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# Principles of bacterial genome organization, a conformational point of view

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## Abstract

Bacterial chromosomes are large molecules that need to be highly compacted to fit inside the cells. Chromosome compaction must facilitate and maintain key biological processes such as gene expression and DNA transactions (replication, recombination, repair, and segregation). Chromosome and chromatin 3D-organization in bacteria has been a puzzle for decades. Chromosome conformation capture coupled to deep sequencing (Hi-C) in combination with other “omics” approaches has allowed dissection of the structural layers that shape bacterial chromosome organization, from DNA topology to global chromosome architecture. Here we review the latest findings using Hi-C and discuss the main features of bacterial genome folding.

## KEYWORDS

3D organization, bacterial chromatin, bacterial chromosome, condensins, nucleoid-associated proteins

## 1 | INTRODUCTION

Bacterial genomes can be small or large, ranging from hundreds of kilobases to 16 megabases (Touchon & Rocha, 2016). While the canonical situation corresponds to a unique circular chromosome, around 10% of bacteria harbor a multipartite genome (e.g., *Borrelia*, *Burkholderia*, *Brucella*, *Rhizobium*, and *Vibrio*). Moreover, some bacterial chromosomes present a linear geometry (e.g., *Streptomyces* and *Borrelia*). Remarkably, *Agrobacterium tumefaciens* showcases both types of geometries in its two chromosomes—one linear and the other one circular. Generally, bacteria are monoploid, but many contain multiple copies of this single chromosome during rapid growth. Moreover, some phylogenetic groups (e.g., *Streptomyces*, *Deinococcus*, and *Thermus*) are polyploid with multiple copies of their genomes independently of the growth rate, up to half a million in the giant bacterium *Thiomargarita magnifica* (Volland et al., 2022).

How does the overall folding of each 3D genome reflect this diversity? This question began to be addressed with the development of innovative techniques, such as the imaging of individual chromosomal loci and genomic approaches, which, when applied in combination, have enabled us to take a significant step forward in understanding 3D genome organization (Le & Laub, 2014). More than a decade ago, the use of chromosome conformation capture coupled with deep sequencing (Hi-C) was a game changer in the field of genome biology (Lieberman-Aiden et al., 2009). Hi-C is a method to study the spatial organization of DNA at different scales. It involves cross-linking DNA fragments close in space using formaldehyde (FA), followed by digestion and labeling with biotinylated nucleotides. These fragments are ligated together, forming chimeric DNA molecules. After reversing cross-links, DNA is purified, sheared, and biotinylated chimeras are pulled down with streptavidin beads. The captured DNA fragments are then sequenced using Next-Generation Sequencing (NGS) technologies. In silico analysis

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identifies chimeric DNA fragments to build a map of DNA contacts, represented as heatmaps showing the relative frequency of contacts between genomic regions (Figure 1a) (Cockram et al., 2021).

In bacteria, Hi-C was used for the first time to unveil the folding of the *Caulobacter crescentus* chromosome with high resolution (Le et al., 2013). The use of Hi-C to study the genome organization of other bacterial species quickly progressed after the first Hi-C study and not only revealed the diversity of 3D-genome architectures in multiple bacterial species across the phylogenetic tree (Figure 1b), but also the different scales of genome organization and the mechanisms of action of key structural factors and processes (Figure 2). In this review, we will revisit the work performed in these bacteria, both at the global scale (chromosome level) and the local scale (chromatin level) and we will discuss the rules of genome folding that can be inferred from them.

## 2 | BACTERIAL CHROMOSOME LARGE-SCALE STRUCTURING

### 2.1 | SMC complexes as global drivers of 3D chromosome folding in bacteria

A look at the Hi-C contact maps performed in diverse bacteria species (Figure 1b) reveals that most of their chromosomes can be folded in one of two main conformations: (i) chromosomes whose Hi-C maps present only a main diagonal, revealing frequent intra-chromosomal DNA contacts; or (ii) those in which their contact maps shows, in addition to the main diagonal, a secondary diagonal unveiling DNA contacts between the two chromosome arms (Figure 1a). Although less frequent, a variation of these two main conformations can also be observed (for instance in *Limosilactobacillus reuteri*, Figure 1b).

