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Review

From structure to function of bacterial chromosomes: Evolutionary perspectives and ideas for new experiments

Marco Cosentino Lagomarsino [a], Olivier Espéli [b], Ivan Junier [c,d,e,*]

[a] Laboratory of Computational and Quantitative Biology, UPMC, CNRS UMR 7238, Paris, France
[b] CIRB-Collège de France, CNRS UMR 7241, INSERM U1050, Paris, France
[c] Laboratoire Adaptation et Pathogénie des Micro-organismes – UMR 5163, Université Grenoble 1, CNRS, BP 170, F-38042 Grenoble Cedex 9, France
[d] Centre for Genomic Regulation (CRG), Dr. Aiguader 88, 08003 Barcelona, Spain
[e] Universitat Pompeu Fabra (UPF), Barcelona, Spain

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ABSTRACT

The link between chromosome structure and function is a challenging open question because chromosomes in vivo are highly dynamic and arduous to manipulate. Here, we examine several promising approaches to tackle this question specifically in bacteria, by integrating knowledge from different sources. Toward this end, we first provide a brief overview of experimental tools that have provided insights into the description of the bacterial chromosome, including genetic, biochemical and fluorescence microscopy techniques. We then explore the possibility of using comparative genomics to isolate functionally important features of chromosome organization, exploiting the fact that features shared between phylogenetically distant bacterial species reflect functional significance. Finally, we discuss possible future perspectives from the field of experimental evolution. Specifically, we propose novel experiments in which bacteria could be screened and selected on the basis of the structural properties of their chromosomes.

1. Introduction

Similarly to their eukaryotic counterparts, bacterial chromosomes perform the complex task of efficiently compacting DNA while supporting gene regulation and proper DNA segregation. Chromosomes are thus shaped at multiple scales by a large number of proteins and DNA enzymes [1,2] (Fig. 1). At the smallest scale, DNA-binding proteins called nucleoid associated proteins (NAPs) participate in the local compaction of DNA (some of them are described as "histone-like" proteins) and in the regulation of specific genes [3]. At a larger scale, transcription, replication and the action of topoisomerases generate supercoiling in the DNA helix, which has been shown to be restricted to domains that range in length from a few kb to a few hundred kb [4,5]. At even larger scales, specific genomic regions on the order of Mb called macrodomains [6,7] confer compartmentalization properties to the chromosomes [8]. The bacterial chromosome is also highly dynamic, with active mechanisms and stress-relaxation systems that continuously modify chromosome conformations at all scales. These dynamic processes impact both the diffusion properties of individual loci and the cell-cycle trajectories of segregating chromosomal loci [9–12].

While the development of genetic tools and visualization techniques has accelerated the understanding of bacterial chromosome organization [18], the functional impact of the chromosome's three-dimensional structure is less well understood [19], largely because chromosome architecture alterations (the deletion of architectural genes or genome reorganization) have pleiotropic consequences. For example, DNA supercoiling can drastically enhance contacts between distant chromosomal loci [20]. Nevertheless, whether bacteria effectively use this type of long-range contacts to coordinate the expression of genes or to specifically regulate them, similarly to eukaryotic enhancers, remains an open question.

One way to address the relationship between structure and function is to perform a comparative analysis of chromosome-related properties across multiple species to identify and analyze conserved patterns [21,22]. As in protein function [23] and protein functional associations [24], these comparative analyses rely on the rationale that structural features that are conserved in

* Corresponding author at: Laboratoire Adaptation et Pathogénie des Micro-organismes – UMR 5163, Université Grenoble 1, CNRS, BP 170, F-38042 Grenoble Cedex 9, France.
E-mail address: ivan.junier@ujf-grenoble.fr (I. Junier).

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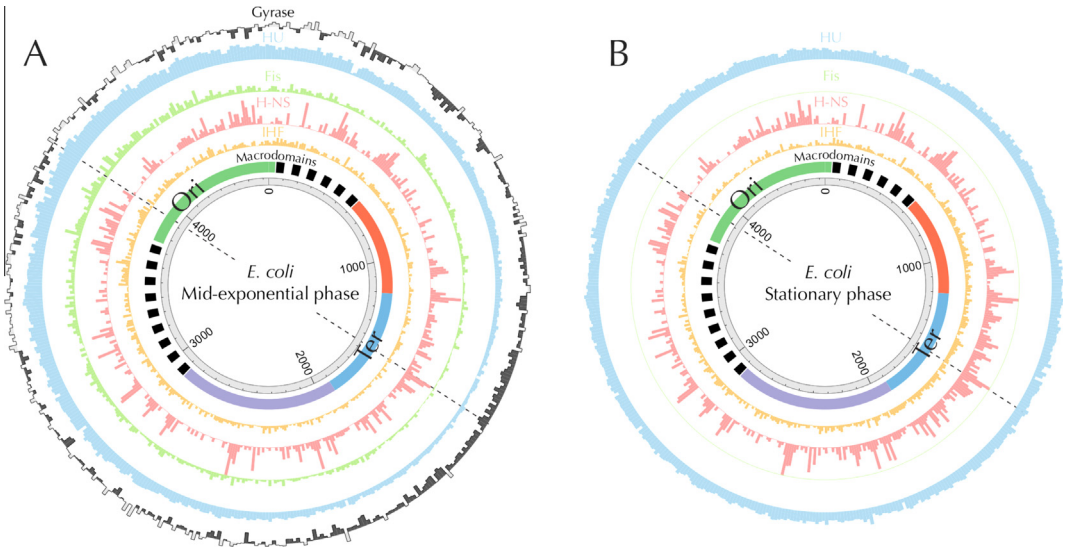

