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Opinion

CRISPR-Cas, DNA Supercoiling, and Nucleoid-Associated Proteins

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In this opinion article we highlight links between the H-NS nucleoid-associated protein, variable DNA topology, the regulation of CRISPR-*cas* locus expression, CRISPR-Cas activity, and the recruitment of novel genetic information by the CRISPR array. We propose that the requirement that the invading mobile genetic element be negatively supercoiled limits effective CRISPR action to a window in the bacterial growth cycle when DNA topology is optimal, and that this same window is used for the efficient integration of new spacer sequences at the CRISPR array. H-NS silences CRISPR promoters, and we propose that antagonists of H-NS, such as the LeuO transcription factor, provide a basis for a stochastic genetic switch that acts at random in each cell in the bacterial population. In addition, we wish to propose a mechanism by which mobile genetic elements can suppress CRISPR-*cas* transcription using H-NS homologues. Although the individual components of this network are known, we propose a new model in which they are integrated and linked to the physiological state of the bacterium. The model provides a basis for cell-to-cell variation in the expression and performance of CRISPR systems in bacterial populations.

Highlights

A novel proposal is made for the operation of CRISPR-Cas systems based on the abilities of physiologically responsive DNA topology, nucleoid-associated proteins, and conventional transcription factors to operate in combination to set and to reset the bacterial transcriptome.

Our model exploits knowledge that CRISPR-Cas systems are dependent on DNA supercoiling for efficient operation, and that their expression and expansion are sensitive to the activities of nucleoid-associated proteins, especially H-NS and integration host factor (IHF).

We describe the mechanisms by which DNA supercoiling and nucleoid-associated proteins influence the adaptation, the expression, and the interference stages of CRISPR-Cas function.

A stochastic regulatory switch, based on the mutually antagonistic activities of the H-NS and LeuO proteins, drives cell-to-cell variation in CRISPR-*cas* transcription within bacterial populations.

Bacterial Defence against Foreign DNA

Bacterial cells employ a defence-in-depth strategy to limit the potential damage that is associated with the horizontal transfer of DNA. Foreign DNA can arrive through bacteriophage-mediated transduction, via conjugation involving self-transmissible plasmids, or by uptake of naked DNA by competent bacterial cells [1,2]. Among the hurdles that incoming DNA must overcome are restriction barriers imposed by endonucleases that can distinguish between 'self' and 'non-self' DNA, transcription-silencing proteins that prevent the immediate expression of newly-acquired genes, and clustered regularly interspaced short palindromic repeats (CRISPR) systems that target DNA incomers with CRISPR-associated (Cas) proteins that are guided by short RNAs derived from an archive of genetic signatures from previously-encountered invaders [3] (Figure 1).

The Principal Operational Steps of CRISPR Systems

CRISPR-*cas* systems have the potential to protect bacteria from horizontally acquired DNA that is imported in plasmid form or that infects the cell following injection by a bacteriophage [4–9]. They do this by identifying the target DNA as foreign and then destroying it. (We are not concerned here with those CRISPR systems that target foreign RNA). Identification is achieved using a memory bank of DNA sequences that is derived from invader DNA that the system has encountered in the past. These 'spacer' sequences form a linear array that is transcribed as a precursor CRISPR RNA (pre-crRNA). This transcript is processed into spacer-length segments (crRNA) that guide the Cas proteins to the newly arrived invader DNA [4,8,10,11]. These events represent the three distinct steps in the operation of CRISPR-Cas systems: spacer integration into the CRISPR array (adaptation), CRISPR-Cas expression, and foreign DNA interference (Figure 2).

