Mini Review

DNA Supercoiling: an Ancestral Regulator of Gene Expression in Pathogenic Bacteria?

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DNA supercoiling acts as a global and ancestral regulator of bacterial gene expression. In this review, we advocate that it plays a pivotal role in host-pathogen interactions by transducing environmental signals to the bacterial chromosome and coordinating its transcriptional response. We present available evidence that DNA supercoiling is modulated by environmental stress conditions relevant to the infection process according to ancestral mechanisms, in zoopathogens as well as phytopathogens. We review the results of transcriptomics studies obtained in widely distant bacterial species, showing that such structural transitions of the chromosome are associated to a complex transcriptional response affecting a large fraction of the genome. Mechanisms and computational models of the transcriptional regulation by DNA supercoiling are then discussed, involving both basal interactions of RNA Polymerase with promoter DNA, and more specific interactions with regulatory proteins. A final part is specifically focused on the regulation of virulence genes within pathogenicity islands of several pathogenic bacterial species.

Contents

1. Introduction

DNA supercoiling (SC) has received considerable attention in recent years as a global and ancestral actor in genetic regulation. This is especially conspicuous in bacteria [1–3], where the chromosome is maintained at an out-of-equilibrium level of negative SC by a finely controlled balance of topoisomerase activity. And yet, in contrast to

classical regulation based on transcription factors, quantitative models of the regulatory mechanisms by SC are essentially lacking. A possible explanation for this shortcoming is that SC affects transcription at several stages of the process, and can also be involved in various and complex interactions with regulatory proteins. As a result, virtually every investigated promoter exhibits a distinct SC response, making it difficult to dissect and model the underlying mechanisms. In this review, we wish to summarise existing evidence and models suggesting a widespread role of SC in bacterial genetic regulation, and more specifically in bacterial virulence. This topic has already been addressed in previous extensive reviews focused either on its role in bacterial growth [4] or on specific promoters that were analysed in detail [5]. Here, we propose a complementary focus on proposed mechanistic and computational models of transcriptional regulation by SC as well as accumulating information obtained from transcriptomic data, which together underline the broad relevance of the investigated phenomenon in bacterial virulence and call for a combined experimental-theoretical research effort.

2. DNA Supercoiling: A Global Regulator of Bacterial Gene Expression

2.1. DNA Supercoiling: A Relay of Environmental Signals to the Bacterial Chromosome

As observed immediately following the discovery of the double-helical structure of DNA, virtually all DNA transactions face substantial topological constraints [6]. In mechanical terms, the latter give rise to a ubiquitous torsional stress, which in turn results in DNA supercoiling (SC), i.e., the deformation of the molecule either by rotation around its helical axis (over- or under-twisting) or by the winding of this helical axis itself (writhing), as illustrated in Fig. 1 [4,7].

Topoisomerases are the global regulators of SC and more generally, the solvers of topological problems associated with DNA transactions [8]. In bacteria, the two main topoisomerases are topoisomerase I (topo I) and DNA gyrase. The latter maintains the chromosomal DNA in an underwound state by introducing negative supercoils in an ATP-dependent manner, while conversely, topo I relaxes the DNA (i.e. removes negative supercoils) without any ATP requirement. The global negative SC level of the chromosome is thus primarily determined by the dynamic equilibrium between these two enzymes (Fig. 1). Additional actors play a more specific role: the ATP-consuming topoisomerase IV is primarily involved in solving topological problems associated with DNA replication and cell division [9], and abundant nucleoid-associated proteins (NAPs) contribute in distributing SC along the bacterial chromosome [4,10].

The negative SC level of the chromosome is finely controlled by the cell in response to environmental conditions, since almost all types of environmental challenges have been associated with SC variations, and in particular those most commonly encountered by pathogens
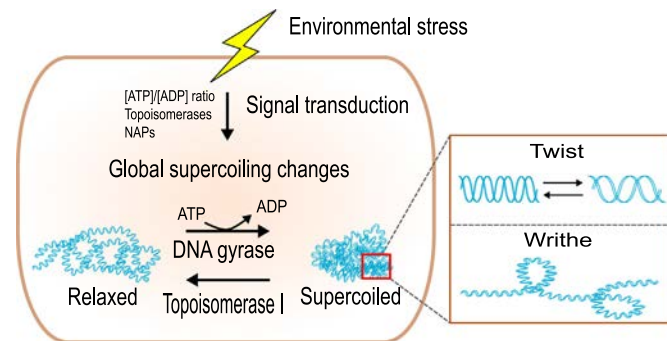

Fig. 1. DNA supercoiling acts as a sensor of environmental stress in the bacterial chromosome. Environmental cues are transduced by different mechanisms into global, stress-specific variations of the SC level. At a smaller scale, SC is distributed as twist and writhe deformations, which directly affect the transcriptional activity.

Table 1
Chromosomal supercoiling response to environmental stress conditions is conserved in distant bacterial species. Phyla: P: Proteobacteria, F: Firmicutes, A: Actinobacteria. SC variations: Rel (+): relaxation, Hyp (−): hyper-supercoiling.