Fig. 1. Protein occupancy landscape of the *E. coli* chromosome. Distribution of the binding sites (bin size = 10 kb) for 4 NAPs (IHF, H–NS, Fis, and HU) in the exponential and stationary phases (data from [13,14]) and for gyrase in the exponential phase; for the gyrase, gray (black) values correspond to an enrichment (depletion) of binding obtained in a ChIP experiment [15]. Macrodomains [7,16] are represented in color in the inner circle; Ter and Ori are blue and green, respectively. Note the gradient of binding in the exponential phase for HU, Fis and gyrase from the origin of replication to the terminus (the origin-terminus axis is indicated by the dashed black line) (see [17] for further details). As shown in the right panel, the binding of Fis has not been detected in the stationary phase. For information regarding the conservation of these proteins, see Fig. 3B.

phylogenetically distant species are under selective pressure and, hence, highlight functional relationships. Compared to eukaryotes, bacteria (and fungi) also offer the possibility to perform evolutionary experiments on laboratory timescales, which can be "long-term" [25] or "short-term" in selective conditions [26,27], allowing functional analyses based on evolutionarily conserved properties to be implemented in the lab.

In this context, our goal here is to examine promising tools to delineate the relationship between structure and function in bacterial chromosomes. To this end, we first briefly introduce some key genetic, biochemical and visualization techniques that have been developed to uncover relevant properties of chromosome structural organization. Second, we discuss how the comparison of genome organization and chromosome structuring among phylogenetically distant bacteria can provide insights into the question. Following the evolutionary line, but with the goal of providing hints to experimentally investigate the relationship between structure and function, we finally propose novel experiments that aim at efficiently screening and selecting bacteria on the basis of the structural properties of their chromosomes.

2. Spatial structuring of bacterial chromosomes

Arguably the first structural insights into bacterial chromosomes came from the isolation of bacterial nucleoids to monitor their supercoiling using DNase treatment [28] and to visualize them using electron microscopy [29]. These experiments revealed that bacterial chromosomes are organized in vivo into *independent* domains of braided DNA (plectonemes) induced by the supercoiling of the DNA helix; evidence of independency came from the necessity of cutting DNA in multiple sites to decondense isolated nucleoids [28].

2.1. Genetic recombination assays reveal the domain organization of chromosomes at multiple scales

Genetic tools have complemented this static picture of the nucleoid by enabling the detection of structural events in vivo, providing evidence of DNA folding from a few kbp to the entire genome length (Fig. 2). Pioneering experiments measured the consequences of supercoiling on DNA folding in *Escherichia coli* by using site-specific recombination assays [30–32]. These techniques rely on the fact that DNA recombinases work as "topological filters", requiring substrates with a specific topology and producing products with a specific topology, too. These experiments revealed that approximately half of the supercoiling density is constrained to nucleoprotein complexes, while unconstrained supercoils form the other half. Researchers also corroborated that topological microdomains of supercoiling fold in vivo into plectonemic loops, rather than into solenoids [30], a finding that was eventually demonstrated a few years later using polymer physics approaches [20].

Next, studies in *Salmonella typhimurium* revealed that microdomain boundaries are frequent (every 10 kb) and dynamic [33]. This result was demonstrated by analyzing the efficiency of site-specific recombination between pairs of γδ transposase binding sites located at different positions within a specific region of the *S. typhimurium* genome. The stochastic nature of these boundaries was corroborated at a genome-wide level in *E. coli* several years later by monitoring the change in transcription activity of >300 supercoiling-sensitive genes under the activation of a rare-cutting restriction enzyme [4]. Microdomain boundaries have since been shown to result from multiple processes, such as transcription itself [34], the action of DNA gyrases [35,36], the bridging of transcription factors (TFs) [37], or the anchoring of DNA to large stable structures. The latter can occur *via* the binding of proteins or, for the expression of membrane proteins, *via* the tethering of RNA polymerases to the sites of protein translocation [38,39].

