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Future Directions of the Prokaryotic Chromosome Field

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DOI
10.1111/mmi.15347

Publication date
2025

Document Version
Final published version

Published in
Molecular Microbiology

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Link to publication

Citation for published version (APA):
Abbondanzieri, E. A., Badrinarayanan, A. B., Barillà, D., Bell, S. D., Blombach, F., Bouet, J. Y., Bulgheresi, S., Cao, Q. A. D., Dame, R. T., Dekker, C., Demuysere, M., Espéli, O., Fogg, P. C. M., Freddolino, P. L., Ganji, M., Gerson, T. M., Grainger, D. C., Hamoen, L. W., Harju, J., ... Zhang, Z. (2025). Future Directions of the Prokaryotic Chromosome Field. *Molecular Microbiology*, *123*(2), 89-100. https://doi.org/10.1111/mmi.15347

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EDITORIAL

Future Directions of the Prokaryotic Chromosome Field

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Received: 22 March 2024 | **Revised:** 27 January 2025 | **Accepted:** 28 January 2025

Funding: The Lorentz Center is funded by the Dutch Research Council (NWO) and Leiden University.

Keywords: archaeal chromatin | archaeal chromosome | bacterial chromatin | bacterial chromosome | nucleoid

ABSTRACT

In September 2023, the Biology and Physics of Prokaryotic Chromosomes meeting ran at the Lorentz Center in Leiden, The Netherlands. As part of the workshop, those in attendance developed a series of discussion points centered around current challenges for the field, how these might be addressed, and how the field is likely to develop over the next 10 years. The Lorentz Center staff facilitated these discussions via tools aimed at optimizing productive interactions. This Perspective article is a summary of these discussions and reflects the state-of-the-art of the field. It is expected to be of help to colleagues in advancing their own research related to prokaryotic chromosomes and inspiring novel interdisciplinary collaborations. This forward-looking perspective highlights the open questions driving current research and builds on the impressive recent progress in these areas as represented by the accompanying reviews, perspectives, and research articles in this issue. These articles underline the multidisciplinary nature of the field, the multiple length scales at which chromatin is studied in vitro and in and highlight the differences and similarities of bacterial and archaeal chromatin and chromatin-associated processes.

1 | Introduction

Understanding the compaction and functional organization of chromosomes of organisms in all three domains of life (bacteria, archaea and eukaryotes) is a question of great biological significance. It is especially fascinating to understand and explore these questions in relation to prokaryotic (bacterial and archaeal) chromosomes, which, different from their eukaryotic counterparts, are not enclosed within a dedicated organelle, the nucleus, underscoring the importance of physico-chemical mechanisms of compartmentalization. Despite unprecedented progress in this field during the last decade, since we organized a previous Lorentz workshop on this topic (Dame et al. 2012), we remain far from a detailed understanding of the structure of the chromosome, the mechanisms responsible, and the role this structure plays in essential cellular processes, including transcription, replication, chromosome segregation, and cell division. Also, chromosome organization is intrinsically connected with genome plasticity and evolution. A central challenge is that determining the structure and function of the chromosome is a problem that spans a vast range of length scales. Especially at larger length scales, the exact mechanisms underlying the structural and functional organization remain unclear. Whereas historically,

All authors contributed equally to this work. Authors are ranked in alphabetical order.

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a focus has been on the involvement and action of specific proteins, physical mechanisms of self-organization likely play important roles.

The field of prokaryotic chromosome organization and dynamics has seen a decade of unprecedented progress due to the development of a number of novel and powerful techniques that have generated structural and functional insights at both molecular and cellular scales. These developments were facilitated by the contributions of researchers from quantitatively oriented fields of biophysics, mathematics, and bioinformatics. This extraordinary progress has been the result of many complementary approaches. The molecular-scale mechanisms of many chromatin proteins have been established in vitro. In vivo approaches determine the population-average occupancy of these chromatin proteins genome-wide. These approaches, in combination with RNA-seq experiments, have elucidated the mechanism of complex regulatory networks in which chromatin proteins play key regulatory roles, while Chromosome Conformation Capture-based approaches and an ever-increasing array of emerging genome-wide approaches, including SisterC, ChiApet, DRIP-seq, etc., have determined the structure of the chromosome. Each of these experimental approaches is used in conjunction with modeling, whether bioinformatic (relying on genome-wide gene expression data and protein binding maps) or biophysical (chromatin modeled as a folded polymer), to provide insight into the principles underlying chromosome organization. These models, in turn, can be tested in vivo employing the power of highly tractable bacterial genetics (Dame, Rashid, and Grainger 2020).

Current developments, such as decreasing costs of next-generation sequencing, the emergence of novel concepts of self-organization, and machine learning approaches providing new analytical tools, suggest that great progress will be made in the coming decade, but also emphasize the need for the formation of new collaborations to apply these novel approaches to prokaryotic systems. In the following sections, the discussions on current topics of interest are summarized.

