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*Curr Opin Infect Dis*. Author manuscript; available in PMC 2016 June 01.

Published in final edited form as:

*Curr Opin Infect Dis*. 2015 June ; 28(3): 267–274. doi:10.1097/QCO.0000000000000154.

# I can see CRISPR now, even when phage are gone: a view on alternative CRISPR-Cas functions from the prokaryotic envelope

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## Abstract

**Purpose—**CRISPR-Cas systems are prokaryotic immune systems against invading nucleic acids that adapt as new environmental threats arise. There are emerging examples of CRISPR-Cas functions in bacterial physiology beyond their role in adaptive immunity. This highlights the poorly understood, but potentially common, moonlighting functions of these abundant systems. We propose that these non-canonical CRISPR-Cas activities have evolved to respond to stresses at the cell envelope.

**Recent findings—**Here, we discuss recent literature describing the impact of the extracellular environment on the regulation of CRISPR-Cas systems, and the influence of CRISPR-Cas activity on bacterial physiology. The described non-canonical CRISPR-Cas functions allow the bacterial cell to respond to the extracellular environment, primarily through changes in envelope physiology.

**Summary—**This review discusses the expanding non-canonical functions of CRISPR-Cas systems, including their roles in virulence, focusing mainly on their relationship to the cell envelope. We first examine the effects of the extracellular environment on regulation of CRISPR-Cas components, and then discuss the impact of CRISPR-Cas systems on bacterial physiology, focusing on their roles in influencing interactions with the environment including host organisms.

### Keywords

CRISPR-Cas; envelope stress; membrane composition; bacterial pathogenesis

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**Conflicts of interest**: TRS and DSW have filed provisional patents based on CRISPR-Cas technological applications. HKR has no conflict of interest.

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## Introduction

Prokaryotic organisms have evolved unique, adaptive, nucleic acid restriction machineries to prevent the uptake of mobile genetic elements, such as those derived from bacteriophages and plasmids(1). Termed CRISPR (clustered, regularly interspaced, short, palindromic repeats) - Cas (CRISPR-associated) systems, these RNA-guided endonuclease machineries canonically act in a sequence-specific fashion to cleave foreign DNA or RNA targets(2-5). This protects cells from exposure to potentially harmful genetic elements(2-4). Beyond this well-established function, CRISPR-Cas systems have been observed to play alternative roles in physiology. These moonlighting functions of CRISPR-Cas systems include roles in oxidative stress tolerance, antibiotic resistance, extracellular structure formation, DNA repair, and host-microbe interactions.

The molecular mechanism of many alternative CRISPR-Cas functions has not yet been fully elucidated, but may utilize a similar activity to that used in canonical targeting of foreign nucleic acids (6, 7). The signature component of CRISPR-Cas systems is the CRISPR array, composed of short, repetitive, and often palindromic sequences(8). These repeats are interspaced by short, unique, spacer sequences that are complementary to different nucleic acid targets (2, 9, 10). In most systems, the CRISPR array is transcribed as a single transcript (the pre-crRNA array) and is cleaved into small targeting RNAs (crRNAs) (11-14). These crRNAs form complexes with Cas proteins, which are encoded in adjacent, conserved operons (4). The complexes are capable of sequence-specific interaction with foreign nucleic acids (6). Upon hybridization of the crRNA to its target sequence, endonuclease activity of the associated Cas protein(s) is triggered, resulting in target cleavage (6). CRISPR-Cas systems are diverse and can be grouped into three main subtypes (types I,II, and III) defined by the unique Cas proteins used in crRNA processing and targeting/cleavage(1). While the type I and III systems use multimeric protein complexes for these processes, the type II system requires a single Cas protein, Cas9, as well as a unique accessory RNA, the trans-activating CRISPR RNA (tracrRNA) (1, 13, 15, 16). Uniquely, CRISPR-Cas systems can also acquire new spacer sequences within the CRISPR array as the nucleic acid threats (such as bacteriophages) in the environment change (2, 17).