Because of its robustness and high dependency on the spatial distance between its attachment sites (attL and attR), λ recombinase was used to probe the 3D folding of the *E. coli* chromosome at a higher scale. This effort revealed the presence of hundreds of kb-long domains, also called macrodomains (Fig. 1), in which the frequency of contact between chromosomal loci is abnormally high, thus indicating a tendency for these genomic domains to define statistically separate "compartments" [7]. Genomic analysis then revealed that the macrodomain surrounding the terminus of replication (Ter) contains approximately 20 similar motifs (*matS*)

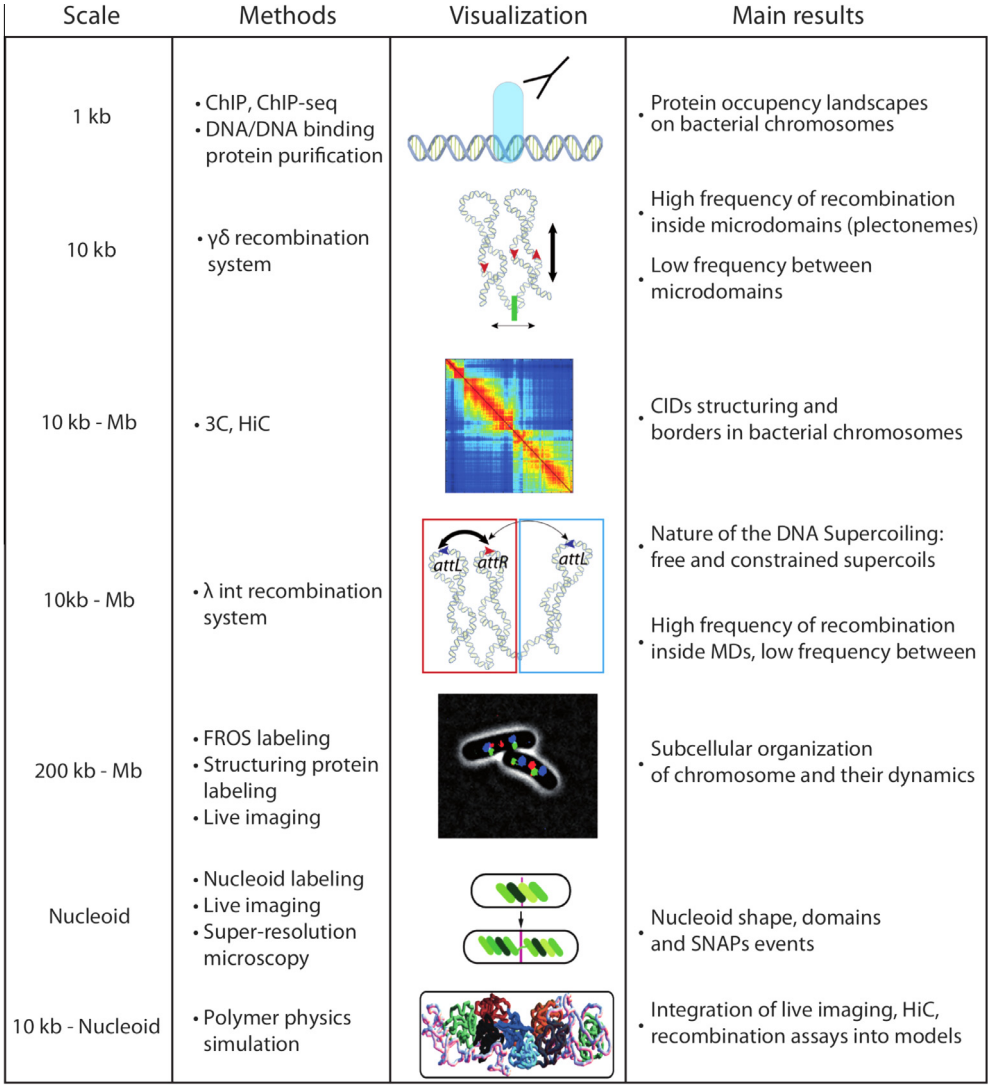

Fig. 2. Typical tools used for describing bacterial chromosome structures at length scales ranging from 1 kb to the whole nucleoid. Building a coherent picture that integrates in a unified model information at these multiple scales remains an open challenge.

absent from the rest of the genome [16]. ChIP, ChIP-seq and in vitro assays revealed that the *matS* sites were specifically bound by MatP. MatP proteins were also shown to tetramerize, strongly suggesting that a MatP–MatP DNA-bridging mechanism is responsible for Ter condensation [40]. The equivalent of *matS* sites has not been identified in other macrodomains, which are thus expected to rely on different structuring processes. For example, cellular and molecular biology experiments suggest that the structuring of the domain surrounding the origin of replication in *Bacillus subtilis* resembles that of *E. coli* and is related to the action of the *parS*/ParB/SMC system, which tethers the Ori region to the nucleoid edge [41–44]; this system is actually absent from the *E. coli* chromosome.