DNA Supercoiling and R-loop Formation

The CRISPR guide crRNA recognizes its target (a 'protospacer' that is accompanied by a protospacer-adjacent motif, or PAM) marking it for the attention of the Cas proteins by base-pairing with the complementary DNA sequence within an R-loop [12]. Because PAMs are present in target molecules and not in the CRISPR arrays, making their presence a requirement for target recognition avoids CRISPR auto-destruction. R-loop formation in the target molecule requires one DNA strand to be displaced to allow the other to pair with the complementary crRNA sequence [13]. This process is more likely to occur in negatively supercoiled DNA than in DNA that is either relaxed or positively supercoiled

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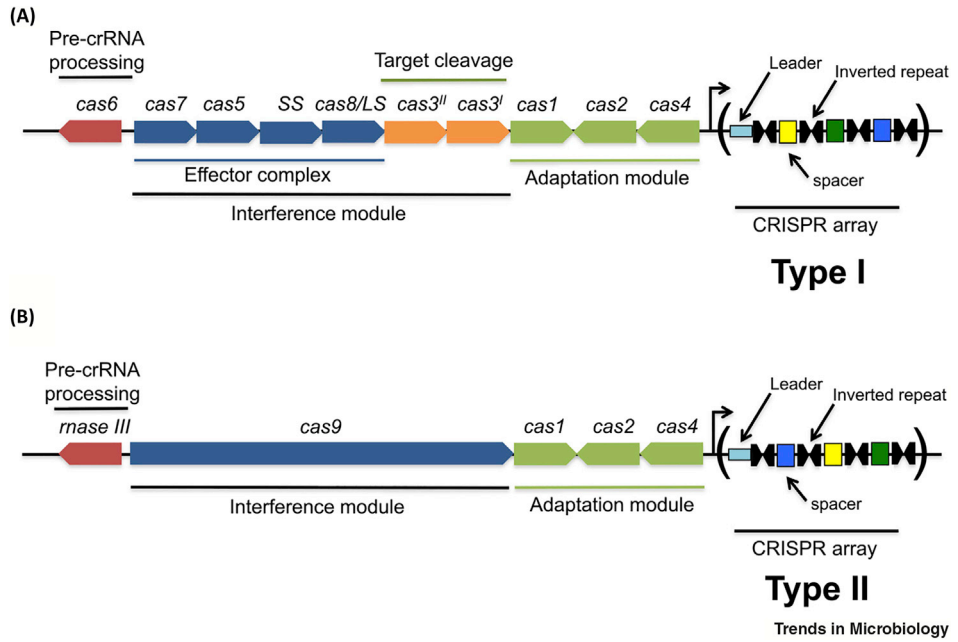

Figure 1. CRISPR-*cas* Structures.

Summaries are provided for class I (A) and class II (B) CRISPR-*cas* genetic loci. The main distinction lies in that many interference-related functions are found among a set of Cas proteins in class I systems while these are combined in a single protein (e.g., Cas9) in class II. The two classes are further subdivided into a series of types, creating quite a complex picture [93,94]. The spacer sequences that make up the memory of CRISPR systems are in the arrays at the right. Here, each spacer is separated from its neighbours by copies of an inverted repeat; adaptation proteins integrate the spacers. Transcription of the array occurs from a common promoter (represented by the angled arrow), and the resulting pre-crRNA is processed to generate the crRNAs that will be used by the interference system to locate a complementary sequence in an invading mobile genetic element (typically a bacteriophage or a plasmid) (see Figure 2 for details). Abbreviations: LS, large subunit; SS, small subunit. Not to scale.

[14–16]. The underwinding of the duplex in negatively supercoiled DNA encourages strand separation and facilitates the entry of the guide RNA. Factors that enhance or inhibit DNA underwinding might be expected to influence the R-loop formation at CRISPR targets, just as they influence R-loop formation in other scenarios [17,18] (Figure 2).