Shock	Phylum	Species	SC change	Mechanism	Ref
Heat	P	*Escherichia coli*	Rel (+)	Gyrase and topoI activities	[12]
		Yersinia enterocolitica		Gyrase activity decrease	[13]
		Dickeya dadantii			[14]
	F	*Bacillus subtilis*			[15]
Cold	P	*Escherichia coli*	Hyp (−)	Gyrase activity increase / HU	[16]
	F	*Bacillus subtilis*			[15]
Acidic	P	*Escherichia coli*	Rel (+)		[17,18]
		Salmonella typhimurium		Gyrase activity decrease	[18,19]
		Dickeya dadantii			[20]
Osmotic	P	*Escherichia coli*	Hyp (−)	[ATP]/[ADP] increase	[21]
		Salmonella typhimurium			[22]
		Dickeya dadantii			[23]
	F	*Bacillus subtilis*			[15]
		Staphylococcus aureus			[24]
	A	*Streptomyces lividans*			[25]
Oxidative	P	*Escherichia coli*	Rel (+)	TopA activation by Fis	[26]
		Dickeya dadantii			[23]
Anaerobic	P	*Escherichia coli*	Rel (+)	[ATP]/[ADP] decrease	[27]
		Salmonella typhimurium			[28]
	F	*Bacillus subtilis*			[15]

during infection (Table 1). Depending on the applied stress, the chromosomal DNA experiences either a partial relaxation (+) or an increase in negative SC (−), which is usually rapid and transient. Importantly, in spite of strong differences in terms of phylogeny or lifestyle, the response to each specific stress is qualitatively similar in all investigated species, although these exhibit quantitatively different SC levels in standard growth conditions [3]. This observation suggests that SC is used in a wide range of bacteria to quickly transduce environmental signals toward the chromosome, with ancestral control mechanisms. Interestingly, environmental stresses have also been correlated with SC changes in archaeal species [11], suggesting that this notion could be extended to an even wider range of microorganisms.

What are the underlying mechanisms? The most clearly described pathway involves the modulation of gyrase activity by the energy charge of the cell through the [ATP]/[ADP] ratio [21,27,29]. When the latter is increased, gyrase introduces supercoils in the chromosomal DNA more actively and its negative SC level increases. Environmental stresses usually alter cellular metabolic fluxes and the energy charge in various ways [30], and this relatively simple, quick and general mechanism is indeed involved in the chromosomal response to a variety of conditions (Table 1). Other invoked mechanisms include (1) the action of NAPs (e.g. HU, FIS) either by direct interaction with DNA or as modulators of topoisomerase activity; (2) the regulation of topoisomerase expression (*topA*, *gyrA* and *gyrB* genes encoding topoI and gyrase subunits respectively); (3) the modulation of topoisomerase activity through post-translational modifications [31].

It should be underlined that most bacteria from Table 1 are pathogenic or have pathogenic serotypes. Since the infection process can be assimilated to successive environmental changes to which the pathogen needs to adapt quickly, SC appears as a general candidate for the efficient transmission of stage-specific environmental signals toward the bacterial chromosome and thus also for its

transcriptional response, which in turn is critical for the subsequent steps of the infection.

2.2. Global Transcriptional Response to Variations in DNA Supercoiling

The transcriptional response to various stress conditions can be readily analysed from transcriptomic data, but it is then difficult to disentangle (1) the generic effect of the stress-induced SC variation on transcription and (2) the transcriptional effect of stress-specific pathways. The former contribution can however be analysed separately in transcriptomes obtained with gyrase inhibiting antibiotics. These are either aminocoumarins (novobiocin, coumermycin) which block the ATPase activity of class-II topoisomerases (gyrase and topoisomerase IV), or quinolones (ciprofloxacin, norfloxacin, nalidixic acid, oxolinic acid) which block their ligase activity, the latter resulting in many double-strand breaks and triggering a SOS response of the cell with pleiotropic effects [32]. When applied at a sublethal dosage, these drugs induce a sudden global relaxation of the chromosomal DNA, and the transcriptional response is then measured after a short time (usually 5–30 min), assuming that the latter then mostly reflects the direct effect of SC, rather than indirect effects influenced by the cell's response. Such data were obtained in many organisms (Table 2). Note that the reported number of affected genes is strongly variable; this variability might partly reflect actual differences between organisms, but is strongly affected by the experimental conditions and methods (relaxation level, transcriptomics technology, statistical analysis). Altogether, these data consistently demonstrate a very broad response to chromosomal relaxation, with a significant effect on more than one quarter of the genes. This response is complex, with some genes being upregulated and others downregulated. These affected genes are functionally diverse, including genes involved in essential functions (e.g. DNA replication, cell division), stress responses and metabolic pathways (e.g. stringent response, DNA repair pathway), as well as virulence. They are also usually scattered throughout the chromosome, highlighting that SC-mediated regulation acts in a global way, but follows a spatial organisation pattern involving large-scale responsive domains related to structural properties of DNA [20,33].

Since this global transcriptional response is observed in a wide range of species from different phyla (Table 2), SC might be considered as an ancestral and widespread mode of regulation in bacteria. This notion can be related to the fundamental and highly conserved character of topoisomerase enzymes themselves [8], and may even be extended to eukaryotes, albeit with different rules [7,34]. It does not mean however that the mechanism is identical in all bacteria. The longest-running evolution experiment [35] emphasized that mutations affecting SC

constitutes a "quick and efficient" way to modify the global expression pattern and gain substantial fitness, in this case by a mutation reducing topo I efficiency in less than 2000 generations [36]. It is therefore no surprise that fluctuations in topoisomerase structure and SC level were pointed in the close relatives *E. coli* and *S. typhimurium* [3] which have different lifestyles, and this is probably also frequent in different strains of the same species [19]. Changing the chromosomal SC level is thus a fundamental and generic way by which bacteria adapt to new environmental challenges, according to common ancestral rules. This extends in particular to genome-reduced bacteria almost devoid of TFs such as *Mycoplasma* or *Buchnera* [37–39], where transcriptional regulation remains poorly understood.

