exploit this unique niche. While these organisms are technically challenging to study in the context of an infected host cell, recent advances have led to an improved understanding of how the intracellular environment impacts bacterial gene expression. We recently demonstrated that chromatin immunoprecipitation (ChIP) can be used to quantify transcription factor binding in the obligate intracellular pathogen *Chlamydia trachomatis* within infected cells. Furthermore, we showed it was possible to experimentally modulate transcription factor binding while simultaneously measuring changes in transcription. Here we discuss these findings as well as other recent work that has used ChIP to study intracellular pathogens within infected cells. We also discuss technical considerations associated with this approach and its possible future applications.

Keywords Intracellular pathogen · Chromatin immunoprecipitation · ChIP · *Chlamydia*

Introduction

A number of important pathogenic bacteria are able to infect and survive within eukaryotic host cells (Casadevall 2008). For example, *Salmonella*, *Mycobacterium*, *Yersinia*, and *Legionella*, are called facultative intracellular bacteria because they can live inside or outside a host cell. In contrast, *Chlamydia*, *Coxiella*, *Rickettsia* and *Mycobacterium leprae* are obligate intracellular bacteria because they require a host cell for replication. With the exception of *Coxiella*, these obligate intracellular bacteria cannot be grown in axenic culture, but can be studied in the laboratory with a cell culture infection model.

The effects of the intracellular environment on bacterial gene expression have not been fully explored. The majority of gene regulation studies for facultative intracellular bacteria have been performed in the absence of a host cell because it is easier to grow these organisms in axenic culture. Gene regulation in obligate intracellular pathogens has been mostly studied with in vitro assays or heterologous in vivo systems. However our understanding of gene regulation during an intracellular infection is not complete unless it is analyzed within an infected host cell.

Chromatin immunoprecipitation (ChIP) is a method for studying transcriptional regulation in vivo. This approach involves cross-linking of DNA-binding proteins to their target DNA sequences within bacterial cells, fragmentation of the DNA into small segments, and then isolation of specific protein-DNA complexes with antibodies to the protein. The amount of bound DNA can then be quantified with target-specific or genome-wide methods. For example, with ChIP-qPCR, binding of a transcription factor to a particular target gene can be quantified by using specific antibodies against the transcription factor to isolate the in vivo complexes, and then measuring the amount of bound target DNA with

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quantitative PCR. To study genome-wide targets, the DNA isolated with the ChIP procedure can be analyzed by massively parallel sequencing (ChIP-seq) or by hybridization to a microarray (ChIP-chip). These latter methods do not require prior knowledge of the target sequences and yet can identify target genes and quantify the level of binding.

ChIP has many advantages over other methods for studying transcriptional regulation because it directly measures the *in vivo* binding of a transcription factor to its target DNA. The activity of this transcription factor in modulating transcription can be assayed for individual target genes as well as over its entire regulon. In addition, binding of the transcription factor can be analyzed and compared under different *in vivo* conditions. *In vivo* reporter assays, in contrast, are best suited for the analysis of individual target genes. Transcriptional profiling methods, such as qRT-PCR for individual genes, or DNA microarrays and deep sequencing for genome-wide analysis, provide information about transcript levels, but not about the underlying mechanisms of transcriptional regulation. These methods for measuring transcript levels can complement ChIP, however, by allowing the effect of transcription factor binding on gene expression to be assayed under the same conditions.

ChIP and intracellular pathogens

ChIP with infected cells has been used to investigate modification of host gene expression by bacterial factors. For example, intracellular bacteria regulate host gene expression by modifying histones and host chromatin structure to silence expression at specific gene loci. The RomA protein of *Legionella pneumophila* uniquely methylates histone H3 with a tri-methyl group at lysine 14 (Rolando et al. 2013). ChIP-seq analysis of H3 in *L. pneumophila*-infected cells revealed that H3 methylation by RomA occurred at over 4,800 promoter regions (Rolando et al. 2013). Furthermore, RomA methylation of H3 resulted in repression of transcription, indicating that *L. pneumophila* silences a large number of host genes during infection (Rolando et al. 2013). In a related manner, ChIP with infected cells demonstrated that the phosphatase OspF of *Shigella flexneri* prevents phosphorylation of histone H3 at serine 10, impairing recruitment of NF- κ B to the *IL8* promoter (Arbibe et al. 2007). ChIP was used to show that the secreted protein AnkA, from the obligate intracellular bacterium *Anaplasma phagocytophilum*, binds directly to host DNA (García-García et al. 2009; Rennoll-Bankert and Dumler 2012; Sinclair et al. 2014). Similarly, ChIP with infected cells demonstrated that the proteins Ank200 and TRP120 of the obligate intracellular pathogen *Ehrlichia chaffeensis* bind host DNA in infected cells (Zhu et al. 2009, 2011). It is currently unclear how AnkA, Ank200, and TRP120