2.2. High-throughput biochemical methods reveal binding domains of nucleoid-associated proteins and generate interaction maps genome wide

NAPs play a crucial role in structuring the chromosome because they can bend, wrap and bridge DNA [3]. Knowledge of their operating modes has come from a combination of biochemistry, electron microscopy, AFM, single-molecule molecular tweezer experiments and the crystal structures of DNA–protein complexes. Information regarding their properties in vivo has been derived from the impact of mutations on their control of gene expression and, later, from the identification of their binding sites at a genome-wide level using high-throughput biochemical techniques. In particular, ChIP-seq revealed the binding sites of most NAPs [13,14,40,45,46]. Another ChIP-related technique revealed Extended Protein Occupancy Domains (EPODs) that cover >2 kb-long genomic regions [47], with transcriptionally silenced regions strongly enriched in the NAP H–NS and highly expressed regions strongly enriched in RNA polymerases. Integrating these binding data in the case of *E. coli* reveals strong variations between NAPs for the positioning of their binding sites (Fig. 1), suggesting that each NAP plays a specific functional role, e.g., the role of H–NS in silencing foreign genes [48,49]. Although the genomic patterns of certain NAPs such as H–NS are compatible both with the known macrodomain organization of chromosomes [50] and, at shorter scale, with their expected organization in topological microdomains [51,52], chromosome properties associated with these NAPs still remain poorly understood in vivo.

High-throughput techniques based on biochemical methods can also provide statistical information regarding the spatial

structure of chromosomes. The first step in this direction began with the development of the chromosome conformation capture (3C) method [53]. This technique allows the quantification of the frequency of interaction between any two genomic loci by ligating loci that are sufficiently close in space and by performing DNA sequencing. This method, together with its various improvements, has provided unprecedented detail concerning the "folding" of eukaryotic chromosomes [54]. In the case of bacteria, an early derivative of 3C-based experiments captured known features of chromosome interaction maps [55]. More recently, the HiC method, a high-throughput derivative of 3C [56], has revealed in *Caulobacter crescentus* a novel structural layer, which consists of a succession of tens of kb-long domains named "chromosomal interaction domains" (CIDs) (Fig. 2) [5]. CID boundaries frequently overlap highly expressed genes, with additional ectopic boundaries that may be created by the insertion of highly expressed genes [5]. Using various mutants, CIDs have been shown to be sensitive to supercoiling and NAPs. CIDs thus appear to lie between the topological microdomains that they contain and the macrodomains that contain them. In particular, compared to previous genetic analysis that detected approximately 400 *stochastic* microdomains in *E. coli* [4], only 23 CIDs that are *very stable* across the cell-cycle have been found in *C. crescentus* [5].

2.3. Fluorescence labeling techniques capture both dynamic and static properties of the nucleoid

In parallel with genetic and biochemical techniques, single-cell studies using advanced fluorescence microscopy techniques have revealed important dynamical features of nucleoid structuring. Such techniques employ either fluorescent tags inserted in the genome, as in fluorescent repressor operator sequences (FROS) [57], or fluorescent probes that are able to bind DNA, as in fluorescence in situ hybridization (FISH) [6]. In the former case, labels can be tracked dynamically, whereas in the latter case cells are fixed to allow probes permeating the cell walls, while preventing cell lysis.

The first insights into the cellular organization of the whole *E. coli* chromosome in vivo were thus obtained by FISH [6]. Labeling in vivo the entire chromosome of *E. coli* has further revealed a condensed structure that is significantly smaller than the cell volume and that tends to be chiral [58–61]. Labeling multiple chromosomal loci with tags of different colors has also revealed the specific sub-cellular organization of chromosomal loci depending on their coordinates along the genome [6,62–65]. In particular, the chromosome of *C. crescentus* has been shown to be longitudinally organized, with the origin and terminus of replication located at opposite poles of the cell and the position of chromosomal loci linearly correlating with their genomic position [66]. In slow-growing *E. coli*, the chromosome is transversally organized with the origin and terminus of replication located at the cell center, the chromosomal arms are located on each side of this transversal axis, and the spatial position of loci from each replichore recapitulates the genetic map [62–64]; at faster growth rates (when cell cycles overlap), *E. coli* chromosome organization is consistent with a model in which the nucleoid is a constrained ring polymer, and the replichores are partitioned in different territories along the radial axis [67]. Strikingly, similar analyses have revealed that slow-growing *B. subtilis* oscillates between two types of organization [44], one that is similar to the transversal organization of *E. coli* and one that is similar to the longitudinal organization of *C. crescentus*, suggesting the existence of a small number of nucleoid configurations that may be ubiquitous in the bacterial kingdom [12]). Live imaging, which consists in dynamically tracking fluorescent labels in non-fixed cells, further revealed a compartmentalization property of chromosomes in slow growing *E. coli*, with in particular a marked sub-diffusion law in macrodomains, and a motion close to Brownian dynamics in non-structured domains (black, dashed domains in Fig. 1) [8]. Dynamical studies in these cells have also revealed that the nucleoid does not segregate progressively but rather performs rapid active movements over short time periods [12] and displays abrupt internal loss of cohesion several minutes after initiation of replication [68].