Interestingly, many of the alternative activities (not involving the targeted degradation of foreign nucleic acid) of CRISPR-Cas systems are linked to processes occurring at the bacterial envelope. Herein, we present a CRISPR view of how CRISPR-Cas systems monitor and respond to stresses at the cell envelope, allowing bacteria to counteract not only bacteriophage infection, but also diverse insults such as antibiotics and host defenses. First, we discuss the transcriptional regulation of CRISPR-Cas systems in response to environmental changes signaled by the status of the bacterial envelope. We then describe the current understanding of how CRISPR-Cas systems regulate bacterial physiology, largely through changes at the cell surface, to promote resistance to environmental stresses. Finally, we highlight unanswered questions in the field of CRISPR-Cas biology, the exploration of which will provide insight into the evolution of CRISPR-Cas systems and the origins of their increasingly broad functions in bacterial physiology.

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## Activation and function of CRISPR-Cas systems in response to envelope stress

Since CRISPR-Cas systems target nucleic acids that have entered the cell through the envelope, it is interesting to note that their transcriptional activation often occurs directly, and indirectly, in response to envelope stresses (Figure 1). The most explicit example of this occurs during bacteriophage infection. It is logical to think that upon bacteriophage adsorption and DNA injection the envelope is disrupted, resulting in an envelope stress response(18-20). Concomitantly, activation of CRISPR-Cas transcription has been observed, suggesting that the cell actively senses the status of the envelope in order to respond to invading threats(21, 22). Furthermore, it has been observed that membrane protein dysregulation is capable of inducing the increased expression of CRISPR-Cas systems. For instance, in *Escherichia coli*, the BaeSR extracytoplasmic stress response regulator acts to activate its CRISPR-Cas system when the bacterial envelope is perturbed (23). Furthermore, the transcriptional regulator H-NS is an inhibitor of CRISPR-Cas expression. Upon an envelope stress response, H-NS is inhibited, leading to an upregulation of a CRISPR-Cas system in *Salmonella enterica* and *E. coli* (24, 25). Additionally, high temperatures result in misfolding of membrane proteins and an envelope stress response leading to activation of heat shock protein G (HtpG) (26, 27). HtpG has subsequently been shown to activate transcription of CRISPR-Cas systems in *E. coli* (27). Thus, CRISPR-Cas systems can be primed by stress at the envelope, likely at least in part to counter actin coming foreign nucleic acids.

In line with this idea, a recent study of *Streptococcus mutans*, a cause of tooth decay, revealed that expression of the Type II-A CRISPR-Cas system was negatively affected by the stress response regulator VicK/R two-component system, which also positively regulated the expression of its Type I-C system (28-30). Additionally, it was observed that both of these CRISPR-Cas systems play a role in temperature stress tolerance. CRISPR-Cas locus deletion mutants exhibited reduced survival after heat exposure, and surprisingly, double mutants in both loci had a greater sensitivity to high temperature than mutants from either locus alone, suggesting independent activity of each system (30). Furthermore, CRISPR-Cas mutants in the type II-A system, but not the Type I-C system, displayed reduced growth upon exposure to membrane stress (detergents) as well as oxidative stress (paraquat and hydrogen peroxide) (30). Together, these data directly link CRISPR-Cas function to envelope stresses, and further suggest that VicK/R may differentially regulate each CRISPR-Cas system under specific conditions. This raises the questions of whether these systems work together in nucleic acid defense as well, if they have distinct defense activities beyond adaptive immunity, or if they diverged in function to fulfill distinct regulatory roles, perhaps by altering the envelope. Exactly how these CRISPR-Cas systems regulate stress tolerance remains to be elucidated, and continued study of this phenomenon in diverse bacteria will be necessary to identify common themes. It is reasonable to postulate that this occurs through physiological changes at the envelope, which acts as the frontline to counteract environmental stressors.