function to modulate host gene expression, but they may induce global transcriptional changes by recruiting chromatin remodeling enzymes to specific chromosomal locations (Sinclair et al. 2014).

In a few instances, ChIP has been used to study gene regulation within obligate intracellular pathogens, most notably in protozoa, such as *Plasmodium falciparum* and *Toxoplasma gondii*. For example, the *P. falciparum* protein PfBDP1 was enriched near promoters of its invasion genes and was associated with their activation in infected erythrocytes (Josling et al. 2015). Similarly, the *T. gondii* protein gAP2XI-5 was enriched at promoters of important virulence genes within infected cells (Walker et al. 2013). In contrast, very few studies have used ChIP to study how bacteria regulate their genes during an intracellular infection. The best example is a ChIP study showing that the *Salmonella* response regulator PhoP bound its target *ssrB* promoter in infected macrophages (Bijlsma and Groisman 2005). This study demonstrated that a bacterial transcription factor can regulate target genes that control type III secretion in the context of an intracellular infection. However, to our knowledge there have been few other studies that have taken advantage of ChIP to analyze bacterial transcription factors within an infected cell.

Intra-ChIP

We have developed a ChIP method to study the function of a transcription factor within a bacterium residing inside an infected host cell. While the intracellular context is important for studying the bacterium in its native environment, it brings along host proteins and DNA that can affect the sensitivity and specificity of the ChIP assay. Our method, which we call intra-ChIP, utilizes the conventional ChIP approach to measure bacterial protein-DNA binding, but is modified to minimize background from host proteins and DNA. We did not have to change the cross-linking step for locking in *in vivo* protein-DNA interactions, because we used the cross-linking agent formaldehyde, which diffuses into both the infected cell and the intracellular bacteria. We also retained the basic immunoprecipitation step, using specific antibodies to isolate protein-DNA complexes for the transcription factor of interest. However, we found that it was important to optimize the conditions for DNA fragmentation and the amount of starting material for the intra-ChIP assay. The presence of host DNA is also an important consideration for the DNA analysis step to identify or quantify DNA binding targets.

The DNA fragmentation step has to be optimized because the material from the infected cell may contain much more host cell DNA than bacterial DNA. This imbalance is due to the size of the bacterial genome (1 to 5×10^6 bp),

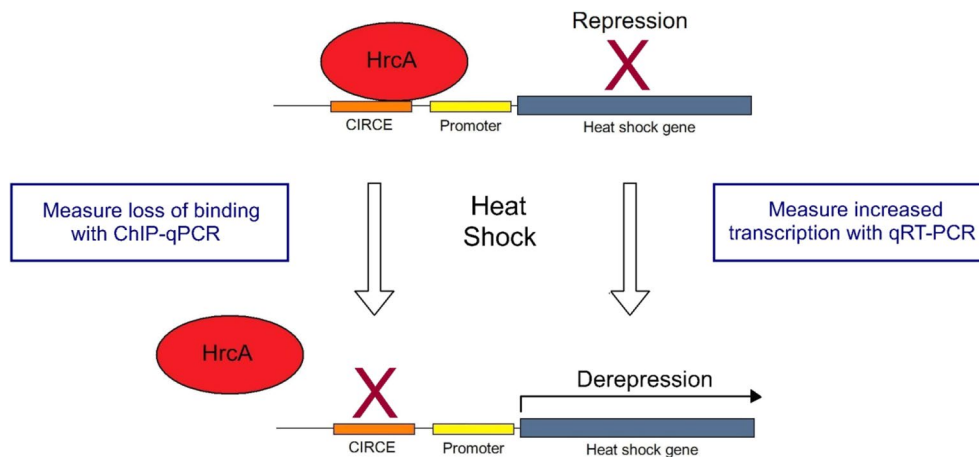


Fig. 1 Model of HrcA regulation. Under non-stress conditions HrcA binds the CIRCE operator and represses transcription of heat shock genes. With stress conditions, such as heat shock, there is derepression with loss of HrcA binding which leads to increased transcription