2 | Horizontal Gene Transfer (HGT) and Chromosome Dynamics

In the context of this theme three questions emerged:

1. How does horizontal gene transfer shape chromosome organization?
2. What is H-NS really for?
3. What is the role of horizontal gene transfer in segregation of the genome in polyploid organisms?

The text below covers some of the issues repeatedly encountered when considering these questions.

2.1 | Why Is So Much Horizontally Acquired DNA AT-Rich?

A major topic covered by two of the discussions was the high AT content of many horizontally acquired genes. In particular, it was questioned why this is indeed the case. That AT-rich DNA is horizontally acquired seems unchallenged in the literature, but explanations are incomplete. It was concluded that there may be many reasons why we now see such "AT-rich islands" in genomes. First, cytosine deamination can lead to C to T conversions in DNA. This means that there may be a constant drift of DNA sequences to a more AT-rich state. Additionally, there have been reports of different mutation rates for horizontally acquired DNA that is silenced (Higashi et al. 2016). This was work presented at the first iteration of this Lorentz workshop in 2012 (Dame et al. 2012). Second, we know that cells have a mechanism to deal with the harmful consequences of harboring AT-rich sequences, associated with the binding of H-NS and similar factors (Grainger 2016). This may be why so many such sequences can be retained. As far as we know, cells do not have an equivalent mechanism to counter the negative properties of GC-rich DNA, and there have been reports that such sequences are more costly to cells (i.e., less likely to be retained than AT-rich DNA) (Dietel et al. 2019). Lastly, we know that many horizontally acquired mobile DNA elements (phages and plasmids) tend to be AT-rich, making them natural targets of nucleoid-associated proteins such as H-NS. One suggestion for future work was the labeling of mobile DNA elements (e.g., plasmids or transposons) with fluorescent tags so their movements and interplay with nucleoid-associated proteins could be tracked in real time. An alternative approach was suggested in another of the discussions: the use of droplet digital PCR to measure the acquisition, loss, and spread of plasmids in single cells in a population.

2.2 | What Is the Real Role of H-NS?

Two of the discussions focused extensively on the role of H-NS in horizontal gene transfer. Originally discovered as an abundant DNA-binding protein (Cukier-Kahn, Jacquet, and Gros 1972), the function of H-NS was, almost by default, assumed to be that of a global chromosome organizer. While H-NS can clearly organize DNA regions in specific ways (Rashid and Dame 2023; Rashid et al. 2023), on a chromosome-wide scale, H-NS only has local impacts on chromosome folding (Lioy et al. 2018). This fits well with observations that deleting H-NS has no evident effects on the nucleoid at a slow growth rate but reduces nucleoid compaction of fast-growing bacteria (Helgesen, Fossum-Raunehaug, and Skarstad 2016). Our discussions focused on the idea that H-NS is a DNA-binding factor with a very specialized role: to handle mobile genetic elements. Traditionally, the role of H-NS was thought to be limited to transcriptional silencing, and we discussed exactly how transcription of H-NS-bound genes is achieved (remodeling vs. removal of H-NS filaments). Previous works based on the high-throughput analysis of sister chromatid contacts in *Vibrio cholerae* chromosome unveiled that H-NS acts as a cohesive factor within the *Vibrio* Pathogenicity Island 1 and the O-antigen region (Espinosa, Paly, and Barre 2020). In agreement with this, new results presented at the workshop indicate that the DNA bridging activity of H-NS could play a role during the acquisition of mobile DNA: chromosomally bound H-NS might make bridges with incoming DNA to direct it towards insertion at specific sites of the chromosome (Cooper et al. 2024). In this regard, long-term evolution experiments may provide useful information. For instance, genome sequencing of bacterial populations at different

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time points could reveal how H-NS controls the position at which new DNA sequences are integrated into the genome. Does H-NS play a role in inactivating some such sequences (e.g., by driving the insertion of transposons)? Is H-NS responsible for the accumulation of insertion sequences in chromosomal "graveyards" where they cause least harm?

2.3 | What Is the Impact of Horizontal Gene Transfer on Chromosome Dynamics?

The question of whether and how HGT impacts chromosome dynamics was also discussed. For instance, does HGT impact transcription? We know that, by virtue of their high-AT content, such DNA sequences are proficient at driving transcription (Singh et al. 2014). While H-NS often binds across extended regions, Chromatin immunoprecipitation-sequencing (ChIP-seq) does tell us that, in some cases, H-NS binding is restricted to promoter regions of genes (Kahramanoglou et al. 2011). Could these regions be the final remnants of an ancient HGT event? That is, could it be that just a small region of the originally acquired DNA has been retained, which is now used as regulatory DNA? This might be determined by testing the similarity of the translated sequence in all three reading frames to that of known proteins.