3. Mechanisms and Models of Transcriptional Regulation by DNA Supercoiling

The abovementioned transcriptional responses induced by SC variations differ qualitatively from those induced by classical transcriptional factors (TFs). The latter recognize, bind and regulate a specific subset of the genome defined by a well-defined (although often degenerate) target sequence motif, and their action can be modelled using classical thermodynamic models of activation or repression [55]. In contrast, as noted above, SC affects the transcriptome of all investigated species globally, without any identified promoter sequence determinant. And yet strikingly, regulatory models comparable to those involving TFs are essentially lacking. There are two reasons for this: first, experimentally, SC regulates gene activity in a continuous "more or less" manner as opposed to the stronger "on or off" mode of regulation by TFs [56]; second, SC can modulate transcription in a variety of ways, making them difficult to decipher. In the following, we discuss such mechanisms, most of which will be illustrated on the promoter of *pelE*, one of the major virulence genes of the phytopathogen *Dickeya dadantii* where SC-mediated regulation was studied extensively.

3.1. Basal Regulation of the RNA Polymerase-DNA Interaction

The ancestral and global mode of regulation by SC results, in the first instance, from it affecting the interaction between DNA and RNA Polymerase (RNAP) itself, independently from any additional regulatory protein. But this basal regulation already involves distinct mechanisms occurring at successive steps of the complex process of bacterial transcription: closed-complex formation, open-complex formation, promoter clearance, and elongation [57].

The most clearly identified – and possibly strongest - effect of SC on transcription initiation results from the requirement for RNAP to open

Table 2

Transcriptomic response to variations of DNA supercoiling in bacteria. Phyla: P: Proteobacteria, F: Firmicutes, A: Actinobacteria, T: Tenericutes, C: Cyanobacteria. SC variations: Rel (+): relaxation, Hyp (−): hypersupercoiling. Transcriptomics technology: M: DNA Microarray, S: RNA Sequencing

Phylum	Species	SC change	Method	Genes significantly affected (% genome)	Technology	Ref
P	*Escherichia coli*	Rel (+)	Norfloxacin	613 (15%)	M	[40]
		Rel (+)	Novobiocin / pefloxacin	1957 (48%)	M	[41]
		Rel (+)	Genetic engineering	740 (18%)	M	[42]
		Rel (+)	Genetic engineering / norfloxacin / novobiocin	306 (7%)	M	[43]
	Salmonella typhimurium	Rel (+)	Genetic engineering	499 (10%)	M	[44]
	Dickeya dadantii	Rel (+)	Novobiocin	1461 (32%)	M	[20]
		Rel (+)		1212 (27%)	S	[45]
	Haemophilus influenzae	Rel (+)	Novobiocin / ciprofloxacin	640 (37%)	M	[46]
F	*Streptococcus pneumoniae*	Rel (+)	Novobiocin	290 (14.2%)	M	[47]
		Hyp (−)	Seconeolitsin	545 (27%)	S	[48]
	Staphylococcus aureus	Rel (+)	Novobiocin	280 (11%)	M	[49]
	Bacillus subtilis	Rel (+)		1075 (24%)	M	[50]
A	*Streptomyces coelicolor*	Rel (+)	Novobiocin	121 (1.5%)	S	[51]
	Mycobacterium tuberculosis	Rel (+)		Not provided	M	[52]
T	*Mycoplasma pneumoniae*	Rel (+)	Novobiocin	469 (43%)	S	[37]
C	*Synechocystis*	Rel (+)	Novobiocin	Several genes	M	[53]
	Synechococcus elongatus	Rel (+)		Not provided	M	[54]

the DNA strands and stabilize a "transcription bubble", in order to gain access to the DNA bases in the template strand. In torsionally unconstrained DNA, this melting transition represents a substantial free energy cost of around 10 k_BT (6 kcal/mol), which in eukaryotes is provided through ATP hydrolysis by the basal transcription factor TFIIH [34]. Crucially, this cost reduces drastically when the double helix is destabilised by negative torsion at SC levels physiologically relevant in bacteria (Fig. 2A, upper panel), thus providing the physical basis for the bacterial transcription process that does not require any external energy consumption [57]. Interestingly, the same can become true of eukaryotic RNA Polymerase II when operating on a comparably negatively supercoiled template [34], whereas temperature may replace SC as the source of melting energy in thermophilic archaea relying on reverse gyrase [58,59]. This strong regulatory effect of SC can be observed in well-controlled in vitro transcription assays (Fig. 2A, lower panel), where plasmids carrying a model promoter are prepared at different supercoiling levels, resulting in a drastic variation of expression strength without any modification of the DNA sequence or regulator concentration. This activation curve is quantitatively reproduced [45] using thermodynamic models of DNA opening [60–62] relying on knowledge-based enthalpic and entropic parameters for base-pairing and stacking interactions of all base sequences.