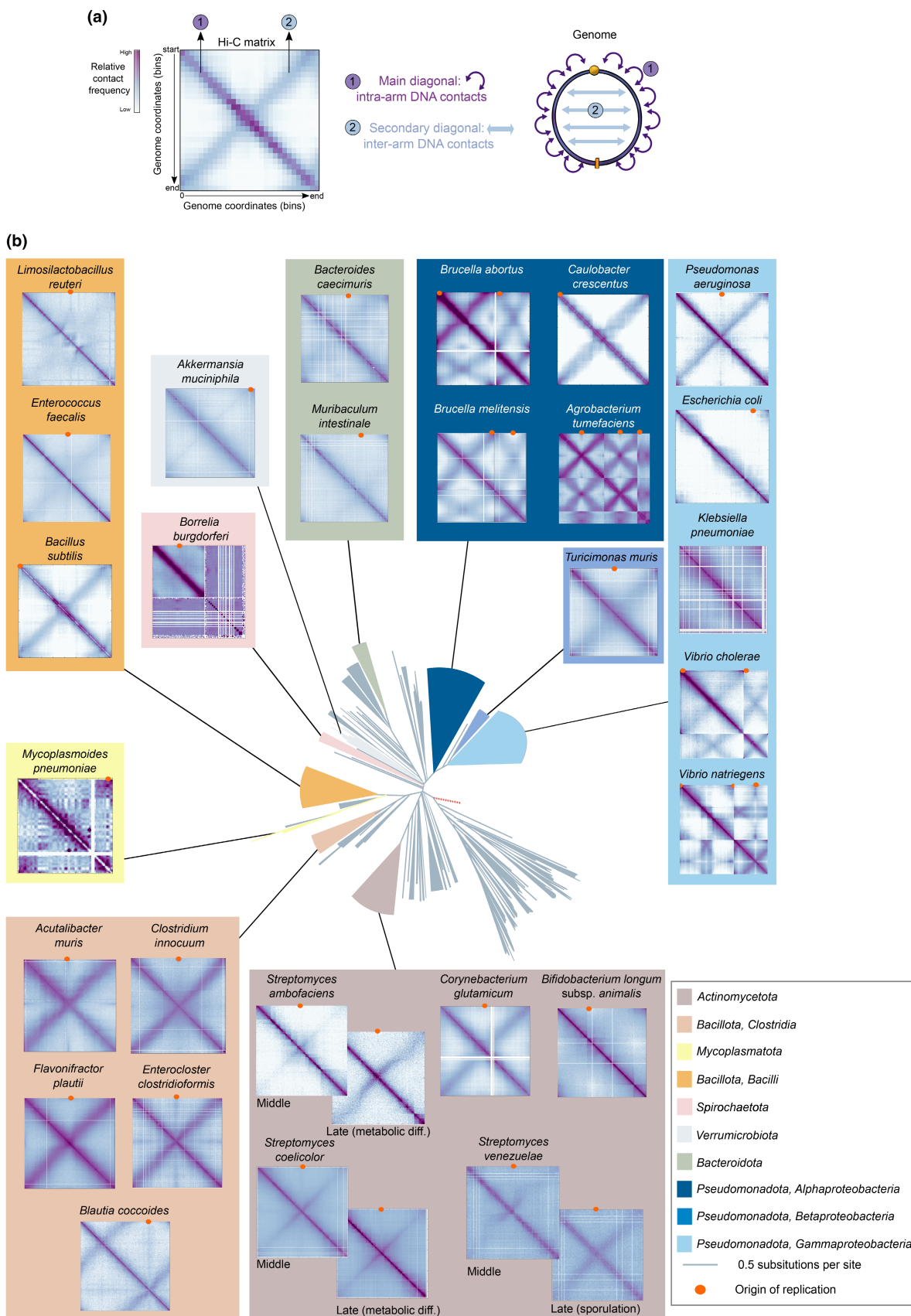
The first Hi-C study in *C. crescentus* showed that the secondary diagonal was a consequence of the activity of the Structural Maintenance of Chromosomes (SMC) complex (also known as condensin complex), Smc-ScpAB (Le et al., 2013). In *C. crescentus*, this genome folding was consistent with the longitudinal disposition of the chromosome inside the cell, whereby the origin of replication

(*ori*) and *terminus* (*ter*) regions are localized at the cell poles and the two arms of the chromosome lie longitudinally (Viollier et al., 2004). In *Bacillus subtilis*, similarly to *Caulobacter*, the Smc-ScpAB condensin complex was shown to promote inter-arm DNA contacts (Le et al., 2013; Marbouty et al., 2015; Tran et al., 2017; Wang et al., 2011, 2015). It is interesting to highlight that in *Caulobacter*, the translocation of Smc-ScpAB along the chromosome extended around 600 Kb from the *parS* sites, while in *B. subtilis*, this condensin complex translocates all the way from *ori* to *ter* (Tran et al., 2017; Wang et al., 2017), indicating that the processivity of the Smc-ScpAB complex activity can vary between species. Interestingly, the presence of Smc-ScpAB does not always imply the existence of inter-arm contacts (Lamy-Besnier et al., 2023). For instance in *Streptomyces*, the relative frequency of contacts between the two chromosomal arms is rather low in exponential phase even though the frequency increases after metabolic or sporogenic differentiation (Deng et al., 2023; Liroy, Lorenzi, et al., 2021; Szafran et al., 2021), when the global folding of the chromosome changes (Figure 1b). Altogether, this indicates that condensin activity can be modulated depending on the genetic background and/or as a function of growth phase.

In addition to Smc-ScpAB, two other types of condensin complexes have been identified so far: MukBEF, which is found in some  $\gamma$ -proteobacteria, and MksBEF ("MukBEF-like SMC protein"), which is structurally similar to MukBEF but scattered over the phylogenetic tree. MksBEF can co-exist with other Smc-ScpAB or MukBEF complexes (Petrushenko et al., 2011), raising the question of the role of condensin interplay in the management of bacterial chromosome architecture. Remarkably, MukBEF and MksBEF were shown to increase the frequency of long-distance intra-arm DNA contacts (>900kb), mainly acting on newly replicated DNA (Liroy et al., 2018, 2020; Seba et al., 2023). MksBEF long-range activity is revealed in the absence of Smc-ScpAB or in genetic backgrounds in which it can act before this complex, indicating the existence of a hierarchy of condensin activities (Liroy, Junier, & Boccard, 2021).