Another topic discussed was the maintenance of elements acquired through HGT in polyploid organisms with multiple copies of the genome (especially archaea). We envisage the presence of a heterozygous population (due to presence of plasmid/chromosome copies containing and lacking the element) before the stabilization of the element in the whole genome. In such a scenario, how would transcription levels between the different copies be (coordinately) regulated? Moreover, in organisms with high copy number genomes and a high tendency to acquire elements by HGT (like haloarchaea), would there be a preference to maintain acquired DNA with certain elements in a dormant state? For example, if a beneficial element is repressed and expressed only in certain stress conditions, would the cells prefer heterozygosity? This scenario could be tested by inserting different selection markers on otherwise identical plasmids. Monitoring the dynamics of plasmid spread through a population, across multiple generations, in the presence and absence of stress would be informative. Even if mobile DNA sequences contain beneficial elements, organisms with a tendency to retain them run the risk of redundancy and DNA accumulation.

This is also interesting with respect to partitioning systems being acquired into the genome via HGT and driving the selection of certain plasmids over others in the organism. ParA-like proteins are encoded in bacteria and archaea. The proteins are involved in the segregation of the genome, among other functions. Long-term evolution experiments would be quite revealing on this aspect of chromosome dynamics in organisms with high copy number genomes.

3 | The Role of NAPs on Local Chromosome and Plasmid Dynamics and Regulation

Further discussions focused on the interplay between combinations of nucleoid-associated proteins (NAPs). It was noted that we have little understanding of how these factors act in unison to control the interplay of supercoiling, chromosome architecture and dynamics, and transcriptional regulation (Blombach and Werner 2024; Villain and Basta 2024; Hustmyer and Landick 2024; Cody et al. 2025). How do cells achieve distinct local chromosome folding states? What is the stability and structure of these states? It is difficult to get a unifying view and derive universal principles given the considerable variability and redundancy in bacterial and archaeal chromatinization. Significant challenges include the large and variable repertoire of NAPs (Baglivo et al. 2024; De Kock, Peeters, and Baes 2024; Erkelens et al. 2024; Santoshi, Tare, and Nagaraja 2024; Schwab and Dame 2024; Hustmyer and Landick 2024) and our lack of knowledge of functionally significant post-translational modifications (PTMs) (Dilweg and Dame 2018). The emergence of new technologies, including methods that can identify local chromosome folding states in vivo and approaches that integrate in vivo and in vitro methods, are sorely lacking. Future directions are considered below.

3.1 | What Is the Structure of NAP-DNA Complexes?

X-ray crystallography has been crucial to provide high-resolution insight into NAP:DNA interactions (see e.g. Rice et al. 1996; Swinger et al. 2003), but protein:DNA crystallization studies are limited to short DNA templates, leaving us with an incomplete picture of the effect of NAPs on DNA conformation. High-resolution structures with longer DNA templates and multiple NAPs are therefore critically needed. The rapid development of Cryo-electron microscopy might hold the key to fill this gap, but this requires us to overcome technical challenges regarding the variable size and structural heterogeneity of NAP:DNA complexes. An alternate approach could involve combining single-molecule experiments with molecular dynamics simulations. These methods are well-suited for characterizing conformational variety and are mutually reinforcing, with experiments validating simulations and simulations enhancing the resolution of experiments (van der Valk et al. 2017; Yoshua et al. 2021). Enhanced sampling approaches can be used to extend the time scales accessible to conventional molecular dynamics simulations (Van Heesch, Bolhuis, and Vreede 2023). To next extend these structural studies to include combinations of NAPs (reflecting the situation in vivo) is an even higher bar, which is, however, also aimed at (Birnie and Dekker 2021; Holub et al. 2022). Nevertheless, the reductionists approach to building up structures of increasing complexity might be preferred over aiming to isolate and visualize "native chromatin".

3.2 | What Is the Interplay Between DNA Supercoiling and NAP Binding?

Several recently developed methods shed light on the in vivo supercoiling status along the chromosome. (i) Psora-seq, a method that relies on crosslinking DNA with biotinylated Psoralen that preferentially binds negative supercoiled regions and allows for their enrichment (Visser et al. 2022). (ii) A recently established method termed GapR-Seq is based on the heterologous expression of the GapR protein from *Caulobacter*

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crescentus (Guo et al. 2021). GapR is a NAP that preferentially binds positively supercoiled DNA and stabilizes the twist in the DNA (Guo et al. 2018). Studies in which heterologous GapR expression was combined with ChIP-seq robustly revealed positively supercoiled genomic regions (Guo et al. 2021). (iii) ChIP-seq mapping of Topoisomerase I and Gyrase occupancy on the genome is another established method to identify genomic regions of negative and positive supercoiling (Jeong, Ahn, and Khodursky 2004; Ahmed et al. 2017; Sutormin et al. 2022).

In combination with genomic occupancy maps of NAPs, these new methods will help us to understand what role NAPs play in constraining local supercoiling domains. Are silencers such as H-NS able to function analogously to insulators in eukaryotes? Does NAP chromatinization of DNA sterically exclude Topo1 and Gyrase?

Importantly, the above-mentioned NGS-based techniques provide population averages. The link between NAP occupancy and local supercoiling status might often be obscured in these data due to heterogeneity in the cell populations. An example was presented in the workshop in which, for genomic regions that are controlled by bistable switches, gating distinct subpopulations by Fluorescence-Activated Cell Sorting (FACS) is critical to perform genomics studies. Single-molecule experiments and simulations have already addressed some of the questions above (van Noort et al. 2004; Kim et al. 2018; Watson et al. 2022; Kolbeck et al. 2024; Janissen et al. 2024) and are expected to continue being significant in the future.