DNA Supercoiling and Bacterial Physiology

DNA in bacterial cells is maintained in a negatively supercoiled state, but the degree to which the DNA duplex is underwound changes throughout the growth cycle, with DNA in cells undergoing exponential growth being more negatively supercoiled than DNA in stationary-phase cells [19,20]. Changes to the linking number of DNA have consequences for the operations taking place in the DNA, such as replication fork movement and transcription initiation, elongation, and termination (Box 1). These processes also influence the supercoiling of the DNA at a local level, contributing to phenomena such as transcription bursting [21,22]. A snapshot of DNA topology in a particular genomic neighbourhood will reveal a level of supercoiling that is the outcome of the actions of topoisomerases working on a DNA topological landscape that is set and reset by DNA-based processes. The activities of the ATP-dependent type II topoisomerases DNA gyrase and topoisomerase IV will also respond to the [ATP]/[ADP] ratio in the cell, which is a reflection of metabolic flux. In this way, the degree to which DNA is underwound is a reflection of metabolism and will change with the physiological circumstances of the bacterium (Box 1). We now propose that the operation of CRISPR-Cas systems is networked with this physiologically sensitive system.

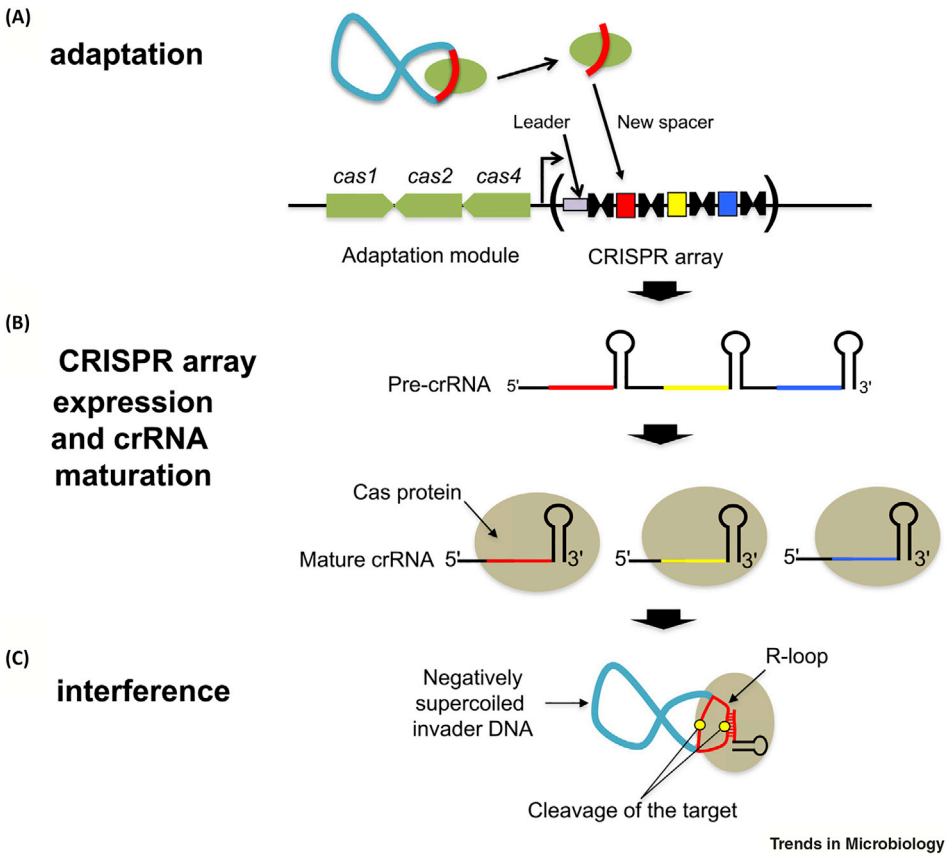

Figure 2. The Stages in CRISPR-Cas Immunity.

The three stages of CRISPR-mediated immunity are adaptation (A), expression and maturation of the CRISPR (cr) RNA (B), and interference with the invading mobile genetic element (C) [3]. In the adaptation stage, the proteins of the adaptation module (green) select and remove a section (red) of the invading mobile genetic element for integration at the leading end of the CRISPR array. Successful integration (A) requires that the CRISPR DNA is negatively supercoiled or appropriately bent by the integration host factor (IHF) protein [46,48]. Transcription of the array (B) produces the long pre-crRNA, and the Cas proteins process this precursor to generate the mature crRNA guides. These form an RNA:DNA hybrid in an R-loop in the negatively-supercoiled invader DNA molecule by base-pairing with their complementary sequence in the DNA (C). This marks the invader for destruction by the Cas nuclease activity.