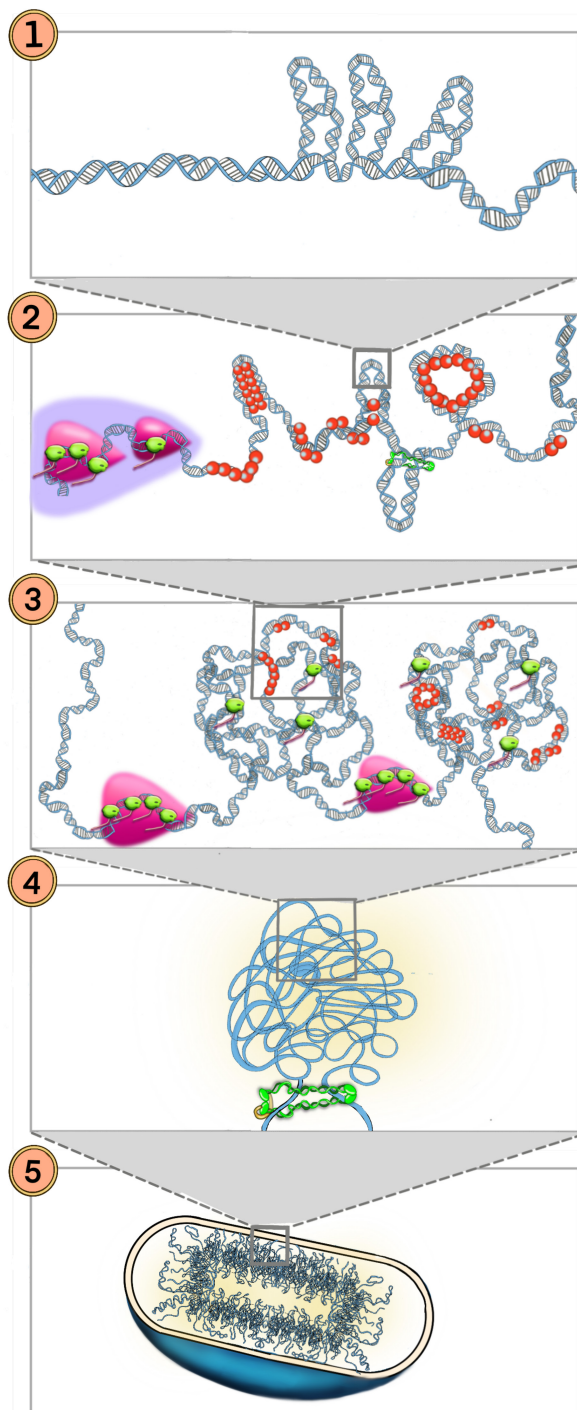
Studies performed in *Borrelia burgdorferi* have revealed that this bacterium harbors the genes encoding Smc and ScpA, but not for ScpB. In addition to these genes, this bacterium harbors a second SMC gene, *mksB*, but not the genes encoding the MksE or MksF subunits (Takacs et al., 2022). Interestingly, Smc is loaded by the

**FIGURE 1** Hi-C contact maps in bacteria. (a) Schematic interpretation of a Hi-C contact map. The Hi-C contact map is built using the genome of the bacteria of interest segmented into windows of a defined size (bins). The frequency of contacts between bins is then represented using a heat map. In this heat map, bins with a high frequency of contacts are represented in dark purple, while bins with a low frequency of interactions are represented in light blue. In a canonical contact map, the main diagonal reflects the contacts between genomic loci *in cis* (1, intra-arm DNA contacts), while the secondary diagonal represents the contacts between genomic loci between the two chromosome arms (2, inter-arm DNA contacts). (b) Diversity of bacterial 3D genome folding in the tree of life. The bacterial tree of life is represented in gray, and the red line corresponds to the archaea and eukaryota branching (tree adapted from Zhu et al., 2019). Within this tree, Hi-C maps of wild-type bacteria with characterized 3D chromosome folding are displayed. The dynamics in 3D folding along the cell cycle is shown for *Streptomyces* species in early stages of growth (Middle) versus metabolic differentiation (Late). Hi-C maps shown here were obtained from published works (Barton et al., 2023; Böhm et al., 2020; Deng et al., 2023; Huang et al., 2023; Lamy-Besnier et al., 2023; Le et al., 2013; Liroy et al., 2018, 2020; Liroy, Lorenzi, et al., 2021; Ren, Liao, Karaboja, et al., 2022; Ren et al., 2023; Szafran et al., 2021; Trussart et al., 2017; Val et al., 2016; Wang et al., 2015, 2022; Yin et al., 2020). Except for *C. crescentus*, *B. subtilis*, *A. tumefaciens*, *S. coelicolor*, and *S. venezuelae*, for which contact maps were publicly available, all contact maps were built from the available FASTQ files using the OHCA pipeline (Serizay et al., 2024).



ParB/parS complex, without promoting inter-arm DNA contacts, but favoring long-range intra-arm interactions (presumably in a complex with ScpA). In a similar way, MksB was shown to promote long-range

intra-arm DNA contacts (Ren et al., 2023). In addition, in the absence of smc and mksB, the long-range interactions within the telomeres of the chromosome are increased, suggesting that these proteins might



**FIGURE 2** The layers of 3D chromosome organization in bacteria. Schematic representation of the distinct layers of bacterial chromosome organization. Gray squares show a layer of magnification, from the naked DNA sequence to the bacterial nucleoid. (1) DNA and supercoiling domains; (2) The bacterial chromatin containing Nucleoid Associated Proteins (NAPs), SMC complexes bound to DNA, and Transcriptionally Induced Domains (TIDs). NAPs are shown as red beads, capable of bending, bridging, wrapping, or oligomerizing onto DNA. The SMC complex is represented as a dimeric ring associated with the kleisin and kite subunits (green and subunits in yellow). Transcribing RNA polymerase is shown as a green complex, with transcribed RNA shown in red. TIDs are illustrated in pink, with inter-TID interactions represented by a light purple cloud. Other DNA binding proteins were omitted for simplicity; (3) Chromosome Interacting Domains (CIDs) are domains of variable size that change according to the growth conditions, usually defined by long and highly expressed genes or TIDs; (4) SMC complexes are loaded onto the chromosome and promote the long-range DNA contacts in an ATP-dependent manner in most bacteria by bringing in close proximity distant genomic regions. (5) The integration of these layers with other out-of-equilibrium processes, such as DNA transactions, molecular crowding, and interactions with cellular structures, converges in the formation of the bacterial nucleoid.