3.3 | What Are Examples of Functional PTMs That Regulate NAP Function?

While the role of posttranslational modifications (PTMs) in the function of prokaryotic NAPs is probably not as extensive as its eukaryotic counterpart in the form of the histone code, PTMs are likely to play a role in NAP function, and numerous PTMs on NAPs have been identified. It is important to establish which modifications of NAPs are enzymatically deposited (by "writers"), regulatable/regulated, and functionally important to the cell due to effects on DNA binding, oligomerization, and/or recognition by "reader" proteins. Many PTMs of NAPs in bacteria have been identified (Dilweg and Dame 2018) and we know that PTMs can affect the DNA binding properties of NAPs. For example, *M. tuberculosis* Lsr2 phosphorylation by Protein kinase B and lysine acetylation of *M. tuberculosis* MtHU, a HU variant with a C-terminal extension, both reduce DNA binding (Ghosh et al. 2016; Alqaseer et al. 2019). This holds true also for PTMs of archaeal NAPs such as Cren7 (Ding et al. 2022). However, a role of these PTMs in transcription regulation remains to be demonstrated. Clear examples of epigenetic regulation via NAPs will be critical to establish models allowing us to distill fundamental concepts. To achieve real progress in this field, we will need to develop new techniques, including creative pulldown methods based on ChIP-seq and nuclease-deficient Cas9-based technologies (dCas9) allowing for locus-specific enrichment of chromatin proteins (Waldrip et al. 2014; Liu et al. 2017; Tsui et al. 2018; Fujita, Yuno, and Fujii 2018) that will allow us to identify the direct connection between post-translational NAP modification and transcription state of the genes that they chromatinize. However, the development of tools like PTM-specific antibodies for NAPs is not realistic until the marks are better defined.

3.4 | Is Bacterial and Archaeal Chromatin Remodeled and, If So, How?

Chromatin remodeling in eukaryotes requires ATP-dependent molecular machines to evict, deposit, and slide their cargo, the nucleosome, which is wrapped in its track (DNA). Bacteria and archaea, lacking these well-defined structures, may still require active mechanisms to remodel chromatin locally. There are relatively few examples of remodelers that act specifically on DNA-bound NAPs, aside from RNA polymerases and the DNA replication machinery. The energetic cost of removing specific NAPs in bacteria and the possible requirement of chaperones to achieve distinct chromatin states are also not clear. Supercoiling has emerged as a key force for chromatin remodeling in bacteria, and supercoiling-mediated remodeling is likely to result both from NTP-hydrolysis dependent and purely binding-mediated processes (Picker et al. 2023; Gerson et al. 2023).

3.5 | In Vitro Versus In Vivo: How to Ensure the Physiological Relevance of Reconstituted Systems?

Developing informative in vitro assays of bacterial chromatin requires the characterization of functionally relevant, distinct in vivo states. Comprehensive Hi-C maps and ChIP-seq maps of different NAPs in clearly defined growth conditions form an important first step, but they still show limitations due to the significant phenotypic heterogeneity at the single-cell level, even under steady-state growth conditions, in particular regarding the lack of cell cycle synchronization. Genetic approaches to identify the functional consequences of individual NAPs are often compromised by the considerable functional redundancy between NAPs that usually show broad binding specificities and are often present as multiple paralogues in a single species (e.g., see Castang et al. 2008). Combinatorial knockdowns of individual NAPs using methods like CRISPRi (Bikard et al. 2013; Qi et al. 2013; Zink et al. 2021) may be a way to avoid secondary effects, but the considerable timescale required for depletion with the existing methods in bacterial and archaeal models and possibly longer half-life of small NAPs means that discounting secondary effects is challenging. Another approach might be to express NAPs genetically fused to degradation tags. In vivo supercoiling maps, while semi-quantitative, hold promise in providing key information for the design of model templates for in vitro reconstituted chromatin interaction domains. Assembling uniformly chromatinized states from these templates in vitro or developing model synthetic templates with NAP binding or nucleation sites presents unique challenges that are absent in eukaryotic reconstituted systems, where nucleosome deposition can be tightly controlled using synthetic positioning sequences (the "Widom code"). If synthetic templates that recapitulate the in vivo properties at the population level can be established as model systems for prokaryotic chromatin, single molecule methods will be able to capture variation within such model templates. However, such assays present the challenge of getting meaningful N when

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looking at a population across multiple aspects (i.e., supercoiling, transcription, and NAP occupancy). Synthetic plasmids may serve as a powerful bridge between in vitro and in vivo studies and may enable a better understanding of the common molecular signatures of both chromosomes and plasmids and how chromatinization shapes their evolution.