Regulation of CRISPR-Cas Systems

The LeuO LysR-type transcription factor is a tetrameric DNA-binding protein found in many Gram-negative bacteria where it influences the expression of many genes, including its own gene, *leuO* [23]. LeuO is a positive regulator of CRISPR promoters [24,25]. In the case of the *leuO* promoter, the LeuO protein acts as an antirepressor to prevent encroachment by the H-NS nucleoid-associated protein, a pervasive silencer of transcription in Gram-negative bacteria [26]. H-NS is also a silencer of CRISPR promoters, as is its paralogue, StpA [27,28]. The mutually antagonistic activities of LeuO and H-NS [24,29] provide a basis for a genetic switch whose operation is controlled through the supply of LeuO; H-NS is present throughout growth and will bind to its genomic targets unless this interaction is prevented, reversed, or remodelled [30]. In the switch that we envision at CRISPR promoters, H-NS binds to a nucleation site near the target promoter, polymerizes along the DNA until the H-NS polymer intrudes into the promoter sequence, excluding RNA polymerase and preventing transcription initiation. LeuO counteracts H-NS polymerization by erecting a barrier between the H-NS nucleation site and the target promoter. This barrier is composed of the tetrameric LeuO protein bridging two

Box 1. DNA Supercoiling in Bacteria

DNA in bacterial cells is maintained in an underwound state due to a deficit in linking number, the number of times that one DNA strand in the duplex crosses the other, compared with fully relaxed DNA [54–56]. This deficiency introduces torsional stress into DNA, and the molecule seeks to adopt a minimum energy conformation through the writhing of the axis of the already-coiled DNA duplex ('supercoiling'), by loss of DNA twist through local unzipping of the two DNA strands, or some combination of the two [57]. The movement of the replisomes during chromosome replication [58,59] and the process of transcription elongation during gene expression [22,60–67] generate positive and negative DNA supercoiling at a local level. The type II topoisomerase DNA gyrase introduces negative supercoiling using an ATP-dependent DNA cleavage-and-double-stranded-DNA passage mechanism; it also eliminates positive supercoils by the same mechanism. Negatively supercoiled DNA is relaxed by the type I topoisomerases topo I and III and the ATP-dependent type II topoisomerase, topo IV. A homeostatic balance between these supercoiling and relaxing activities is thought to keep the average linking number of DNA in the genome within limits that are appropriate for DNA metabolism [68]. However, DNA supercoiling varies with the growth cycle: bacteria in the lag and the stationary phases of growth have DNA that is, on average, more relaxed than DNA in bacteria undergoing exponential growth [20,69,70]. This is thought to reflect a link between metabolic flux, the [ATP]/[ADP] ratio, and the activity of DNA gyrase. Environmental shocks, such as thermal, osmotic, acid, nutritional, oxidative stress, anaerobic stress all produce changes to the linking number of bacterial DNA that are accompanied by changes to the expression of genes involved in responding to those environmental insults [71–92]. Thus, DNA topology is a barometer of cellular health, with rapidly growing and nongrowing cells being distinguishable by the superhelicity of their genetic material.

LeuO binding sites at the exit of a DNA loop that is created by the LeuO protein [26,31]. A competition between H-NS and LeuO for access to their respective binding sites provides the basis for the switch: each would have to rebind following passage of a chromosome replication fork or other protein-displacing event, and this rebinding would be stochastic. Intervention by LeuO at CRISPR promoters will depend on the available supply of this protein, which in turn reflects the expression pattern of the *leuO* gene, which is under complex, multifactorial control [28,31,32] (Figure 3).