The advent of super-resolution microscopy [69,70] has increased the spatial resolution of these techniques by breaking the diffraction barrier. In short, these techniques consist of stimulating the emission of fluorescent labels such that they emit light intermittently. Respective identification of the average centers of labels then allow meshing the cell volume in a more precise way than when using constantly emitting light sources, at the cost of a lower time resolution. These techniques have thus been used to quantify the sub-cellular localization of chromosomal DNA [71], the major NAPs (HU, Fis, IHF, Stpa, and H–NS) [72–74], RNA polymerases, ribosomes [75,76], and transcription/replication machineries [77].

3. Functional insights into chromosome structuring using comparative genomics

Although genetic, biochemical or "cell biology" experiments can provide insights into the structural properties of chromosomes, they are usually not designed to address the question of the function of the corresponding structures. Moreover, the exploration of the alterations to chromosome architecture through perturbations such as the deletion of architectural genes or artificially induced genome reorganization is complicated by the pleiotropic consequences of the perturbations.

Knowing that features that are shared between distant species generally reflect strong selective pressures and, therefore, functional significance, one possible method to circumvent these difficulties is to use evolutionary conservation as a proxy of functionality. In the case of bacteria, this principle is all the more robust, since the composition and organization of their genomes is well known to evolve quickly [78]. Therefore, just as the analysis of protein sequence has provided insight into the functional implications of protein folding [23], the analysis and comparison of genomic patterns among distant bacteria may provide insights into the functional impact of chromosome structure. Small DNA loops associated to the bridging of certain transcription factors (TFs) provide an inspiring example of such genomic footprints of functional structures (Fig. 3A). These loops are typically repressors that trap promoters so that to prevent the RNA polymerase from initiating transcription [79,80]. Importantly, TF-bridging can occur only in the presence of multiple binding sites properly positioned along DNA so that they can face each other in space (Fig. 3A). As a consequence, due to the helical structure of DNA, below 200 bp the thermodynamic stability of loops is a periodic function of the distance that separates the binding sites, with a repression level that can vary over two orders of magnitude and a typical period on the order of one helix turn [81,82]. From the perspective of comparative genomics, this implies that specific distances (modulo one helix turn) between binding sites associated to the formation of these functional loops are overrepresented.

3.1. Conserved chromosomal clustering of genes: toward a functional role for topological microdomains

Beyond the gene scale, the tendency for specific long linear genomic regions to be conserved on evolutionary time scales, a property termed "synteny" [87–89], suggests that the bacterial genome is organized into functionally distinct chromosomal domains that extend beyond operons [51,52,90–92]. Such