4 | The Chromosome and Its Environment

Instead of focusing on individual protein factors and the interactions between them and DNA, other discussions considered the wider cellular environment. Prokaryotic chromatin positioning, configuration, and folding are dynamic. Concerning positioning, prokaryotes with a low nucleocytoplasmic ratio (e.g., *Escherichia coli*, *Bacillus subtilis*) localize their nucleoid at mid-cell, and the ribosomes are excluded from it. On the other hand, in prokaryotes with a high nucleocytoplasmic ratio (e.g., *C. crescentus*, *P. aeruginosa*) the nucleoid occupies the whole cell, and ribosomes diffuse throughout the nucleoid freely (Gray et al. 2019). Inside the nucleoid, replicating chromosomes are arranged with specific patterns (longitudinal or transversal), changing as a function of the cell cycle, dictating the average localization and dynamics of loci according to their position on the genetic map (Badrinarayanan, Le, and Laub 2015; Wang, Llopis, and Rudner 2013; Wang et al. 2014).

Concerning chromosome conformation, dynamic sub-megabase domains of chromatin are a common denominator among prokaryotes (Lioy, Junier, and Boccard 2021; Ponndara et al. 2024). Recent work showed that transcription defines the smallest domains around expressed genes and operons (Bignaud et al. 2024). These Transcription Induced Domains (TIDs) present folding, localization, and dynamics that suggest compartmentalization. Interaction between expressed regions allows the formation of looped domains 10–50 kb in size. Long and highly expressed genes form strong interaction borders that define chromosome interaction domains (CID) that can coalesce together into megabase-long macrodomains. Finally, in archaea such as *Sulfolobus solfataricus*, compartments resembling eukaryotic heterochromatin and euchromatin were observed (Bell 2022; Pilatowski-Herzing et al. 2024). However, it is not known how chromatin intracellular positioning, configuration, or conformation changes when cells adhere to a substrate or to a host cell. We also do not know how prokaryote-virus interaction affects the chromatin of either the prokaryotic host or the virus. Finally, we do not know how the chromatin of the host bacterium is affected by an invading predatory bacterium, either during the attack phase or when the bacterium has already settled inside the host cell. We believe that this knowledge gap should be filled as a specific localization, configuration, or conformation might be required for host cell attachment or to establish symbiotic or pathogenic long-term associations.

4.1 | In Our Discussion a Few Key Research Questions Were Identified

1. How does chromatin change when prokaryotes transition from planktonic/free-living to host-attached and from being host-attached to being intracellular or in a biofilm?
2. How is invading extrachromosomal DNA, including phage DNA, folded once it is in the cytoplasm of the host cell or once it is integrated into the host chromosome?
3. How can phages alter host chromatin folding to their advantage?

We hypothesize that mechanosensing, chromosome segregation mechanisms (e.g., ParABS) and NAPs underlie specific chromosome positioning, configurations, and/or conformations that, in turn, may favor substrate attachment, biofilm formation, or intracellular invasion.

In order to address the above questions, we propose the following approaches. We will need to compare the chromosome localization, configuration, and conformation of free-living prokaryotes (exponential and stationary phase) and viruses with the chromosome localization, configuration, and conformation of their counterparts when attached to a substrate or in a biofilm or when present inside the host cell. The conformation of the bacterial and host chromosome should be captured dually (i.e., in parallel). This approach would concurrently require performing dual transcriptomics, including measuring small (regulatory) RNA and analyzing the metabolomics of prokaryotes interacting with the host. Finally, it would be important to sequence membrane-associated nucleic acids (mem-seq) in prokaryotes that are free-living, attached, or intracellular.

It will be essential, but particularly challenging, to ensure that the selected prokaryotic population is synchronizable. Visualization of bacterial chromosome localization and configuration when bacteria are in a biofilm, attached to the host cell, or inside it may be difficult (especially if prokaryotes are not genetically amenable). High-resolution microscopy of extremophilic archaeal cells or obligate anaerobic bacteria is still largely under development. Also, an (animal) infection model would be needed to determine the medical relevance of specific chromatin positioning, configuration, or conformation.

5 | Chromosome Evolution and Dynamics Across the Tree of Life

Understanding how chromosomes are organized and what functionality the organization imparts is a fundamental quest in prokaryotic chromosome biology. Our discussions highlighted the need to obtain knowledge of chromosome organization and function in much more diverse settings compared to those currently available for standard laboratory conditions and model organisms, and to extract the underlying evolutionary, organizational, and functional principles.

5.1 | Limitations of Current Model Systems

To date, most studies have been conducted almost exclusively at steady state and in traditional model organisms (Wang, Llopis, and Rudner 2013; Badrinarayanan, Le, and Laub 2015; Barillà 2016; Jalal and Le 2020). However, various experiments performed in stress conditions revealed that chromosome and nucleoid plasticity

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may be involved in stress tolerance (Cabrera and Jin 2003; Bakshi et al. 2014; Passot et al. 2015; Yang, Blair, and Salama 2016; Cho et al. 2017; Vickridge et al. 2017; Banneville et al. 2022; Walker, Abbondanzieri, and Meyer 2024). To date, chromosome folding and dynamics of the vast majority of bacteria and archaea and particularly those living in stressful environments, have not yet been investigated. We expect many adaptations to take place and dramatic differences compared to the current models based on studies of model organisms to be discovered.