The cAMP-dependent CRP DNA-binding protein binds to a site that overlaps with a LeuO binding site in CRISPR of *Escherichia coli* [33]. CRP binding represses CRISPR, linking its transcription to cAMP and glucose levels. High concentrations of glucose relieve cAMP-CRP-mediated repression of CRISPR transcription [33]; they also correlate with increased negative supercoiling of DNA in *E. coli* [19]. In *Salmonella enterica* serovar Typhi, the leucine-responsive regulatory protein, LRP, is a repressor of CRISPR transcription, linking it to branched-chain amino acid metabolism [31].

Silencing of CRISPR Systems by Mobile Genetic Elements

Some mobile genetic elements encode factors that can neutralize the effects of CRISPR-Cas systems. These include phage-encoded anti-CRISPR (Acr) proteins that operate by inhibiting the functions of CRISPR-encoded nucleases [34,35]. Other mobile genetic elements carry genes that encode members of the H-NS protein family: proteins that can silence CRISPR promoters [36,37]. The orthologous Sfh protein encoded by plasmid R27 acts as an auxiliary to H-NS, much as the chromosome-encoded StpA paralogue does [38]. Sfh and StpA production profiles exhibit a reciprocal pattern during the growth of a bacterial culture, such that H-NS is always accompanied by one or the other [39]. This partnership results in a minimal disturbance to the competitive fitness of bacterial cells that have just acquired the R27 plasmid; in contrast, derivatives of R27 that lack the *sfh* gene produce dysregulation of the H-NS regulon, probably due to titration of the protein by the large, A+T-rich plasmid [40]. Production of the R27-encoded H-NS orthologue Sfh enhances the survival of the new plasmid–bacterium combination in the microbial population [40]. We now propose that the presence of genes encoding H-NS-like proteins on plasmids also benefits those plasmids by reinforcing the silencing of CRISPR transcription, helping the plasmid to evade CRISPR-mediated destruction (Figure 3).

The acquisition of new spacer sequences by a CRISPR array is an infrequent event, occurring in 1 in 10^7 cells [41–45]. Site-specific insertion of new spacer sequences into the array requires the integration

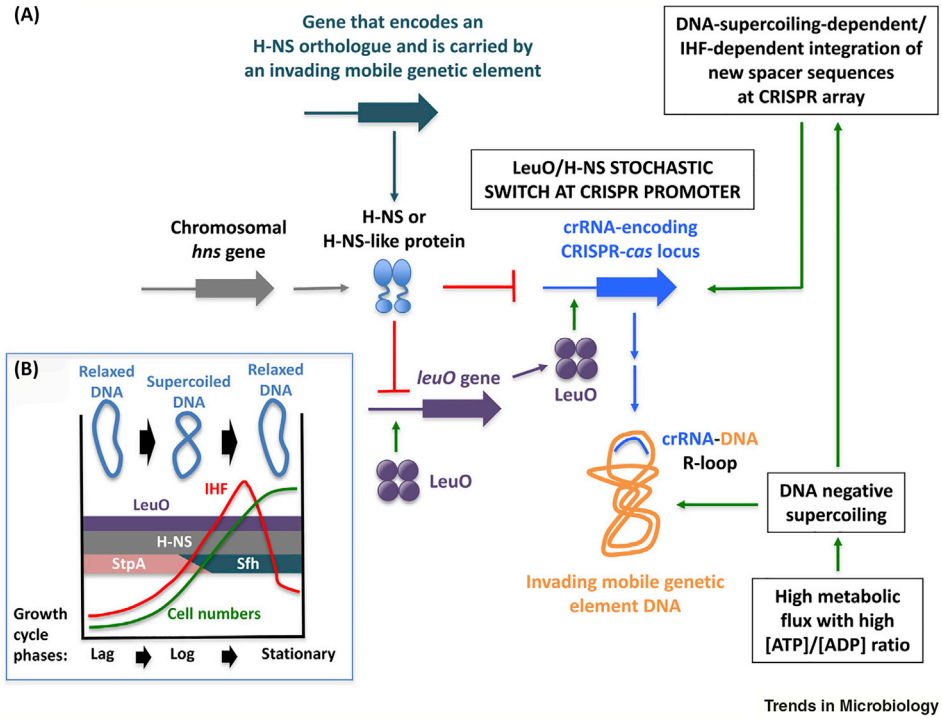

Figure 3. The Interconnections between CRISPR, H-NS-like Proteins, LeuO, DNA Supercoiling, Cas-Nuclease-Mediated Target Destruction, Integration Host Factor (IHF), and Spacer Acquisition.