Is condensin activity essential to bacteria? The answer depends on the bacterial species studied. In bacteria such as *C. crescentus* or *P. aeruginosa*, deletion of the genes encoding Smc-ScpAB has no major impact on cell physiology, although the effect on overall genome folding is not negligible (Lioy et al., 2020; Tran et al., 2017). On the contrary, deletion of the condensin complex in *B. subtilis* or *E. coli* has important consequences for fitness and genome folding, and is associated with an increased frequency of anucleated cells (Nolivos & Sherratt, 2014). The difference likely resides in the role of these condensin complexes on chromosome segregation. For instance, the generation of elongated or shortened Smc mutants in *B. subtilis* failed to promote inter-arm contacts in the presence of native *parS* sites, but supported growth (Anchimiuk et al., 2021). In addition, a recent study in *E. coli* shows that in the absence of replication, thus no need for chromosome segregation, MukBEF is capable of promoting long-range DNA contacts (Seba et al., 2023), illustrating how the segregational and conformational activities of condensins are able to work independently in chromosome management (Badrinarayanan et al., 2012). These results raised the yet unanswered question of the functional role of long-range DNA contacts (Yáñez-Cuna & Koszul, 2023).

Many studies have shown that the activity of condensins is tightly controlled in vivo. Smc-ScpAB is usually loaded by the ParB/*parS* complex at the origin region, while MukBEF has no preferential loading in the chromosome of *E. coli* (Gruber & Errington, 2009; Seba et al., 2023; Sullivan et al., 2009). In *B. subtilis*, control of Smc-ScpAB activity is achieved by the specific number and distribution of *parS* sites, the affinity of Smc-ScpAB to the loader ParB/*parS* complexes, but also by its translocation rate (Anchimiuk et al., 2021). In *E. coli*, MukBEF activity is prevented by MatP binding to *matsS*, likely acting as an unloading factor in the *terminus* region (Bürmann et al., 2021;

reduce contacts between telomeres (Ren et al., 2023) and that these SMC proteins present non-canonical activities. Whether these SMC proteins form complexes with other unknown accessory subunits or how the conformational activity of these two SMC complexes can be accommodated in the same chromosome remains to be investigated.

Altogether, these studies underline the fact that condensin complexes are ATP-dependent molecular motors that promote long-distance DNA interactions, likely acting as loop extruders (Bürmann & Löwe, 2023) and that they (or an analogous system) are key contributors to the higher order organization of bacterial genomes (Figure 2).

Liroy et al., 2018; Nolivos et al., 2016). The activity of Smc-ScpAB is also prevented at the *terminus* region in *B. subtilis* (Karaboja et al., 2021). In this case, the XerD protein is responsible for unloading Smc-ScpAB. Remarkably, the inter-arm contacts also decrease toward the ends of the linear chromosome of *Streptomyces* (Szafran et al., 2021). In *Streptomyces venezuelae*, Smc-ScpAB is excluded from the terminal arms, which are enriched in the Nucleoid Associated Protein (NAP) HupS (Szafran et al., 2021). All in all, the mechanisms by which condensin activity is prevented within the *terminus* region, involves dedicated systems (i.e., MatP/*matS* or XerD/*XDS*). Does the absence of condensin activity in the *terminus* region confer selective advantages? We can speculate that inhibiting condensin activity may be crucial to avoid interference with several machineries driving critical processes before cell division (termination of replication, decatenation, segregation in combination with the unclosed divi-some ring). Alternatively, the *terminus* region of many bacteria may present specific emerging properties that are still uncharacterized since most of the studies developed so far specifically focused on the properties of the individual components of this region. Further experiments are needed to confirm the *terminus* region emergent behavior in bacteria.

## 2.2 | Inter-replicon contacts are key features in the 3D folding of segmented bacterial genomes

Large scale chromosome structuring of only a few bacterial species with more than one replicon have been characterized so far (Barton et al., 2023; Huang et al., 2023; Liao et al., 2022; Ren, Liao, Barton, et al., 2022; Ren et al., 2023; Val et al., 2016) (Figure 1b). In all of them, inter-replicon contacts were revealed in the Hi-C map, indicating a protein-mediated physical interplay between genetic elements.

*V. cholera*'s chromosomes present a tightly regulated replication process. The initiation of replication of Chr2 is orchestrated by the initiation protein RctB encoded in Chr2 and the replication of DNA sequence *crtS* (Ramachandran et al., 2018; Val et al., 2016) found in Chr1. This ensures replication of Chr2 only once per cell cycle and in coordination with Chr1. Specifically, Chr1 is two-thirds replicated before Chr2 begins its replication upon binding of the RctB protein to the *crtS* locus (Val et al., 2016). Hi-C maps performed in replicating *V. cholera* revealed contacts between *ori2* and the *crtS* site of Chr1 (Val et al., 2016). Thus, contact maps revealed for the first time a "communication" between two replicons that may play a role in the coordination of the initiation of replication of Chr2, although this remains to be shown experimentally. In addition to these contacts, other inter-chromosome contacts were revealed by Hi-C and they stretched out from *dif* sites and formed a cross shaped inter-chromosome pattern (Val et al., 2016). *V. cholerae* encodes the MatP/*matS* system, which seems to be a likely candidate responsible for *ter1-ter2* contacts (Demarre et al., 2014). These contacts could provide a physical validation for the synchronized termination of replication in *Vibrio*, but experimental data is needed to support this hypothesis.