Furthermore, the relation between chromosome domain structure and function should be explored taking into consideration subcellular organization, supercoiling, and chromosome segregation. Yet, despite many years of investigations, some chromosome processes remain poorly understood even in well-established model systems, such as *E. coli*, due to the limited available tool set. This bacterium does not harbour a ParABS system; however, it deploys SMC-family proteins (MukBEF) and a dedicated controller, MatP, in the terminus region for chromosome organization and segregation (Mercier et al. 2008; Gruber 2018; Mäkelä and Sherratt 2020; Mäkelä, Uphoff, and Sherratt 2021; Sadhir and Murray 2023). In addition to dedicated factors, entropy (Jun and Wright 2010) and mechanical forces may also play a role (Hadizadeh Yazdi et al. 2012; Kleckner et al. 2014). New methods allowing to modify and sense mechanical forces inside live microbial cells will be critical to decipher these mechanisms.

5.2 | Where Should We Be Looking and What Tools Will We Need?

The next challenge for the field is establishing and investigating new model systems across the tree of life from both the bacterial and archaeal domains to better understand the evolution of chromosomes, their organization, and how DNA transaction processes and DNA-binding proteins have been shaped through evolutionary history. A vast amount of knowledge of genome primary sequence currently available derives from metagenomic data related to organisms that are not yet cultured. Thus, biomass availability becomes a key challenge. As a consequence, technologies that enable meaningful analyses and interpretations of low cell numbers and heterogeneous mixed cultures need to be refined and made generally accessible. Community sharing of newly cultured species should be commonplace. With these technologies in hand, it should become possible to generate an encyclopedia of chromosome conformations from a broad phyletic distribution of species. Effort should be made to ensure that all major phyla are covered, ideally with more than a single representative species.

The encyclopedia generated as described above will allow identification of key target species for further in-depth investigation. To extend the project beyond the purely observational and to establish the molecular forces and players underpinning genome conformation in novel candidate model organisms selected from the triage process, new genetic tools need to be developed. This task can be time-consuming and can require several years of dedicated efforts from multiple laboratories. Microscopy studies require thermostable fluorescent proteins to investigate thermophilic microbes, and these tools are not currently available. Development of high-throughput super-resolution approaches (Xiao and Dufrêne 2016) will also be necessary for microscopy studies. Ideally, the next 10 years will see the development of tools for at least one organism from each phylum. It is also important to study organisms that are phylogenetically related to observe differences in various environments and temperatures. Comparative studies should shed light on evolutionary patterns, possible convergent evolution of protein function, and common principles. Replicon organization, ploidy, replication mode, and genome maintenance processes and mechanisms should also be studied in parallel with chromosome organization mechanisms.

5.3 | Understanding the Relationships Between Chromosome Folding and Other DNA Transactions

A key and unresolved issue lies in the causality relationship between chromosome conformation and transactions that act upon this three-dimensional polymer (Liu et al. 2021; Bell 2022). To what extent does form define function and does function define form? The core functions associated with chromosomes are replication, transcription, and repair. Thus, it is crucial to understand how these processes play a role in chromosome organization and in turn how chromosome organization affects these DNA-associated transactions. In this direction, genome maintenance processes and mechanisms can be studied in parallel with chromosome organization. For example, how do NAPs affect DNA repair mechanisms such as mismatch detection during replication? Understanding this interplay can provide insights into how chromosome organization influences genome maintenance (and *vice versa*).

In addition to studying the organisms that 4 billion years of evolution present us, the above studies could be complemented by studies on synthetically-derived minimal cells—this could be particularly informative in determining how readily NAPs can cross complement functionally, influence chromosome conformation, and impact gene expression (Dame, Rashid, and Grainger 2020).

A broader array of functional outputs should be explored, such as subcellular organization of cytoplasmic proteins and organelles, supercoiling states of topological domains, and chromosome segregation. A broader array of non-model organisms under different conditions and beyond should also be employed to address this essential question in the larger context of prokaryotic chromosome biology.

Technological advances in high throughput super-resolution microscopy, Hi-C studies using Nanopore sequencing, cryo-EM/cryo-ET investigations, simulations, and AlphaFold2 structure predictions should make a very significant contribution to advancing our knowledge and solving some of the challenges of the field in the next 10 years.

6 | Outstanding Challenges

Addressing the outstanding challenges required for understanding the physical and biological principles that drive the

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functioning of the prokaryotic chromosome requires a multi-disciplinary approach. Just as the chromosome uses bridging connections to unlock novel cell behaviors, our field needs to form bridging connections to tie together the work performed by theorists and experimentalists, labs focused on in vivo and in vitro techniques, and researchers working at the nanoscale up to population-level studies. Below, we delineate several key topics that will require researchers in the field to work together to combine techniques and expertise. We conclude by identifying key strategies to encourage research teams to find new ways to combine and concentrate their expertise to tackle these problems.