How may this mechanism lead to transcriptional regulation, i.e., the selective activation of a subset of promoters by global SC variations? The opening curve is strongly dependent on the promoter base sequence (mostly though its GC content) in the −10 region and that immediately downstream referred to as the "discriminator" [4,63–65]. Remarkably, although the latter region does not harbour any consensus binding signal for RNAP, mutation studies showed that it plays a predominant role in the SC-sensitivity of promoters: GC-rich discriminators are typically more activated by negative SC than AT-rich ones, the canonical example being those of stable RNAs strongly induced during exponential growth [4,64,65]. And yet, a systematic model of this regulation mechanism is still lacking. A possible obstacle is that the thermodynamic description used above might be insufficient, if the expression level of investigated promoters is limited not by the rate of promoter opening but of promoter escape. As an example, some promoters might attract RNAP and easily form an open-complex, but still exhibit low expression levels

if the latter is too stable, resulting in abortive rather than processive transcription [4,66,67]. Since the influence of SC on these subtle kinetic steps was not dissected in detail, little can be predicted from such a scenario that may explain why promoters respond differently to SC variations [68], and some even in opposite ways [69–71]. Yet we still note that many in vitro investigated promoters do follow the behaviour expected from thermodynamic modelling (Fig. 2A, lower panel), which might thus account for at least a significant fraction of transcriptomic responses to SC variations.

A regulatory action of SC was proposed at least at two other transcription steps. RNAP binds promoters by recognising the −10 and −35 elements, which are separated by a spacer of variable size (15–20 nt, with an optimum at 17 nt). Because of the helical structure of DNA, these variations are associated to different relative angular positions of the recognition sites around the helical axis, possibly placing them out-of-phase for RNAP binding and closed-complex formation. A suitable (spacer length-dependent) level of torsional stress could then modulate this binding rate by untwisting the spacer DNA toward a favourable orientation [73,74]. This scenario was invoked to explain the response of several promoters to SC variations, including ribosomal RNAs which usually exhibit a suboptimal spacer length of 16 nt [73].

SC may also affect transcription at the elongation stage, where positive torsion hinders the progress of RNAP, as demonstrated from single-molecule experiments [75]. Such positive SC levels are not observed in vivo by usual techniques involving plasmids, but might be transiently generated locally downstream of transcribed genes (see paragraph 3.3 below). However, most *E. coli* genes were found to be transcribed at a comparable speed [76], suggesting that positive supercoils are readily eliminated by topoisomerases and do not play any regulatory role during elongation of these moderately expressed genes, whereas mechanical stalling of RNAP might occur at the most strongly expressed genes such as those of ribosomal RNAs [77].

3.2. Specific Regulation Involving DNA Regulatory Proteins

SC affects transcription via RNAP itself, but also via promoter-specific regulatory proteins (TFs). In classical thermodynamic regulation models [55], the latter act (as activators or repressors, depending
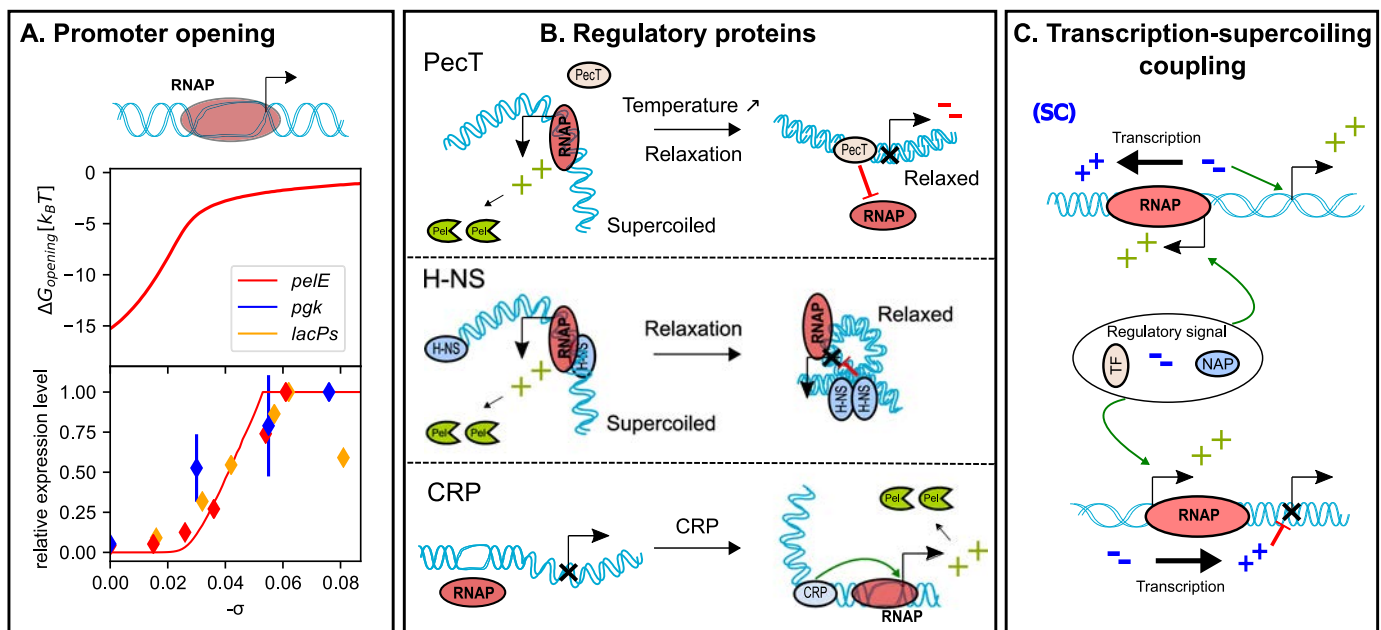
Fig. 2. Mechanisms of transcriptional regulation by DNA supercoiling. (A) Opening free energy of the *pelE* promoter of *D. dadantii* (upper panel) and expression level of three promoters from different species (lower panel, solid line for the model prediction) [23,45]; (B) Various mechanisms involving SC and NAPs/TFs at the *pelE* promoter [14,23,72]; (C) Local SC-mediated regulatory interaction: transcription from a given gene (left) can activate (top) or repress (bottom) its neighbour depending on their relative orientation [45].