Similarly to *V. cholera*, studies performed in the  $\alpha$ -proteobacteria *B. abortus* and *Brucella melitensis*, revealed significant inter-replicon contacts (Barton et al., 2023; Huang et al., 2023). The Hi-C map revealed contacts along the two chromosomes in both bacteria, with a relatively high frequency of contacts between *ori1-ori2*. The specific *ori1-ori2* interplay is in agreement with microscopy data in which *ori1* and *ori2* of *B. abortus* are localized in close proximity, while *ter1* and *ter2* never co-localize (Deghelt et al., 2014). Similarly to *Brucella*, Hi-C studies performed with the *Agrobacterium tumefaciens* (Ren, Liao, Karaboja, et al., 2022) multipartite genome also revealed inter-replicon contacts between their *ori* regions. This interplay is consistent with previous fluorescent in situ hybridization (FISH) experiments showing polar co-localization of all the *ori* regions of the four replicons inside the cell (Kahng & Shapiro, 2003). Interestingly, *ori* clustering in these bacteria is mediated by a direct interplay between the centromeric proteins ParB from Chr1 and RepB from Chr2. While the subcellular positioning of *ori1* and *ori2* is strictly dependent on polar organizers, *ori* clustering is independent of them (Ren, Liao, Barton, et al., 2022). Moreover, the contacts promoted by *ori* clustering of the four replicons are essential for maintenance of the secondary replicons (Chr2, pAT, and pTi), independently of *ori* polar localization. Clustering thus reveals an important biological function in genome stability (Ren, Liao, Barton, et al., 2022; Ren, Liao, Karaboja, et al., 2022). Finally, the large-scale genome rearrangement observed in a strain of *A. tumefaciens* in which Chr1 fused with Chr2, did not impact the overall genome folding (Liao et al., 2022), highlighting the robustness of *A. tumefaciens* 3D genome organization.

## 3 | BACTERIAL CHROMATIN ARCHITECTURE

### 3.1 | Interplay between transcription and chromatin folding

Another breakthrough in the field was the discovery of "Chromosomal Interaction Domains" (CIDs), revealed along the main diagonal of Hi-C maps (Le et al., 2013). CIDs represent regions of the chromosome within which the frequency of contacts is higher than between loci in other domains. CIDs are frequently formed as a consequence of long transcriptional units (longer than 2 Kb), that are insulated from the chromosome when highly expressed, generating boundary regions (Le et al., 2013; Le & Laub, 2016). These boundaries often include operons encoding ribosomal RNA (*rrn*) genes or membrane proteins (Le & Laub, 2016; Liroy et al., 2018). The latter observation suggests that transcription-translation coupling could play a role in genome folding. In *C. crescentus*, it was proposed that boundaries were open chromatin regions driven by active transcription and that not all CID boundaries were super-coiling diffusion barriers (Le & Laub, 2016). Later, in other bacterial species, CID boundaries were shown to be regions rich in local DNA-DNA contacts (Deng et al., 2023; Liroy et al., 2018)

(Figure 2). Since their first identification in *Caulobacter*, CIDs and boundaries were observed in all bacterial genomes studied, including very distant bacteria, indicating that these are conserved structural features of bacterial chromosomes. Furthermore, CID boundaries vary according to the growth conditions and the cell phase, and CID sizes are quite variable, ranging from ~15 to 700 Kb (Böhm et al., 2020; Deng et al., 2023; Huang et al., 2023; Le et al., 2013; Lioy, Lorenzi, et al., 2021; Marbouty et al., 2015; Ren, Liao, Karaboja, et al., 2022; Ren et al., 2023; Trussart et al., 2017; Val et al., 2016; Wang et al., 2015) (Figure 1). Although a few examples of boundaries without highly expressed genes exist (Dugar et al., 2022; Lioy, Lorenzi, et al., 2021; Marbouty et al., 2015), most studies pinpoint long and highly transcribed operons (or a succession of genes) as master architects of genome folding by inducing local chromatin constraints.

Reciprocally, does the organization of a bacterial genome in CIDs influence gene expression? In most bacterial models, CIDs were not associated with a role in gene regulation (Böhm et al., 2020; Deng et al., 2023; Huang et al., 2023; Le et al., 2013; Lioy, Lorenzi, et al., 2021; Marbouty et al., 2015; Ren, Liao, Karaboja, et al., 2022; Ren et al., 2023; Trussart et al., 2017; Val et al., 2016; Wang et al., 2015). However, in exponentially growing *S. ambobaciens*, the linear chromosome is organized in large 3D-compartments correlated with both gene persistence and gene expression (Lioy, Lorenzi, et al., 2021). The central compartment is enriched in conserved and highly expressed genes (thus, rich in boundaries) and the terminal compartments are rich in horizontally acquired sequences that tend to be silent (poor in boundaries). These “active” and “silent” compartments present different structural features, indicating that transcriptional activity influenced the large-scale structuring of the chromosome (Bury-Moné et al., 2023). Interestingly, in this bacterial species, the distal *rrn* operons delimit these compartments (Lioy, Lorenzi, et al., 2021), raising the possibility that *rrn* operons have a role in emergence of the genetic compartmentalization of *Streptomyces* (Bury-Moné et al., 2023).