6.1 | Protein-DNA Interactions

Both specific and non-specific interactions of proteins with DNA play a major role in shaping bacterial chromatin at all scales discussed over the course of the sessions (Dame 2005). An ideal understanding of protein-DNA interactions in the context of prokaryotic chromosome biology would include (i) the ability to quantitatively predict the binding affinities of DNA binding proteins for specified DNA sequences and to design—either de novo or by mutagenesis—DNA binding proteins with binding affinities of the designer's choice; (ii) integration of those idealized binding affinities with external factors such as DNA supercoiling states and metabolite/ion concentrations; (iii) proper thermodynamic treatment of the effects of neighboring and overlapping binding sites and possible cooperativity or competition between factors capable of binding a region; and (iv) prediction, at both a local and global scale, of the effects of protein binding on local DNA structure, transcriptional regulation, potential phase separation of specific loci, and long-range contact formation. A multitude of both theoretical and experimental approaches must be employed to meet these challenges: experimentally, measurements of protein binding (generally ChIP-seq) must be cross-referenced with other modalities such as Hi-C, (Crémazy et al. 2018; Cockram, Thierry, and Koszul 2021), GapR-seq (Guo et al. 2021) and NET-seq (Churchman and Weissman 2011) correlating protein binding states to their functional implications. In many cases, it may be necessary for these experiments to be stratified across subpopulations of cells to capture distinct, physiologically relevant cellular states. On the theoretical-computational side, atomistic and coarse-grained molecular simulations provide a clear path towards the prediction of key parameters for the challenges described above (Van Heesch, Bolhuis, and Vreede 2023), but work is needed to achieve sufficient simulation time- and length-scales to overlap with experiments (Hollingsworth and Dror 2018). Extremely coarse, lattice-based simulations of protein occupancy and chromosomal conformations provide a fertile bridging ground for bringing together computation and experiment on key questions such as the partition function for combined protein occupancy states at loci of interest or the large-scale structural effects of protein binding. However, progress will require cross-disciplinary collaboration between experimentalists and theorists in order to obtain sufficiently complex, informative, and tractable models.

6.2 | Studying NAP Bridging With Hi-C

Studies presented at the workshop on how Rok mediates long-range contacts in *B. subtilis*, visible as *dots* on the Hi-C map (Dugar et al. 2022), motivated a discussion on whether bridging by other NAPs could also be studied using Hi-C methods. This would help connect knowledge about bridging in vitro to what happens at larger scales in vivo. Further use of this approach may require the development of new experimental methods since NAPs can protect DNA from restriction enzymes, preventing the use of conventional Hi-C protocols. Identifying NAPs that exhibit specific and long-range bridging behavior could help find candidates for interactions that are clearly visible on Hi-C maps. Finally, it was noted that conventional normalization procedures for Hi-C data might complicate the interpretation of experimental results. To address this issue, a theoretical understanding of what kind of biases Hi-C normalization might introduce and potentially the development of new normalization methods would be welcome. All in all, further collaboration between groups studying NAPs and groups using and analyzing Hi-C data could create new insight into bridging interactions in vivo.

6.3 | Phase Separation of the Nucleoid

The nucleoid forms a distinct phase-separated compartment within the cell (Dame 2005; Racki and Freddolino 2025) and shares some features with canonical liquid phase-separated systems from eukaryotes (Hyman, Weber, and Jülicher 2014). Additionally, there are more localized and specific phase-separated protein complexes present in different prokaryotic systems, from droplets (Guilhas et al. 2020; Babl et al. 2022; Alaoui et al. 2024) to clusters of RNA polymerase (Ladouceur et al. 2020). Progress in this direction is made difficult by challenges specific to prokaryotes, such as their small size, the difficulty of labeling specific NAPs, and heterogeneity within the nucleoid (Azaldegui, Vecchiarelli, and Biteen 2021). Understanding how these phase-separated structures organize bacterial chromatin and give rise to observable bacterial functions requires imaginative new assays and new conceptual frameworks that can only succeed by bringing together researchers from diverse backgrounds (Hoang et al. 2023). In eukaryotes, canonical liquid–liquid phase-separated systems can be defined by the ability of droplets to merge, Ostwald ripening, and the round shape of the droplets. Unfortunately, these properties can be much more difficult to measure in live bacteria using conventional light microscopy. Additionally, the nucleoid itself has viscoelastic properties that can lead to slow kinetics for some of these processes that are difficult to observe (Javer et al. 2014). Moving forward, the field needs to share new techniques and probes to interrogate the nature of compartmentalized regions within the nucleoid. Additionally, theoretical models are needed to interpret how the complex viscoelastic properties of different condensates can influence their functional roles in the cell.