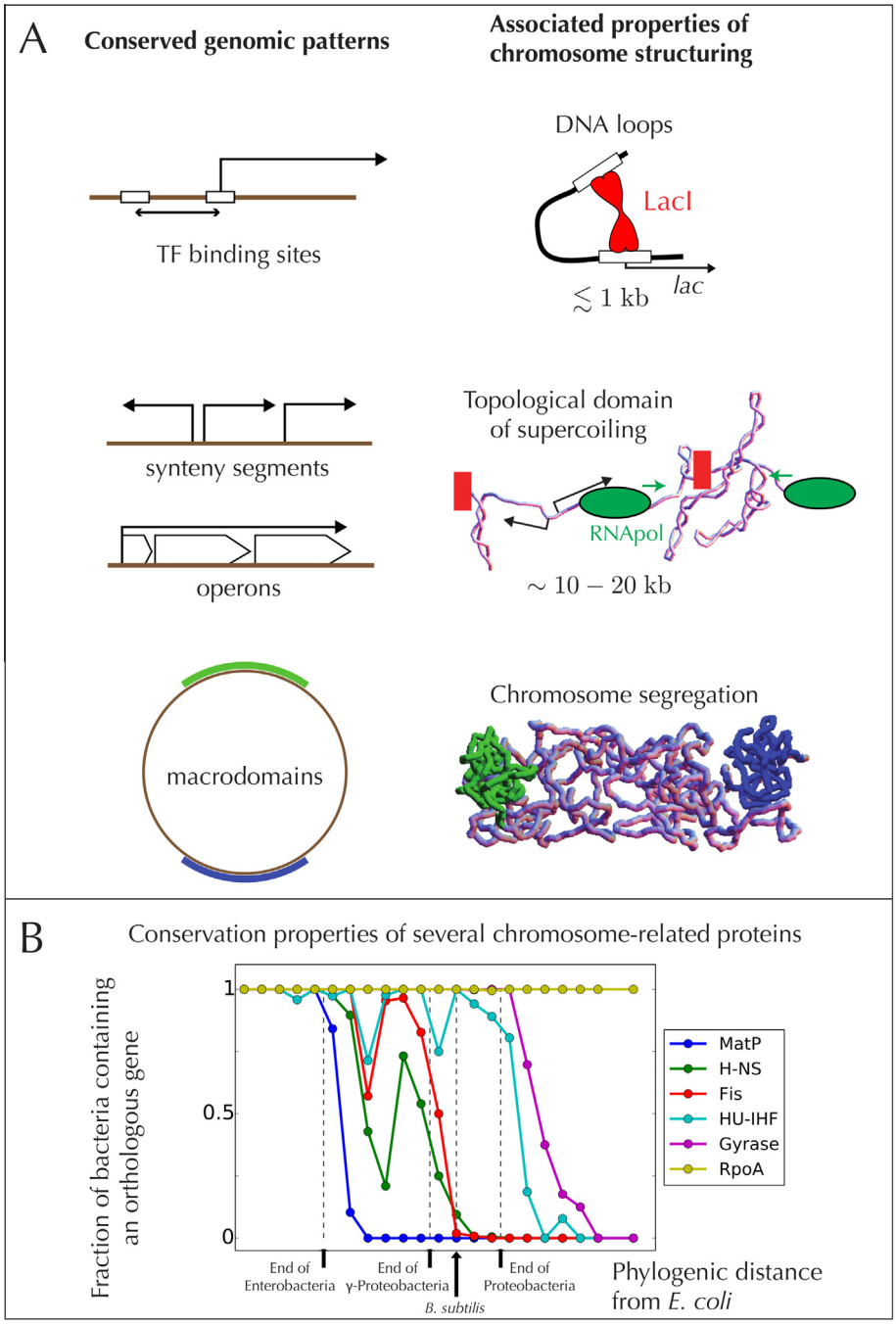

Fig. 3. Conserved genomic properties and their relationship with chromosome structuring. (A) The left column indicates genomic patterns that are conserved in phylogenetically distant bacteria. The right column indicates the structural features of the bacterial chromosome that are known or believed to be associated with these genomic patterns. *Top*: the helical structure of DNA locally constrains distances that separate multiple sites involved in DNA bridging [81,82]. *Middle*: at 10–20 kb scales, genes and operons gather in large clusters that are conserved across species, with a maximal size that is reminiscent of topological microdomains of supercoiling [83]. Insulation barriers (schematically indicated by the red boxes) can prevent supercoiling from diffusing along DNA and may thus efficiently prevent the undesired impact of transcriptional activity of one topological domain on its neighbors. At scales on the order of Mb, macrodomains of interactions close to the origin and terminus of replication appear to be highly conserved [6,7,16,84]. Their presence may be necessary to ensure the proper cellular organization of chromosomes [85]. (B) Conservation of the major NAPs and the gyrase that are found in *E. coli*; for comparison, we also indicate the conservation across all species of the RNA polymerase alpha subunit. Conservation was computed using the COG datasets for orthologous genes [86], by questioning whether at least one COG corresponding to the gene of interest in *E. coli* was present in other phylogenetically distant strains. Evolutionary distances were computed by considering 10 genes that are known to reflect phylogenetic distances between bacteria (as described in [52]).

functional organization of the genome can be observed for instance in the optimized organization of operons with respect to their participation in common biological pathways [92], or by the presence of long genomic regions harboring autonomous pathways that may be transferred horizontally between bacteria [91].

Interestingly, comparative analyses of genome organization further suggest the existence of a parallel between these functional domains and the structural units of organization described in the first part of this essay. First, an analysis of the sets of genes that tend to be present in the same genomes and to remain proximal

along DNA reveals that the conservation of strict chromosomal proximity is limited to groups under approximately 20 genes [83], a size that is reminiscent of topological microdomains. Next, conserved clusters of proximal genes have recently been shown to be preferentially bound at their border by H–NS [52], a highly conserved NAP (Fig. 3B) that has previously been connected to topological microdomains [47,51]. These clusters have also been shown to be enriched in pairs of highly co-expressed genes, and in pairs of genes that are arranged divergently along the genome [52], i.e., in pairs of adjacent genes whose transcriptional properties are known to be particularly sensitive to supercoiling [93–96]. Along the same lines, supercoiling has previously been shown to affect the transcriptional activity of genes beyond the operon scale [15]. Moreover, it is a key target of selection in evolving bacterial populations [97,98].

Altogether, and in accordance with the hypothesis that supercoiling represents a fundamental mechanism for the control of gene expression in bacteria [99,100], these observations suggest the existence of a strong relationship between topological microdomains (structure) and domains of co-regulated genes (function) (Fig. 3A) with, in particular, a role of supercoiling in the regulation of the genes whose proximity is conserved across evolution.