(A) H-NS, its paralogues and orthologues, repress the expression of the *leuO* gene (a red 'T' represents negative regulatory inputs). The *leuO* gene encodes the tetrameric LeuO DNA-binding protein. LeuO is an antagonist of H-NS-mediated transcription silencing at *leuO*, CRISPR, and many other genetic loci in Gram-negative bacteria. At the *leuO* promoter, the H-NS and LeuO proteins determine promoter activity through a mutually antagonistic binding competition, and the outcome of this contest will vary from cell to cell in the bacterial population. Similarly, the supply of these proteins has the potential to produce a stochastic transcriptional switch at CRISPR. The supply of H-NS can be supplemented by the expression of orthologous genes on invading mobile genetic elements that encode H-NS-like proteins. These have the potential to tip the H-NS/LeuO balance in favour of the transcription silencer, downregulating CRISPR and other H-NS targets, including the *leuO* gene. The action of the CRISPR system that degrades invader DNA molecules (orange) depends on the formation of an R-loop consisting of crRNA (blue) and its single-stranded DNA complement, exposed in a DNA bubble. Green arrows represent positive regulatory inputs; blue arrows represent the expression and maturation of crRNA; grey and turquoise arrows indicate the production of H-NS and its orthologues, respectively; the purple arrow represents LeuO production. The blue cartoon represents the H-NS dimer, with a flexible linker connecting the dimerization and the nucleic-acid-binding domains in each monomer. (B) A typical growth curve is shown (green) with the principal phases of the growth cycle indicated on the X-axis. The growth-phase-associated production patterns for the DNA-binding proteins discussed in the text are indicated by filled rectangles (H-NS, StpA, Sfh, LeuO) or by a curve (IHF). The production of the R-loop shown in (A) is assisted by DNA negative supercoiling, and the ATP-dependent supercoiling activity of DNA gyrase is linked to the metabolic flux of the cell. Thus, cells in the exponential phase of the growth cycle have a greater potential to present the invader DNA in a negatively supercoiled conformation that lends itself to R-loop formation. Similarly, the DNA-supercoiling-dependent integration of novel spacer sequences at the CRISPR array is likely to be enhanced in exponential growth, with the window for recruitment being extended by the IHF protein whose production is maximal at the transition from the exponential to the stationary phase of growth. Bacteria in the lag or the stationary phases of growth have DNA that is less negatively supercoiled. The H-NS protein is present approximately at a constant level per chromosome through the growth cycle; the StpA and Sfh homologues of H-NS have individual, growth-phase-specific patterns of production [39]. The LeuO and H-NS proteins act antagonistically at many loci, including CRISPR, providing the basis for a stochastic genetic switch.

host factor (IHF) [46], a nucleoid-associated protein whose expression peaks at the transition from exponential growth to stationary phase [47]. Efficient integration of a protospacer requires a supercoiled CRISPR locus; however, IHF can provide the requisite DNA deformation in the absence of supercoiling [48]. Thus, expansion of the CRISPR genetic memory bank may be modulated by bacterial cell physiology as represented by negative supercoiling of the DNA and the intracellular concentration of IHF (Figure 3).