on their action on RNAP activity) at distinct binding sites characterised by their affinity, the latter being inscribed in the genomic sequence and usually described by sequence motifs. In this simplistic view, the cell has limited regulatory freedom, since the only tunable parameter is the TF concentration. In the classical example of the global regulator CRP which binds (and usually activates) hundreds of promoters in *E. coli*, increasing its concentration (or rather that of its cofactor cAMP) may activate many of these target promoters, but in this model, the *relative* amount of CRP bound at these promoters, and thus also their relative activities, is out of the control of the cell. This view is increasingly challenged since additional layers of complexity were identified, including epigenetic modifications of the regulators or promoter DNA and post-transcriptional regulation. However, we note that many regulatory proteins recognize not only the base sequence, but also the DNA shape, a mechanism often referred to as indirect readout [78]. In that case, the activity of classical regulators is intrinsically modulated, not only by chemical (epigenetic) modifications requiring dedicated enzymes, but also by the ubiquitous mechanical deformations. A computational estimation of this recognition mode suggests that, in the case of CRP, the latter may in fact be the strongest determinant of its loose sequence selectivity [79]. Crucially, in contrast to the classical "static" sequence-motif model, this selectivity is now dependent on the mechanical state of chromosomal DNA, and thus subjected to cellular control through SC. Considering that the SC distribution itself is non-uniform and locally affected by NAPs, this additional mechanical dimension is probably a key contributor in the complexity of the binding selectivity of regulatory proteins.

This mechanism has not been investigated experimentally in many systems. One example is the thermoregulation of *pelE* by the repressor PecT, which is achieved not by a change in regulator concentration but rather by an increase of its binding affinity for the promoter resulting (at least partly) from temperature-induced chromosomal DNA relaxation (Fig. 2B, top) [14,80]. PecT belongs to the LysR-like family of TFs relying on indirect sequence readout, which is the largest TF family in enterobacteria [81], and similar mechanisms are used by other families [81–83]. Altogether, the role of DNA mechanics in TF binding selectivity is thus likely much more important than generally considered, although the precise mechanisms remain to be identified and modelled. Because most dimeric TFs are smaller than RNAP, they are probably less sensitive to the untwisting of bound DNA than the latter [79], but this sensitivity might be augmented substantially in the case of several binding sites arranged in helical phase. For example, H-NS was found to repress *pelE* on relaxed DNA (as most of its targets), but it is not the case on negatively supercoiled DNA [23]. A proposed explanation is that H-NS traps RNAP in a small loop at this promoter by bridging its two binding sites, which in the latter condition is prevented by their unfavourable helical phasing (Fig. 2B, middle). On the other hand, regulatory proteins also exert twist deformations themselves; a spectacular example is MerR, which in the presence of Hg_{2+} binds and untwists the 19-bp spacer of the mercury resistance operon of Tn501 (a transposable element isolated from *Pseudomonas aeruginosa*), thus enabling RNAP binding [84].

In our opinion, the interplay between regulatory proteins and local SC may mostly rely on 3D modifications of the promoter conformation, i.e., writhe rather than twist. On the one hand, writhe could facilitate the formation of loops required for many regulatory interactions. These loops are favoured by the distinct mechanical properties of promoter DNA sequences [85], and were already included in regulatory models based on the transfer matrix formalism [86]. SC-dependent reduction of looping free energies can thus strongly modify the binding landscape of regulatory proteins, as already described [87,88]. Conversely, many regulatory proteins induce strong bends in DNA [83], e.g., CRP (~90°), LexA (~35°), as well as the NAPs FIS (~45°) and IHF (~180°). Since such deformations drastically reduce the energetic cost of DNA loops [89,90], they are expected to displace the twist-writhe equilibrium within the bound region in favour of the latter, and may thus induce local topological changes similar to those induced by SC [91]. In

summary, just like the NAPs are involved in a complex double-sided interaction with SC that shapes the global structure of the chromosome, a comparable effect probably occurs with many more regulatory proteins at the more local scale of gene promoters, with direct consequences for local transcription.