3.2. Conservation properties at large scales: coordinating DNA replication and chromosome segregation

At a larger scale, the conservation of both MatP and *matS* in enterobacteria (Fig. 3B) [16,84] strongly suggests that the condensation of the Ter macrodomain is functionally important for these bacteria. Similarly, the ubiquitous presence of ParB and *parS* in the bacterial world may be related to the presence of a condensed structure around the origin of replication, playing an important functional role. We also note that although the ParB/*parS* system is absent in the chromosomes of enterobacteria, a macrodomain (Ori) has been identified around oriC in *E. coli* [7] (Fig. 1), where it was first identified [6].

What could be the function of these condensed regions? Interesting insights come from biological studies of Ter in *E. coli*. Segregation defects observed in the absence of MatP indeed suggest that Ter structuring contributes to the efficient partitioning of DNA during cytokinesis [101]. MatP has also been shown to influence FtsK, a DNA translocase associated with the divisome, to specifically segregate Ter [102]. Altogether, these results suggest the existence of a subtle interplay between condensation properties and the active segregation of the terminus region that is at the core of the proper segregation of replicated chromosomes in *E. coli*. Likewise, polymer modeling of the bacterial chromosomes indicates that chromosomes without condensed regions are poorly segregated during their replication [85,103]. In contrast, the presence of macrodomain condensation may generate important organizational forces that can efficiently segregate chromosomes [85].

3.3. The promise of comparative analyses of genomic loci contact maps

One powerful comparative approach to address the functional impact of chromosomes would be to identify folding patterns that are conserved among distant bacteria. 3C-related techniques [53,54] offer this possibility. Indeed, these methods allow the generation of "standardized" contact maps of bacterial chromosomes [5,55,77,104], which can be compared directly. To date, only a single (approximately 10-kb) high-resolution map has been produced in bacteria, for *C. crescentus* [5]. However, enormous potential for this approach was recently demonstrated with a metagenomic 3C method that enabled the simultaneous generation of (low-resolution) contact maps for four different bacteria: *B. subtilis*, *E. coli*, *Vibrio cholera* and *Aeromonas veronii* [104]. Despite the low resolution of these contact maps, CID-like interaction domains can be distinguished in every matrix (Fig. 4). One can then speculate that some of these CIDs may be conserved among bacteria, similarly to the so-called topologically associated domains that have been identified in eukaryotes [105–107], in which case they might correspond to genuine functional units of bacterial genomes.

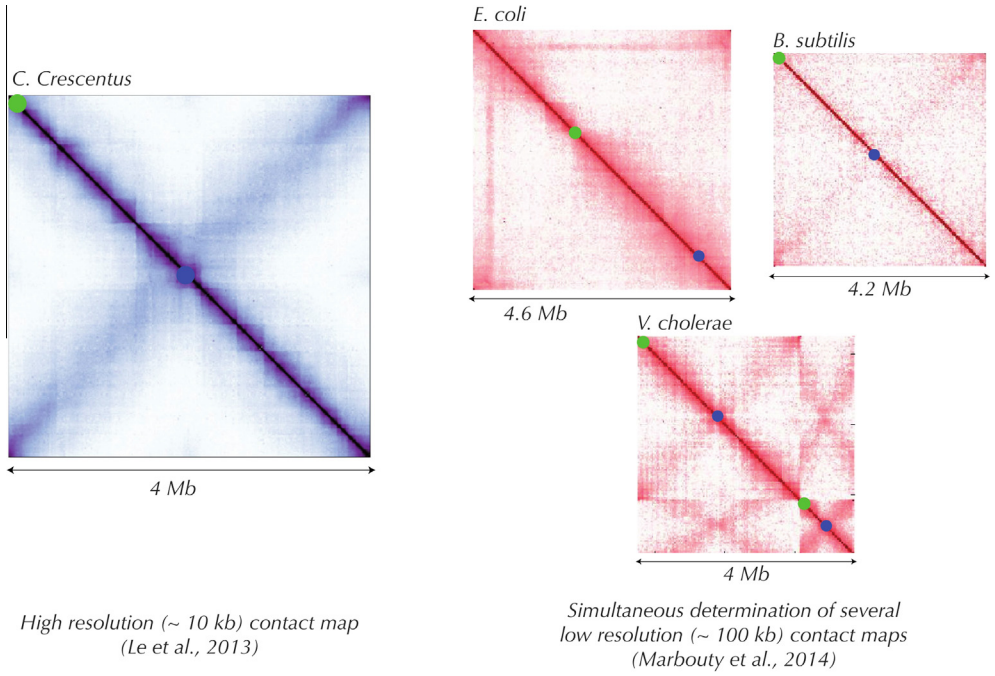

Fig. 4. Comparative analysis of contact maps. The high-resolution HiC contact map of *C. crescentus* shows the presence of stable chromosomal interaction domains (CIDs) [5]. Similar interaction domains can be distinguished in a set of low-resolution maps that were simultaneously obtained using a metagenomic analysis [104]. One can also distinguish the presence of a second diagonal in *C. crescentus*, *B. subtilis* and *V. cholerae* (two chromosomes), in agreement with their longitudinal organization [5].