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## CRISPR-Cas control of population behaviors

In addition to roles in the envelope stress response, CRISPR-Cas systems have been implicated in complex population behaviors that involve extensive envelope alterations, such as biofilm formation and fruiting body development (Figure 2). Before CRISPR-Cas systems were identified, three genes encoded by the Gram-negative saprophytic bacterium *Myxococcusxanthus*, were found to be necessary for sporulation and fruiting body development (31-33). Interestingly, the three genes, *devT*, *devR*, and *devS*, respectively correspond to *cas8*, *cas7*, and *cas5*from a type I CRISPR-Cas system. In the absence of *devT* (*cas8*), *M. xanthus* displayed delayed cellular aggregation, sporulation, and chemotaxis, as well as decreased transcript levels for a fruiting body transcriptional activator(31). While the mechanism of regulation has not been fully elucidated, the *M. xanthus* CRISPR array encodes two spacers that have identity to endogenous sequences on the bacterial chromosome. One has identity to an integrase of a *Myxococcus* bacteriophage, while the other has identity to a *cas*gene in a different CRISPR-Cas locus, raising the intriguing possibility that the CRISPR-Cas system regulates endogenous targets (33). However, whether the CRISPR array itself is required for control of the aforementioned processes remains unknown.

*M. xanthus* regulation of fruiting body formation is further influenced by a type III-B CRISPR-Cas locus, which also regulates exopolysaccharide (EPS) production and type IV pili mediated chemotaxis (34). Not only is crRNA processing required for this regulatory activity, but the associated *cas* genes are as well (34). Further studies are needed to determine if and how the type I and III systems in *M. xanthus* interact to regulate fruiting body formation, as well as the mechanism of CRISPR-Cas mediated EPS regulation. It will be interesting to determine whether these functions evolved due to pressures to restrict mobile genetic elements, broader stresses at the envelope, or from entirely different environmental pressures.

Another population behavior involving extensive envelope changes, biofilm formation, is regulated by the type I CRISPR-Cas system in the opportunistic pathogen *Pseudomonas aeruginosa* (35, 36). A spacer within the *P. aeruginosa* CRISPR array has sequence similarity to a gene within a chromosomally integrated prophage (36). The CRISPR-Cas system interaction with this chromosomal element is necessary to represss warming motility and biofilm formation (35, 36). While it is not known how repression occurs, it is established as a sequence-specific activity requiring all interference components of this CRISPR-Cas system (36, 37). Given the importance of biofilm formation to antibiotic resistance and pathogenesis in *P. aeruginosa*, it is likely that this CRISPR-Cas system plays an important role in mediating infection of eukaryotic hosts.

## CRISPR-Cas mediated regulation of host-pathogen interactions

While all bacteria encounter numerous environmental stresses, those bacteria that interact with eukaryotes, particularly mammalian hosts, are subjected to a variety of microenvironments and stressors as they traffic through the host and encounter the immune system (Figure 2). It is therefore an exciting proposition that CRISPR-Cas systems may be

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utilized in response to these host-derived stresses and ultimately mediate host-microbe interactions.

Recently, it has been observed that CRISPR-Cas systems can modulate host immune evasion. The intracellular pathogen *Francisellanovicida* upregulates its type II-B CRISPR-Cas system in the phagosome of host macrophages, a stressful environment containing a plethora of host defenses that attack the bacterial envelope (38). Components of this system (Cas9, tracrRNA, and a small CRISPR-Cas associated RNA [scaRNA]) regulate the production of an endogenous bacterial lipoprotein (BLP), a process necessary for strengthening the bacterial envelope (38, 39). Loss of these components results in increased envelope permeability and subsequently increases susceptibility to membrane damaging compounds, such as those found in the macrophage phagosome (39). Furthermore, regulation of the BLP dramatically alters how *F. novicida* survives within its mammalian host. In fact, *cas9* mutants are attenuated in a mouse model by $10^3$-$10^4$ fold compared to wild-type bacteria (38). Cas9 and its associated RNAs enable evasion of the host innate immune response through two distinct pathways, both of which originate due to changes at the membrane. In the absence of Cas9, the BLP transcript is de-repressed, and the bacteria are detected by the host pattern recognition receptor (PRR) Toll-like receptor 2 (TLR2), which initiates a proinflammatory response upon recognition of BLP(38). Additionally, repression of the BLP increases envelope integrity and reduces activation of the AIM2/ASC inflammasome, a protein complex involved in a programmed host cell death pathway that results in loss of *Francisella*'s replicative niche (39). This CRISPR-Cas mediated evasion of both TLR2 and the AIM2/ASC inflammasome is critical for the ability of *F. novicida* to cause disease.