Recently, the chromosome of *E. coli* has been studied with ultra-high resolution (sub-kb) (Bignaud et al., 2024). This study revealed that highly transcribed transcriptional units can form discrete Transcriptionally Induced Domains (TIDs) ranging in size from 1 to 20 Kb. Many TIDs coincide with previously identified CIDs boundaries found in lower-resolution Hi-C maps (5kb). In addition, new TIDs that do not match previously identified CID boundaries were also detected. This shows that in low-resolution Hi-C maps, CIDs hinder the identification of TIDs. It is likely that these TIDs present lower transcriptional levels compared to TIDs found at CID boundaries and, as a consequence, impose fewer chromatin constraints (Bignaud et al., 2024; Yáñez-Cuna & Koszul, 2023). Interestingly, TIDs seem to interact with other TIDs found at short distances, forming a “plaid pattern” in the Hi-C contact map. By using a synthetic approach in which DNA folding upon T7 promoter activation was studied, it was proposed that the presence of consecutive units of RNA polymerase along the transcribed track is sufficient to generate TIDs (Bignaud et al., 2024). The study also uncovered the

contribution of supercoiling to TID organization. In the presence of rifampicin, an antibiotic that inhibits the activity of bacterial RNA polymerase but not T7 RNA polymerase, the expression of a *lacZ* gene under the control of the T7 promoter allowed the association of the supercoiled state of the active transcriptional units and their neighboring regions with a Hi-C-specific signature. Regions where negative supercoiling accumulated correlated with an arched striped pattern in the Hi-C map at the 5' end of the transcribed unit. In contrast, the accumulation of positive supercoiling at the 3' end of the transcriptional unit, diffusing over 50–100kb, correlated with the formation of a bundle signal in the contact map. The arched striped Hi-C pattern was observed only when the strong T7 promoter was active, but not in genes with endogenous promoters, suggesting that under physiological conditions, other factors such as gene orientation, density, and expression level are involved in regulating these topological structures in vivo (Bignaud et al., 2024).

Transcribed units seem to be less mobile than low expression regions. This constraint is correlated with a likely relocation of the transcribed region to the lateral edge of the nucleoid (Bignaud et al., 2024). This may favor the interaction with other TIDs, giving rise to the “plaid pattern” observed in the Hi-C maps. It could be speculated that these interactions potentially involve NAPs or other transcriptional factors. TID interplay and other aspects of their formation remain to be further characterized. For instance, it is not known how TIDs are formed in physiological conditions, whether other elements in addition to RNA polymerase can influence their folding or whether they form transcriptional condensates (Bignaud et al., 2024; Ladouceur et al., 2020; Stracy et al., 2019). Resolving these and other questions such as how TIDs are connected to supercoiling barriers or to the long-range organization of the chromosome will allow us to have a broad understanding of the functional organization of the bacterial chromosome.

### 3.2 | Role of nucleoid associated proteins in chromatin folding

A general principle of bacterial genome organization is that NAPs are key components of the chromatin, playing a crucial role in the management of genome topology by bending, looping, coating, bridging, or wrapping DNA (Figure 2). In addition to their role in genome architecture, NAPs affect gene regulation and DNA transactions (Dame et al., 2020). NAPs vary across species, but proteins from the HU family are encoded in all bacteria (Amemiya et al., 2021; Grove, 2011). In some bacteria, there is a single copy, while in others, several homologs can be present (Cases & de Lorenzo, 2002). The impact of deletion varies across bacterial species (Lioy, Junier, & Boccard, 2021), suggesting that the function of HU, and likely of other NAPs, might vary. On the contrary, certain NAPs are characteristic of specific phylogenetic groups, such as the Lsr2-type xenogeneic silencer specifically found in Actinobacteria (Qin et al., 2019). Some NAPs bind non-specifically to DNA while other tends to have sequence bias (Lioy, Junier, & Boccard, 2021).

For many years, NAPs have been considered as key factors involved in the spatial organization of bacterial chromosomes. However, information on the impact of NAPs on chromosome conformation is available in only a few species (Barton et al., 2023; Dugar et al., 2022; Le et al., 2013; Liroy et al., 2018). For instance, the genomes of *C. crescentus* and *E. coli* both encode two HU encoding paralogs whose products can form heterodimers. Remarkably, the effect on chromosome conformation of the deletion of the two HU encoding genes was different in *C. crescentus* and in *E. coli*: while in *C. crescentus* the absence of the HU protein was correlated with a decrease in short-range contacts (up to 100kb) (Le et al., 2013), in *E. coli* the absence of this protein led to a strong decrease in long-range contacts (Liroy et al., 2018). Interestingly, the decrease of long-range contacts outside the *ter* region in *E. coli* is equivalent to that observed in the absence of MukBEF, indicating that this NAP and MukBEF might collaborate to promote the higher order organization of the chromosome (Liroy et al., 2018). An interplay between HU proteins and the condensin complex was also observed in *S. venezuelae* during sporogenic development (Szafran et al., 2021).