of the heat shock genes. Changes in HrcA binding and associated changes in transcription can be measured using ChIP-qPCR and qRT-PCR respectively

which is a thousand-fold smaller than the host cell genome (3×10^9 bp). This disparity will be less of a factor if there are many bacteria per host cell. We fragmented the total chlamydial and host DNA by shearing the sample with a sonicator, which is able to produce a minimum DNA size of 100–500 bp (Elsner and Lindblad 1989; Sambrook and Russell 2006). We found that sonication conditions that sheared total DNA to a size range of 300–1200 bp produced templates that were suitable for ChIP-qPCR analysis. In our intra-ChIP studies of the intracellular bacterium *Chlamydia*, it was not necessary to measure the size of the chlamydial DNA fragments by themselves. Optimal fragmentation conditions may need to be empirically determined for each intracellular pathogen.

The amount of starting material to use for the intra-ChIP assay is affected by the bacterial load in an infected cell. For example, an intracellular *Chlamydia* infection begins with a single bacterium that enters the host cell, but over the course of the 48–72 h intracellular infection, the bacterium replicates repeatedly by binary fission so that there can be a thousand bacteria inside the host cell. It may be necessary to optimize the amount of starting material because too much may lead to high background from non-specific DNA recovery, while too little will compromise the ability to detect specific protein-DNA binding. In our *Chlamydia* intra-ChIP studies, we were able to detect transcription binding with as few as 100,000 bacteria, even at an early stage in the infection where there was only one bacterium per host cell.

The presence of host DNA also affects the subsequent DNA analysis step that measures target DNA bound in the ChIP assay. If qPCR is used to quantify individual DNA targets, it is important to select specific PCR primers that

do not recognize other bacterial or host DNA sequences. The issue with global analysis methods such as DNA-seq or DNA microarrays is that they may detect adventitious binding between the bacterial transcription factor and host DNA that is not relevant to the infection. However, the crosslinking step in the intra-ChIP procedure helps to restrict the analysis to bona fide interactions occurring within the infected host cell.

Chlamydia and intra-ChIP

In a recent study, we used our intra-ChIP method to detect dynamic changes in binding of the chlamydial transcription factor HrcA to its target DNA sequences within human cells infected with *Chlamydia trachomatis* (Hanson and Tan 2015). HrcA is a conserved transcription factor that regulates heat shock genes in many bacteria. HrcA has been shown to bind its cognate operator CIRCE in vitro, and HrcA-CIRCE binding is associated with transcriptional repression of heat shock genes (Minder et al. 2000; Narberhaus 1999; Schulz and Schumann 1996; Zuber and Schumann 1994), (Fig. 1). Stress conditions such as elevated temperature induce transcription of these heat shock genes (Engel et al. 1990) and, for *Helicobacter*, have been shown to cause loss of HrcA binding in vitro (Roncarati et al. 2014) (Fig. 1). Using intra-ChIP paired with qPCR (Fig. 2), we obtained the first direct evidence that HrcA binding within a bacterium can be regulated by heat shock. We detected binding of HrcA to its target promoters in *Chlamydia*-infected cells under normal growth conditions. We then showed that exposure of infected cells to elevated temperature caused loss of HrcA binding, and a concomitant