Consistent with the idea that CRISPR-Cas systems have evolved functions to mediate interactions with eukaryotic hosts, *Neisseria meningitidis* Cas9 is necessary for intracellular survival in human epithelial cells (38). Further, *N. meningitidis* Cas9 is also required for attachment and entry into these cells, processes dependent on surface components, suggesting that it may regulate envelope structures in this bacterium (38). Cas9 is likewise necessary for attachment and intracellular survival of *Campylobacterjejuni*, a cause of diarrheal disease and Guillain-Barré syndrome, in epithelial cells (40). Furthermore, *C. jejunicas9* mutants displayed increased surface antibody binding, as well as increased envelope permeability and antibiotic susceptibility, all potentially linking Cas9 to the regulation of envelope components (40). Finally, it was observed bioinformatically that the presence of envelope sialylation correlates with a loss of the type II CRISPR-Cas system in multiple bacteria (including *N. meningitidis*, *C. jejuni*, and *Haemophilus parainfluenzae*) (40). Taken together, these data provide additional evidence for alternative functions of CRISPR-Cas systems in regulating envelope functions in response to environmental pressures.

Another example of a CRISPR-Cas system promoting host-microbe interactions is observed in the Gram-negative bacterium *Xenorhabdus nematophila*. Here, an orphan CRISPR RNA, termed NilD, is necessary for *X. nematophila* to colonize *Steinemema spp*. nematodes, a symbiotic relationship that facilitates the pathogenesis of these nematodes for their insect hosts (41). This is the first example of a CRISPR-Cas system modulating a mutualistic and

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tripartite interaction, and sheds light on the under explored complexity of CRISPR-Cas functions in broader ecological niches. Interestingly, this CRISPR-Cas system is expressed at a higher level in iron limiting conditions, furthering the concept that these machineries respond to extracellular changes and to events that are tightly regulated at the bacterial envelope (41). Additionally, the role of the crRNA from this system in colonization is independent of the effector protein Cas3, suggesting that the NilD CRISPR RNA has a unique function not involving canonical CRISPR-Cas activity (41). Further studies to elucidate the molecular mechanism of NilD-mediated nematode colonization will shed light not only on envelope changes that facilitate colonization, but also on how orphan crRNAs can potentially function as regulatory elements.

Similar to NilD, it was observed that the *cas2* gene of the type II-B CRISPR-Cas system of *Legionella pneumophila* was required for intracellular survival within amoebae, and that *cas2* was upregulated during intra-amoeba growth (42). Interestingly, no other *cas* gene was required, and *cas2* was not required for growth in broth culture or intracellular infection of macrophages (42). Furthermore, expression of *cas2* in a *L. pneumophila* strain that lacks a CRISPR-Cas system increased the strain's ability to replicate within amoebae, further indicating that Cas2 can act independently of canonical CRISPR-Cas function (43). Cas2 orthologs have RNase and/or DNase activity, depending on the organism, and are involved in spacer acquisition (17, 44-47). Cas2 nuclease activity is dependent on a single catalytic residue, which is also required for *L. pneumophila* intra-ameobal survival (43). In. *L. pneumophila*, not only is Cas2 RNase activity more efficient than DNase activity, but each requires a different divalent ion ($Mg^{2+}$ or $Mn^{2+}$, respectively)(43). Thus, preferred nuclease activity may change with shifts in the bacterial environment. It is unclear which nuclease activity promotes survival in amoebae, and a comparison of the ion concentrations in different growth environments may shed light on this difference. Likewise, the precise role of Cas2 in promoting intracellular survival is still unknown; it is tempting to consider that Cas2 has functions in mRNA regulation, particularly given that residues in its nuclease motif are essential for its role in intra-amoeba survival. Studies to observe which nucleic acids associate with Cas2 in different stages and contexts of *Legionella* growth, as well as determining the environmental cues governing the independent regulation of this Cas protein, will significantly enhance the understanding of CRISPR-Cas function as a regulator of intracellular survival.