A final important ingredient is the widespread occurrence of structural transitions in genomic DNA. The latter can switch from double-stranded B-DNA to, among others, denatured, Z-DNA, G-quadruplex or cruciform states. The rates of these different transitions can be computed by thermodynamic modelling [92], and depend not only on the DNA sequence, but also on torsional stress that destabilises B-DNA and can be accommodated more favourably in alternate states. It was also shown that these transitions occur predominantly at bacterial gene promoters [60] which they regulate according to various mechanisms, some involving TFs. Denatured AT-rich regions located 50–200 bp upstream of the TSS can act as "sinks" for negative SC and impede the proper opening of the promoter by thermodynamic competition. This was shown to occur for *pelE*, which in vitro is not expressed in absence of CRP due to such upstream strand opening; when present, CRP not only favours the correct binding of RNAP, but also "closes" the upstream AT-rich tract, possibly by bending DNA (Fig. 2B, bottom) [23]. Since denaturation bubbles are extremely flexible, they may also strongly facilitate the formation of loops [89] required by TFs [86]. Additionally, some regulatory proteins may selectively bind non B-DNA regions, as occurs at the mammalian oncogene *cMYC* where negative SC triggering DNA melting is provided by adjacent transcription [60,93]. Finally, since rho-independent termination of transcription involves RNA hairpin structures, SC might also favour structural transitions in the DNA template itself at the transcription termination site, which could then modulate the termination rate, as already observed for the B-Z transition [94]. Since transcriptional read-through was highlighted as a widespread feature in bacterial genomes in recent years [95], an additional underestimated layer of regulation might thus also occur at this later stage of the transcription process.

3.3. Spatial Heterogeneities of DNA Supercoiling: The Transcription-Supercoiling Coupling

The intimate relationship between SC and transcription is not single-sided. In the elongation step, the helical structure of DNA imposes a fast rotation of the bulky RNAP relative to it (around two turns per second), but this movement is strongly hindered by the viscosity and crowding of the surrounding medium, resulting in an asymmetric accumulation of torsional stress from back to front, as recognised more than 30 years ago [96]. This phenomenon thus leads to an intrinsic dynamical coupling between SC and transcription highly dependent on gene orientations (Fig. 2C). This coupling regained significant interest in recent years since it was shown to underpin transcriptional bursting in bacteria [97], i.e., the nonlinear auto-induction of a promoter that can typically give rise to phenotypical heterogeneities among isogenic populations of cells [98]. Several theoretical models were proposed [45,77,99–101], most of them focusing on biophysical properties of transcription. A strong obstacle for their application in genomic regulation is the lack of experimental knowledge of the distribution of SC along a bacterial chromosome. A promising method involving the intercalating agent psoralen was developed in eukaryotes [7,102] but did not yet provide high-resolution data in bacteria [1]. Recently, indirect information was provided from binding distributions of topoisomerases obtained by ChIP-Seq at different resolutions [2,103]. These data, together with a systematic analysis of bacterial transcriptomes, confirmed that the distribution of SC along the chromosome is highly heterogeneous and strongly affected by gene orientations [104], leading to a fine-tuned and ancestral regulation of promoters depending on their genomic context [45]. In summary, the *local* level of SC experienced by a given promoter can strongly differ from the *global* (average) level of the chromosome, and depends on the orientation and activity of adjacent

genes, providing a strong mechanistic basis for the co-regulation of co-localised operons [105]. Direct evidence for these effects was obtained from experiments involving supercoiling-sensitive promoters inserted on the chromosome in different artificial configurations [70,104]. Comparable evidence is more difficult to establish for native promoters, but was highlighted in at least two examples of divergently organised operons: the *ilv* promoters of *E. coli* [106] and the *leu-500* promoter of *S. typhimurium* [107]. Interestingly, in these two examples, the local nature of SC (generated by the divergent genes) is combined with a complex regulatory mechanism involving DNA binding proteins. In the first example, the activation of *ilvP_G* is prevented by the denaturation of an upstream AT-rich tract, except when the NAP IHF closes that region and favours the opening of the promoter (like CRP at the *pelE* promoter, Fig. 2B). The pattern is similar for *leu*-500, although the repression is here achieved by H-NS binding at an AT-rich tract, and relieved by the TF LeuO in presence of locally generated negative SC [5,108]. Since divergent genes involved in the same function and simultaneously expressed are commonly found in bacteria, including among those involved in pathogenicity (see below), these examples may only be the first of a large unexplored class.

The high density of bacterial genomes implies that the interaction between neighbouring genes could in fact give rise to a collective behaviour along larger distances, forming "topological domains". Indeed, when promoters were displaced over the chromosome, their expression and supercoiling sensitivity were found to change depending on their location and neighbouring activity [109–112]. These domains, shaped by transcription and architectural proteins, remain a poorly defined notion in bacteria. Proposed lengthscales vary from 10 to 20 kb [109], to 50 kb [112] and up to hundreds of kilobases [20]; while the former may underpin an extension of the notion of operons [105,113], the latter probably reflect a higher order folding of bacterial chromatin involving different actors, and this hierarchical organisation remains to be characterised.