Horizontally acquired sequences frequently encode gene products whose expression can be deleterious for the bacterial cells. Therefore, the propensity of xenogeneic silencers such as H-NS to bind to these rather A/T rich regions and silence them is commonly viewed as a defense mechanism (Lucchini et al., 2006; Navarre et al., 2006). The way H-NS can repress gene expression *in vivo* is still under study. Biochemical characterization of *E. coli* H-NS and its interplay with DNA and RNA polymerase led to two models in which H-NS represses gene expression (Qin et al., 2019). In one model, H-NS oligomerizes onto the regulatory regions where RNA polymerase binds, thereby repressing gene expression. While in the other model, H-NS represses gene expression by bridging DNA and entrapping the RNA polymerase. Conformational studies in *E. coli* showed that around 70% of the loci bound by H-NS presented increased frequency of contacts up to 40kb in a mutant lacking the *hns* gene (Liroy et al., 2018). Furthermore, in the absence of H-NS, the large-scale structure of the *E. coli* chromosome does not seem to be highly impacted (Liroy et al., 2018). In addition, a recent study in which the transcriptional activity of the *proVWX* locus was studied in *E. coli* using 3C-qPCR and Hi-C showed that at low osmolarity, the *proVWX* locus is repressed by an H-NS bridged complex (Rashid et al., 2023). Thus, these results support both models for H-NS repression *in vivo*. Strikingly, in *V. cholerae*, it was recently shown that H-NS acts as a chromatid cohesive factor within horizontally acquired regions in repressed conditions (Espinosa et al., 2020). We can hypothesize that this cohesive activity may reflect the DNA bridging activity of H-NS. In *B. subtilis*, a paralog of H-NS, Rok, was recently shown to be associated with CID boundaries in exponential phase (Marbouty et al., 2015) and involved in loop and CID formation in stationary phase (Dugar et al., 2022). In stationary phase, this NAP seems to be assisted by Smc-ScpAB to promote topological structures. It is important to note that loop formation by Rok is consistent with the bridging activity described *in vitro* for this xenogeneic silencer (Erkelens et al., 2022). Another interesting NAP that

acts as a global transcriptional regulator of the two-chromosome bacteria *Brucella abortus*, is MucR. This protein is a small zinc finger protein that was recently proposed to be an H-NS like protein, acting as a xenogeneic silencer. Hi-C data obtained in this bacterium show that impact of MucR on chromosome conformation is not the same on chromosome 1 (Chr1) and chromosome 2 (Chr2). Strikingly, the activity of MucR seems to be similar to that of *E. coli* H-NS only on Chr2, while MucR in Chr1 affects mid to long-range chromosome organization (Barton et al., 2023). Furthermore, the absence of MucR also altered Chr1–Chr2 interplay, suggesting that MucR evolved additional chromosome organization activities in this bacterium with two chromosomes.

In conclusion, more studies on other NAPs and/or in different bacterial species are necessary to attain a global view of their activities at the organizational level. However, it is very likely that due to their promiscuity in several DNA associated processes, each NAP has evolved specific functions depending on host genetic features and lifestyles. With the exception of HU in *E. coli* and MucR in *B. abortus*, the deletion of NAPs reported so far (Barton et al., 2023; Dugar et al., 2022; Le et al., 2013; Liroy et al., 2018) is not associated with drastic effects on large-scale genome structuration, but seems to play a crucial role at the local level (Table 1). Further experiments are needed to characterize whether these functions are directly linked to NAP impact on genome topology or to other effects indirectly linked to the pleiotropic roles of NAPs in gene regulation.

#### 4 | LIMITATIONS OF HI-C

Although it is undeniable that Hi-C is a key technique to study bacterial genome folding, it has several limitations. First, Hi-C does not reveal absolute information about interacting loci. Thus, we can only obtain the relative frequency of contacts between distant genomic regions and not absolute values. Because of this, when performing comparisons with mutants and/or conditions, it is important to keep in mind that because of the relative nature of the Hi-C data, an increase or decrease of contacts in a given region will always be accompanied by a decrease or increase in another region. Furthermore, Hi-C relies in the use of FA to capture DNA-protein-DNA contacts. FA crosslinking can potentially alter chromosome/chromatin structure. Applying it in combination with other approaches such as fluorescence microscopy or genetic tools (Le & Laub, 2014), when possible, is highly recommended. Second, Hi-C does not allow to detect DNA–DNA contacts between homologous sequences, such as replicated chromosomes. In this case, the development of sister-C methods in bacteria is necessary to gain more insight into the replication process (Mitter et al., 2022). Finally, another important limitation, that is inherent to other genomic techniques, is that it is a whole genome population approach and does not consider cell-to-cell heterogeneity, thus providing an averaged picture of the DNA–DNA contacts mediated by DNA-binding proteins even though the binding is highly dynamic. The development of single cell genomic approaches could be a solution to such problems.



TABLE 1 Summary of the most studied structural factors and processes involved in 3D genome folding in bacteria.

Structural factor	Proteins or processes	Target	Bacterial model	References
Condensin Complexes	Smc-ScpAB	Global/chromosome	<i>C. crescentus</i>	Anchimiuk et al. (2021); Böhm et al. (2020);
			<i>B. subtilis</i>	Le et al. (2013); Liroy et al. (2020); Marbouty et al. (2015); Szafran et al. (2021); Tran et al. (2017); Wang et al. (2015, 2017)
	Smc		<i>B. burgdorferi</i>	Ren et al. (2023)
	MukBEF		<i>E. coli</i>	Conin et al. (2022); Liroy et al. (2018, 2020)
			<i>P. aeruginosa</i> <sup>a</sup>	
	MksBEF		<i>P. aeruginosa</i>	Liroy et al. (2020)
	MksB		<i>B. burgdorferi</i>	Ren et al. (2023)
Transcription	Intense transcription <sup>b</sup>	Local/chromatin	<i>C. crescentus</i> <i>B. subtilis</i> <i>C. glutamicum</i> <i>S. ambifaciens</i>	Böhm et al. (2020); Le et al. (2013); Le & Laub (2016); Liroy et al. (2018); Liroy, Lorenzi, et al. (2021); Marbouty et al. (2015); Val et al. (2016)
NAPs	HU	Local/chromatin	<i>C. crescentus</i>	Le et al. (2013)
		Global	<i>E. coli</i>	Liroy et al. (2018)
	H-NS	Local/chromatin	<i>E. coli</i>	Liroy et al. (2018)
	Rok	Local/chromatin	<i>B. subtilis</i>	Dugar et al. (2022); Marbouty et al. (2015)
	MucR	Global/chromosome (in Chr1) Local/chromatin (in Chr2)	<i>B. abortus</i>	Barton et al. (2023)

<sup>a</sup>MukBEF of *E. coli* was ectopically expressed in *P. aeruginosa*.