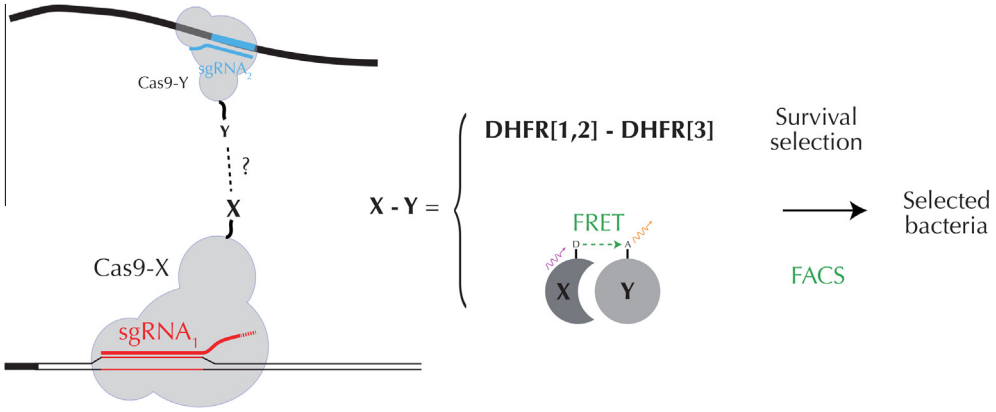

Fig. 5. A possible PCA-Cas9 assay to select bacteria on the basis of physical contacts between pairs of genomic loci. On the one hand, two short guide RNAs ($sgRNA_1$ and $sgRNA_2$) are used to recognize two specific genomic loci (in red and blue) distantly situated along the genome. On the other hand, the Cas9 protein, which is modified to have no endonuclease activity, is used in two different fused systems. Each fused system contains one moiety (X or Y) of a reporter protein (X–Y) that can confer either survival to the bacteria, as in a DHFR system [110], or fluorescence, as in a FRET system [111]. Both cases enable the selection of bacteria that have brought X and Y (i.e., the two genomic loci) into contact. See [109] for a more general discussion about the engineering potential of CRISPR-Cas9 systems.

4. Outlook: exploiting bacterial selection to experimentally address the relationship between structure and function

The most direct way to address the link between structure and function would undoubtedly be to directly modify structures and to analyze the phenotypic impact of such modifications. However, performing such direct manipulations is, in most cases, impossible. Here, we propose that high-throughput screening could bypass this problem, at least in part, by allowing an efficient selection of strains on the basis of their structural properties. To this end, in analogy with devices that select bacteria for their ability to develop drug resistance [27], one would need to develop a selection device that is sensitive to structural properties. This would obviously raise important experimental challenges. Nevertheless, we believe that these are conquerable with current technologies.

The most important challenge is the development of selectable reporters for chromosome structural properties. Consider, for instance, the problem of a selection that depends on the contact between two arbitrary genomic loci. One possible solution to this problem is to adapt a protein-fragment complementation assay (PCA). In a classic PCA assay, two proteins of interest (targets) are genetically linked to two moieties of an enzyme (reporter) that can confer resistance to the bacterium. Because the reporter is fully assembled only when the two target proteins interact, the fraction of surviving bacteria after an antibiotic treatment provides quantitative information regarding the interaction energies between the two proteins [108]. We surmise that this assay may be naturally transposed to the problem of the contact between two genomic loci. To this end, instead of considering two target proteins, we propose to consider a unique target that can recognize two genomic loci. For example, the CRISPR-Cas9 system [109] with two different short guide RNAs (sgRNAs) could be used to direct the target, a modified Cas9 with no endonuclease property [109], to the genomic loci (Fig. 5). The modified Cas9 would then be used in two different fused systems, each one containing one reporter moiety (Fig. 5). A possible protocol would then consist of three stages: (i) activation of the CRISPR-Cas9-PCA system, (ii) antibiotic treatment, and (iii) sequencing of the survivors. By definition, survivors should be enriched in genomes in which the interaction between the two loci is favored because such contact would bring together the reporter moieties. Using a repertoire of sgRNAs covering the entire genome could actually lead to alternative, complementary methods to 3C-related technologies.

A similar protocol could then be implemented to evolve bacteria toward specific structural properties that occur at larger scales, e.g., having a stronger (or weaker) Ter compaction. Indeed, one could use a similar PCA (with sgRNAs associated with the Ter domain in this case) but with a FRET system as the reporter (Fig. 5), a fluorescence-based system that is particularly efficient in locating interacting proteins [111]. One could then select bacteria using fluorescence-activated cell sorting (FACS). By "designing" bacteria with specific structural properties, it should then be possible to investigate the fitness and phenotypes associated with these properties and to precisely characterize the actors that are involved.

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