4. DNA Supercoiling and the Coordination of Virulence Programs

4.1. An Argument for DNA Supercoiling Being an Important Actor in Virulence Genetic Regulation

Most pathogenic bacteria exhibit close genomic proximity to non-pathogenic strains, with differences located at well-defined genomic regions (of a few up to hundreds of kilobases in size) called pathogenicity islands (PAIs), which contain the virulence genes involved in pathogenesis. These regions are harboured either on the chromosome or on plasmids, and are usually acquired by horizontal gene transfer (transformation, conjugation or phage-mediated transduction). As a result, different strains of a single species can present a remarkable diversity of pathogenic phenotypes (more than 10 for *Escherichia coli*), whereas a given virulence factor can be shared between different species [114]. This mechanism explains the rapid evolution of bacterial pathogens,

but also raises the question as to how the transferred genes are properly expressed after their integration into the distinct transcriptional regulatory network of the recipient cell. This problem is particularly acute for the bacterium, since any error in the expression time or strength of virulence factors immediately leads to the recognition and, ultimately, to the destruction of the invader by the host defence system [115,116].

At first glance, such drastic regulation of a few specific promoters seems to deviate from the global and non-specific regulation mode characteristic of SC. However, it appears equally incompatible with the sole action of strongly sequence-specific TFs, which would then be highly unstable during horizontal transfers between species, where these TFs are often evolutionarily distant [117]. As a matter of fact, many TFs involved in virulence indeed exhibit a weak sequence-specificity and are sensitive to the mechanical state of DNA [78], owing to an original regulatory mechanism affecting PAIs. Like other horizontally transferred regions, these usually exhibit a lower GC-content than the chromosomal average, and are therefore normally repressed by extensive binding of the NAP H-NS. Regulators can then activate the genes without any specific contact with RNAP, by competing with H-NS for promoter binding [114,118], which can be strongly dependent on the topological state of the region.

In most investigated species, the key signals triggering a quick activation or repression of virulence genes are precisely those environmental stress conditions that were shown to modulate the chromosome topology in various species (Table 3), e.g., a sharp acidity variation when *S. enterica* is transferred from the stomach to the intestine, or oxidative stress when *D. dadantii* leaves the plant apoplast. It is therefore no surprise that virulence genes from an increasing number of zoopathogenic or phytopathogenic species were shown to be directly regulated by SC, as summarized in Table 3. Does this mechanism play a role during the infection process, as these data suggest? In our opinion, based on the complex regulatory mechanisms illustrated above, SC is a good candidate to play the role of a basal and robust coordinator of virulence gene expression, by (1) modulating the simultaneous action of many (more specific) regulators at virulence promoters, such complexity being a characteristic feature of the latter, (2) co-regulating the adjacent genes of a PAI through the evolutionarily conserved transcription-supercoiling coupling. We present below some examples supporting this hypothesis, keeping in mind that existing results mostly concern individual genes, whereas the topological organisation of entire PAIs and its effect on their expression remain poorly understood [129].

4.2. Widespread Evidence for a Regulatory Role of DNA Supercoiling in Virulence

Salmonella enterica is one of the most studied pathogens, and this is also true of the regulation of its virulence system by SC (mostly in C. Dorman's laboratory). Several key virulence genes within its two largest PAIs (SPI-1 and SPI-2) are supercoiling-sensitive, as well as those of the

Table 3

Virulence genes are regulated by DNA supercoiling in various pathogenic species. Phyla: Proteobacteria (P), firmicutes (F), actinobacteria (A). Lifestyles: F = facultative, I = intracellular, E = extracellular, P = pathogen. Response to chromosomal DNA Relaxation by antibiotics: repressed (−) or activated (+), with corresponding reference(s).

| Phylum	Family	Species	Tissue	Lifestyle	Stress encountered	Gene involved in virulence	Relax response	Ref
P	Enterobacteriaceae	*Salmonella enterica*	gastrointestinal tracts	FIP	acid	*hilD, hilC,ssrAB*	+	[91]
	Enterobacteriaceae	*Shigella flexneri*	intestinal epithelium	FIP	temperature	*virF*	+	[119]
	Enterobacteriaceae	*E. coli (EHEC)*	intestinal epithelium	FIP	temperature	*espADB*	−	[120]
	Pectobacteriaceae	*Dickeya dadantii*	plant apoplast	EP	acid,oxidative	*pelE*	−	[23]
	Pseudomanadaceae	*Pseudomonas syringae*	plant apoplast	EP	oxidative	*avrPphB*	+	[121]
	Vibrionaceae	*Vibrio cholerae*	small intestine	EP	acid	*acfA, acfD*	−	[122,123]
	Alcaligenaceae	*Bordetella pertussis*	lung epithelial cells.	IP	temperature	*ptx*	−	[124]
	Campylobactericeae	*Campylobacter jejuni*	digestive tract	IP	temperature, pH	*momp*	−	[125]
F	Staphylococcaceae	*Staphylococcus aureus*	respiratory tract, skin	EP	osmolarity	*spa, eta*	+	[126,49]
	Streptococcaceae	*Streptococcus pneumoniae*	respiratory tract, skin	EP	oxidative	*fatD*	−	[127]
A	Mycobacteriaceae	*Mycobacterium tuberculosis*	respiratory tract, skin	IP	oxidative	*virR, sodC*	−	[128]
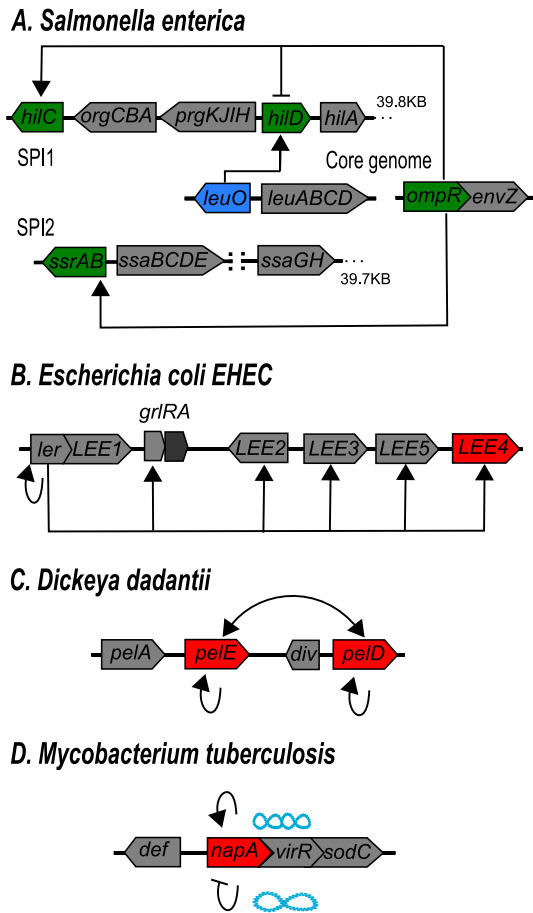
Fig. 3. Regulatory networks within several pathogenicity islands. In all species, key virulence genes are either relaxation-activated (green), relaxation-repressed (red), or regulated via the transcription-SC coupling (blue). Arrows indicate an activating (arrow) and bars a repressing effect. For *M. tuberculosis*, this effect depends on the SC level (repression on a relaxed template, activation on a supercoiled template). In *D. dadantii*, *pel* genes are self- and mutually-inductive via pectin degradation relieving the repression of both genes by the TF KdgR.