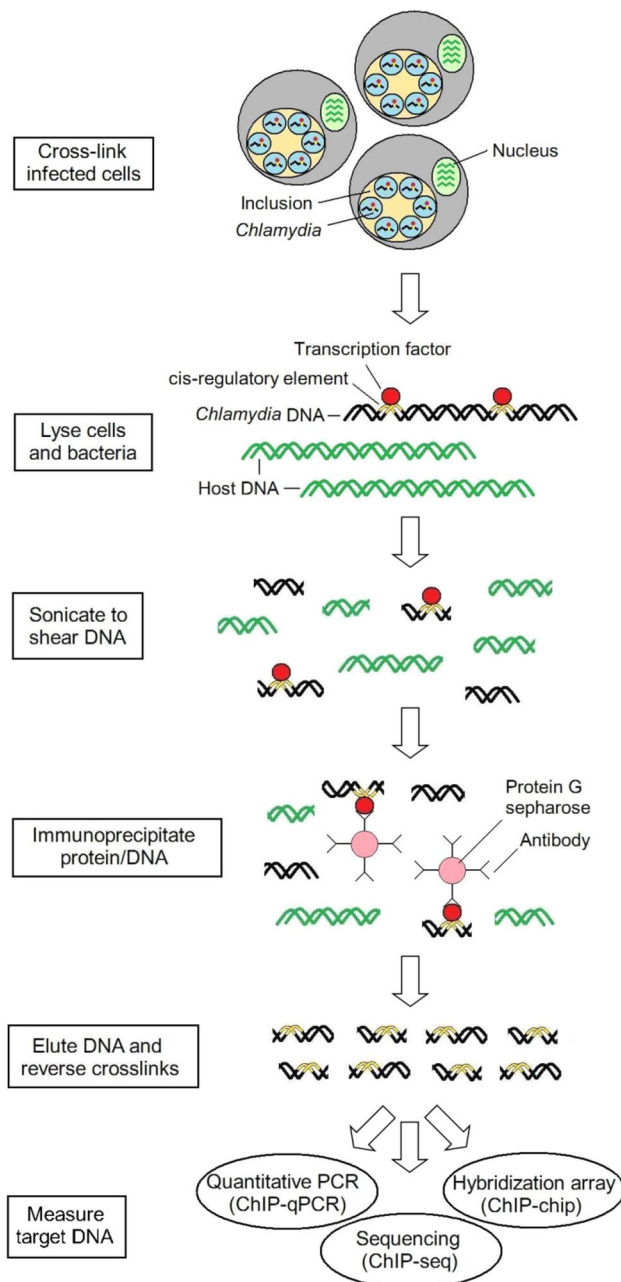


Fig. 2 Workflow for performing chromatin immunoprecipitation with the obligate intracellular bacterium *Chlamydia trachomatis*

increase in transcription from these promoters, as measured by qRT-PCR. We also used this combined ChIP-qPCR and qRT-PCR approach to investigate the kinetics of HrcA binding during heat shock and recovery from heat shock, and the regulation of HrcA binding over the course of the intracellular chlamydial infection. Thus intra-ChIP provided a useful method for measuring the regulated binding and activity of a bacterial transcription factor during an intracellular infection.

Future considerations

Our approach for using intra-ChIP to study bacterial transcription factor activity in an infected cell can be readily applied to other intracellular bacterial infections. For example, an excellent ChIP-seq study identified binding targets for over three-quarters of the transcription factors of *Mycobacterium tuberculosis* when grown in culture medium (Minch et al. 2015). A natural extension of this study would be to perform the ChIP-seq analysis with *M. tuberculosis*-infected macrophages, in which nearly 600 mycobacterial genes are differentially regulated (Fontán et al. 2008). For many other facultative and obligate intracellular bacteria, cell culture infection models exist and could be used to study transcription factors in the context of an intracellular infection. ChIP has even been successfully performed using paraffin embedded clinical tissue samples, and microdissected tissue samples (Fanelli et al. 2010; Murgatroyd et al. 2012), and in the future it would be attractive to perform in vivo ChIP using infected animal or clinical tissue samples.

In summary, intra-ChIP provides the means to study gene regulation of intracellular bacteria within an infected host cell. This intracellular environment is critical because it may affect the transcriptional regulatory networks of the bacterium. Intra-ChIP studies also provide the means to study the in vivo function of transcription factors, especially for obligate intracellular bacteria. These regulatory factors play an important role in controlling bacterial gene expression and they are attractive targets for development of novel antibiotics.

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