6.4 | Bridging Modeling and Experiment

A key goal of the Biology and Physics of Prokaryotic Chromosomes meeting has been a multi-disciplinary approach, especially the

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interchange of ideas between theorists and experimentalists. However, despite years of effort, significant difficulties remain in facilitating fruitful interactions. Participants agreed that ideally there is a closed loop between experiment and theory, with new experimental results inspiring new models and predictions from these models inspiring new experiments (Harju and Broedersz 2024). However, both groups reported that this critical two-way feedback loop was broken. Modelers pointed out that experimentalists often were not following up on their predictions and not making sufficiently quantitative measurements. Experimentalists noted that in their perception modelers were more concerned with theoretical elegance and minimal models than biological applicability and, as a result, they often neglected critical mechanisms that are essential for biological function. It was also pointed out that these models did not make robust, sufficiently qualitative and compelling predictions. Theorists emphasized that models are useful for exploring the null hypotheses and understanding when novel biological mechanisms are required. How do modelers deal with the complexities of in vivo versus in vitro data? Especially in the context of in vivo measurements, modelers complained that very few meaningful parameters could be changed to test their models. Finally, model complexity was also identified as a significant challenge. Theorists emphasized that complex models can fit any data and the parameters that were fit are not predictive of the underlying mechanistic rates. This model sloppiness phenomenon introduces significant challenges (Machta et al. 2013), for example, what do the model parameters mean? Solutions to these problems present an outstanding challenge. On the experimental side, it was agreed that experimentalists should continue to emphasize quantitative methods and be open to testing novel predictions from modeling groups. On the modeling side, solutions include earlier and more meaningful discussions with experimentalists about which mechanistic details are known to be critical to the phenomenon being studied. Modelers should consider multiple competing hypotheses to understand the significance of a model fit. They should perform sensitivity analyses to understand the statistical significance of their predictions and, most importantly, they should look for emergent and robust phenomena and predictions of qualitative changes in phenomenology.

In spite of these frustrations all participants agreed that significant progress has been made: new generations of models are mathematized even in experimental papers. New experiments are increasingly quantitative, and many participants reported fruitful collaborations with their peers from other disciplines.

6.5 | Human Solutions

As noted above, discussions held during the workshop made clear the essentiality of experimentalists and computational/theoretical researchers to each become somewhat versed in the potential and limitations of the others' fields. For example, theorists often severely underestimate the amount of research effort required to perform a single experiment, whereas experimentalists often fail to note that given sufficient parameters, a suitably flexible model can fit nearly any dataset, requiring caution in interpretation and the need to design new experiments to further our state of knowledge. Even simple solutions such as the establishment of new Slack or Discord communities dedicated to maintaining contact and collaboration between workers from different disciplines, nucleated by meetings such as this workshop, could be highly productive.

Another major barrier identified in informal discussions was that academic funding models often do not lend themselves to establishing and maintaining new cross-disciplinary collaborations, especially those emerging spontaneously from interactions such as the present Workshop. Due to the long lag between the inception of an idea and any potential funding decision (often 6 months or longer), and the relative sparsity of academic personnel with excess capacity to take on a new idea, many promising potential collaborations wither before any serious work can be achieved. Advocating for more agile funding mechanisms encouraging inter-disciplinary collaborations, both at institutional levels and those of public grant-funding agencies, will be necessary in order to improve the situation—it is not just that more money is required, but that funds be obtained more quickly and with less administrative overhead in order to spur the new collaborations required to address the key challenges that we have identified here.

6.6 | Computational and Machine Learning Approaches

Solving several of the problems highlighted above will require the integration of multiple data types to predict a complex and inherently quantitative phenomenology. For instance, consider models that integrate (i) the measured binding affinities of multiple proteins with (ii) local supercoiling state to predict the overall protein occupancy profile. In addition to integrating multiple data types, many predictions are inherently probabilistic in nature. For instance, measured Hi-C maps are the population average over many distinct chromosome conformations (Crémazy et al. 2018). How do we simultaneously handle diverse data and probabilistic predictions? Bayesian inference combined with statistical mechanics provides a principled bottom-up approach to inform mechanistically- or structurally inspired models with diverse data to generate probabilistic predictions (Gelman et al. 2004). However, there is now a powerful emerging alternative: machine learning (ML). ML approaches are very flexible and do not depend on mechanistically accurate models but rather learn patterns directly from large data sets. The potential power of these approaches is now clear with the advent of AlphaFold2 (Jumper et al. 2021; Tunyasuvunakool et al. 2021), which uses the ML approach to make predictions of protein structure with dramatically better performance than competing rationally designed algorithms. However, it is important to emphasize that ML approaches are not a one-size-fits-all solution to all data analysis problems. Training these algorithms typically requires very large sets that often must be hand labeled (Cutler et al. 2022). Furthermore, the ML solution to the protein folding problem illustrates both the advantages and shortcomings of this approach: AlphaFold2 makes extremely accurate predictions; however, the algorithm does not provide a mechanistic understanding of why the protein folds as predicted. Despite these cautionary notes, we expect that innovative uses of ML-based approaches will be an exciting focus of our next meeting.

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Acknowledgments

The authors would like to thank the Lorentz Center (Leiden, The Netherlands) for hosting the workshop "Biology and physics of the prokaryotic chromosome" from September 11–15, 2023. Specifically, the "Open Space Technology" sessions facilitated by Lorentz Center staff were instrumental in inspiring and shaping the discussions summarized in this article. The Lorentz Center is funded by NWO and Leiden University.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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