central virulence regulators OmpR and LeuO located in the core genome (Fig. 3A). In this representative example, key virulence functions were thus integrated into the pre-existing transcription regulatory network, whereby OmpR and LeuO, primarily devoted to other functions (the former is an abundant NAP-like protein), are specifically recruited at virulence promoters in a SC-dependent manner [5,91]. Interestingly, these regulators mostly target divergently oriented operons, and the same pattern is observed in *Vibrio cholerae* (*tcpPH*) [78] and *Shigella flexneri* (*icsA*, *icsB*) [117]. While gene orientations are indifferent to classical regulatory models, divergent promoters are the ones most sensitive to the transcription-supercoiling coupling [45], which is also involved in LeuO recruitment at its own (divergent) promoter [108]. Gene orientations within the PAIs are thus likely not accidental, but rather reflect the evolved infrastructure of a local coordinated and SC-coupled gene expression.

The virulence of *E. coli* EHEC (causing severe diarrhoea) depends on a secretion system encoded in the LEE operons (Fig. 3B), including many genes shared with other *E. coli* pathogenic strains, e.g. EPEC [114]. These operons are globally repressed by H-NS, whose binding is antagonised by the activator Ler encoded in LEE1 [118]. Ler itself belongs to the H-NS family and this competition may very well be affected by the topological state of the domain. Although such an effect was not investigated in detail, the expression of LEE4 was indeed found to be SC-sensitive [120], and the same could be true of the other operons.

In the phytopathogen *D. dadantii*, pectinolytic enzymes are the main virulence factors, responsible for the soft rot symptoms [115]. These are encoded by *pel* genes scattered in several PAIs along the chromosome [20]. *pelE* and *pelD*, the two major members of this family, are paralogous genes that evolved from a unique ancestor but exhibit different expression patterns [130]. They are regulated by several NAPs (H-NS, FIS, IHF, Lrp) as well as many TFs (e.g., CRP, KdgR, PecT, PecS) but are also among the most SC-sensitive genes in the chromosome. We illustrated above how different regulatory proteins act in a complex combination, with SC modulating their relative affinities (Fig. 2B). Although no effect of locally generated SC was directly shown here, a divergent non-coding transcript (*div*) was recently identified upstream of the *pelD* promoter, which "feeds" the latter with RNAP with a strong dependence on the 3D conformation of the promoter [130], possibly involving a local transmission of SC (Fig. 3C).

Finally, since topoisomerases are conserved among all bacteria, we may expect SC to play a role in the virulence of widely distant species. This was indeed recently demonstrated in the actinobacterium *Mycobacterium tuberculosis*, where the gene of a new NAP (NapA) was identified in the same operon as major virulence factors/regulators (SodC, VirR) [128]. Interestingly, NapA autoregulates its (divergently oriented) promoter in a SC-dependent manner (Fig. 3D): in this case, global SC variations induced by environmental conditions may act as a switch, turning the operon on or off with high specificity.

5. Conclusion

The discussed examples show that SC plays a direct role in the regulation of virulence in many species, albeit with a remarkable variety of mechanisms involving a combination of additional regulatory actors, and these mostly remain to be characterised. A final and striking example is the remarkably small and poorly characterised tenericute *Mycoplasma pneumoniae*, which is able to infect the human respiratory tract despite being almost devoid of TFs; the ancestral regulatory action of SC is thought to play an even more cardinal role in this case [37]. The increasing interest in SC in the genetic regulation community already results in new experimental techniques facilitating the mapping of supercoil distributions at higher resolution [1,2] as well as the development of computational models at various scales of detail [45,77,101,131], which together will help elucidating the pivotal regulatory action of SC in bacterial genetic regulation advocated here.

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