<sup>b</sup>Either of long transcriptional units or a long succession of genes.

## 5 | CONCLUSIONS AND PERSPECTIVES

Although the main structural layers and key organizers of bacterial chromosome organization are starting to emerge (Figure 2 and Table 1), the complete picture remains elusive. For instance, it is still unclear how the different structural layers are interconnected and which of the main factors are responsible for these connections. Addressing these challenging points will provide deeper insights into bacterial genome dynamics and plasticity, with implications for understanding bacterial evolution, pathogenicity, and antibiotic resistance. Currently, little is known about the importance of bacterial chromosome organization for the acquisition of mobile genetic elements and their regulation (Bobay et al., 2013; Lamy-Besnier et al., 2023; Ren et al., 2023). The interplay with the replication process (Conin et al., 2022) and with different bacterial lifestyles (Bury-Moné et al., 2023) are also poorly understood. In mammals, it is well established that chromosome spatial organization plays a key role in the regulation of gene expression (da Costa-Nunes & Noordermeer, 2023), however, whether the 3D folding of bacterial chromosomes plays a similar role in transcriptional programs remains undetermined. Undoubtedly, the development of the sub-kb resolution Hi-C method will start to shed light on this subject by disclosing the mechanism of formation of TIDs in bacteria. Experiments addressing the formation of TIDs in a physiological situation will certainly add new layers of knowledge in bacterial gene regulation. Furthermore, several studies have reported the existence of non-membrane-bound compartments, whose gel properties can locally

concentrate and/or impact the activity of cellular machineries, such as the ParB/*parS* system in *B. subtilis* (Guilhas et al., 2020). The significance of such compartments and/or transcriptional condensates in the formation of boundaries and/or the regulation of the activity of bacterial chromosome architects remains to be explored.

With respect to the diversity of the spatial organization of bacterial chromosomes studied so far, we can conclude that given the immensity of the bacterial world (Figure 1b), the molecular activities of only a small number of structural factors and biological processes have been characterized so far (Table 1). For instance, a recent study in which the genomes of the synthetic bacterial community stably associated within a gnotobiotic mouse line was analyzed (Lamy-Besnier et al., 2023), revealed that in three bacterial species (*Akkermansia muciniphila*, *Bacteroides caecimuris*, and *Muribaculum intestinale*) no homologs of condensin complexes could be identified, suggesting that homolog machineries (or processes) might be involved in the genome large-scale structuration of these bacteria. Overall, this study revealed novel principles of chromosome organization and highlighted the importance of considering a wide range of models when studying chromosome conformation. In this context, it would be interesting to explore genome organization in non-canonical situations such as bacteria whose nucleoids are surrounded by a membrane (e.g., *Gemmata obscuriglobus*) or organelles composed of DNA and ribosomes as in *T. magnifica* (Volland et al., 2022), to potentially unveil new factors involved in genome architecture.

Remarkably, the use of Hi-C revealed for the first time “communication” between replicons in bacteria with segmented genomes. In

*Agrobacterium*, ParB proteins evolved to promote a physical “communication” between replicons essential for genome stability (Ren, Liao, Barton, et al., 2022). Further research in *Agrobacterium* or in other bacteria with multipartite genomes will allow us to explore how these inter-chromosome “communications” coordinate the stability of the multiple replicons.

Growth conditions can impact chromosome organization, as illustrated by the significant changes observed during the differentiation of *Streptomyces* (Deng et al., 2023; Lioy, Lorenzi, et al., 2021; Szafran et al., 2021) (Figure 1b). Most studies have been conducted under optimal growth conditions, but in their natural environments, bacteria are likely to experience different growth conditions, which could alter the activity and interactions between the cellular machineries involved in genome organization. Therefore, conducting investigations under “more physiological” conditions, such as within biofilms or inside host tissues, may offer a more accurate understanding of chromosome organization.

In conclusion, the majority of the results obtained to date have revealed both common and specific rules in bacterial genome folding, suggesting that bacterial genome architecture is a consequence of its function. However, emerging evidence from bacteria with multipartite genomes indicates that genome folding may also play a determinant role in this function. The development of interdisciplinary (i.e., genomics combined with polymer physics, artificial intelligence, and *in vivo* biochemistry) and interspecies approaches will enable us to address this discrepancy and establish new principles of genome organization and evolution.

#### AUTHOR CONTRIBUTIONS

**Sokrich Ponndara:** Writing – review and editing; resources; visualization; investigation. **Mounia Kortebi:** Writing – review and editing. **Frédéric Boccard:** Writing – review and editing. **Stéphanie Bury-Moné:** Writing – review and editing; resources; visualization. **Virginia S. Lioy:** Conceptualization; funding acquisition; writing – original draft; writing – review and editing; visualization; resources; supervision.

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#### DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

#### ETHICS STATEMENT

No human or animal subjects were used in this review.

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