Linkage of CRISPR Systems to Cellular Metabolism

We propose that the expression, the destructive activity, and the genetic expansion of CRISPR systems are all linked to cellular metabolism via transcription factors, nucleoid-associated proteins, and variable DNA supercoiling. Negative supercoiling of the target DNA enhances R-loop formation through the production of a crRNA–DNA hybrid, and this is an essential step in preparing the invader DNA for nuclease-mediated destruction. Similarly, the rare integration of novel protospacer sequences into the CRISPR array is dependent on DNA negative supercoiling, or, if this is lacking, the IHF NAP binding at the leader sequence of the array [46,48]. Negative supercoiling is growth-phase-dependent and is associated with the exponential stage of the growth cycle. Therefore, bacteria in this phase of growth may be more capable of destroying an invader DNA element and expanding their repertoire of spacer sequences than bacteria in other metabolic states, such as those in the stationary phase of growth. The co-option of IHF, a protein whose concentration peaks at the end of exponential phase, may extend the period of spacer recruitment into the early stages of stationary phase (Figure 3).

Based on these considerations, it appears that invading genetic elements would be least likely to evade CRISPR host defences during the exponential phase of growth. However, one must also consider the negative influence of the H-NS/StpA protein family on CRISPR transcription, something that is likely to be exerted at all stages of growth. The antagonistic relationship of LeuO and H-NS at CRISPR (and other) promoters provides the basis for a stochastic switch that can overcome H-NS-mediated transcription silencing in at least a subset of the cells in a bacterial population (Figure 3). Other transcription factors can add species- or strain-specific features to the basic switch, as in the case of cAMP-CRP in *E. coli* [33] and LRP in *S.* Typhi [31]. The invaders can themselves influence the outcome of the host/parasite interaction by using H-NS-like proteins to reinforce the silencing of CRISPR promoters and/or Acr proteins to inactivate CRISPR-*cas*-encoded nucleases [34,35].

Pathogenic bacteria experience changes in the expression of their CRISPR-*cas* systems during interactions with their hosts. Examples include upregulation of these defence systems in *S.* Typhi while in the macrophage vacuole [49,50], and in the plant pathogen *Pectobacterium atrosepticum* while interacting with potato [51]. Given that H-NS, IHF, LeuO, Lrp, and variable DNA topology have all been implicated in virulence gene regulation in pathogenic bacteria [24,52,53], the nexus between CRISPR-Cas function, horizontal gene transfer, and bacterial virulence would appear to be a fruitful area for future investigations.

Concluding Remarks

CRISPR systems do not appear to be fully active at all stages of bacterial growth, and they may not be 'on' in every cell in a population of genetically identical organisms. Sensitivity to changes in DNA supercoiling and to DNA-binding proteins whose expression and/or activities are linked to bacterial physiology, provides a mechanism to tune the expression and activity of CRISPR systems, so that they are vigilant in bacteria undergoing a high rate of metabolic activity but 'turn a blind eye' to the arrival of foreign DNA in less active cells. This tuning process opens a window of opportunity for bacterial evolution through foreign gene uptake because the receiving bacterium lowers its guard. This tendency towards permissiveness is enhanced when the arriving DNA is a mobile genetic element that can express its own CRISPR-silencing activity in the form of an H-NS-like protein. Deeper knowledge of the barriers to, and the facilitators of, DNA transfer between organisms will aid our understanding of microbial evolution in natural settings and enhance our ability to impede the spread of genetic traits such as those responsible for antimicrobial resistance (see Outstanding Questions).

Outstanding Questions

Are CRISPR promoters sensitive to DNA supercoiling? If DNA supercoiling modulates spacer sequence recruitment and the preparation of R-loop targets for Cas nuclease action, then the possibility that the production of crRNA is also supercoiling-sensitive is an attractive hypothesis because it places all of these aspects of CRISPR function under the influence of physiologically responsive, variable DNA topology.

Is CRISPR expression growth-cycle-dependent, and is this why DNA uptake is more efficient in stationary-phase cells in at least some horizontal gene transfer systems?

The details of CRISPR promoter regulation differ between bacterial species. What is the significance of these differences, and what does it tell us about the expression of CRISPR immunity in the environments typically inhabited by these bacteria?

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