## Are CRISPR-Cas systems more broadly involved in stress responses?

Intriguingly, CRISPR-Cas systems are also regulated by a broad range of environmental conditions not necessarily linked to envelope stress (Figure 1). For instance, in nutrient rich conditions, the leucine-responsive protein (Lrp) represses CRISPR-Cas expression in *Salmonella enterica* serovar Typhi (24). However, upon starvation, Lrp is inactivated and may de-repress CRISPR-Cas transcription (24). Additionally, the regulator LeuO is an activator of CRISPR-Cas expression in *S. enterica* and *Escherichia coli* (24, 25, 48). LeuO is active under low phosphate and stationary phase conditions, further suggesting that starvation responses can increase CRISPR-Cas expression (49, 50). It is interesting to speculate that expression of CRISPR-Cas systems may also be tied to nutrient conditions since prokaryotic organisms may actively seek out nucleic acids as a nutrient source (51).

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While starvation is a stress in itself, it can indirectly result in dysregulation of membrane composition, as well as serve as a signal for prophages to become lytic (52, 53). The same is true for oxidative and osmotic stress, which have been shown to activate CRISPR-Cas systems and cause broad stress to the cell, including at the membrane (54, 55). Therefore, it is unclear whether there is a universal link between induction of CRISPR-Cas systems and envelope stress, or if these machineries may more broadly be induced by diverse stresses. In total, these examples provide further links between CRISPR-Cas activation and the response to environmental cues, which may occur through either their canonical or alternative functions.

In addition, CRISPR-Cas systems may act to regulate the cell's response against other diverse environmental stresses(38, 41, 54-57). For example, in *E. coli*, both the CRISPR array and Cas1 can participate in mediating DNA repair, while in *Thermoproteustenax*, a CRISPR-Cas system is activated in response to DNA damaging UV light (55, 56). Therefore, CRISPR-Cas systems may be responsible for alleviating the effects of stresses that damage the chromosome. In another example, the orphan CRISPR locus in *Listeriamono cytogenes*, *rliB*, acts to upregulate the production of the iron transport system *feoAB*, further demonstrating that CRISPR-Cas systems mediate physiological changes that are likely in response to environmental stress (57). Overall, these observations demonstrate that CRISPR-Cas systems may have evolved multiple functions to not only be activated in response to diverse environmental stress, but also to play active roles in preventing stress-promoted damage.

## Conclusion

CRISPR-Cas systems are complex machineries that act to protect the cell against potentially harmful mobile genetic elements. As such, it would be efficient to regulate expression of these systems to times when the threat of such elements is imminent. Accordingly, there are now multiple examples of increased activation of CRISPR-Cas systems in response to envelope stress, such as bacteriophage binding and envelope disruption, ultimately enabling cells to activate defenses against potential genetic threats.

We have summarized numerous examples of CRISPR-Cas systems having functions beyond defense against foreign nucleic acids, many of which involve regulation of envelope physiology and how the cell interacts with its host and environment. It is interesting to consider how these non-canonical functions may have arisen. These observed roles could have appeared due to independent pressures, or stochastically due to accidental acquisition of spacers targeting self. Furthermore, the relationships between CRISPR-Cas system subtype and their non-canonical functions are poorly understood. Since some bacterial species encode multiple CRISPR-Cas subtypes within the same genome, each unique system may represent a fine-tuning of nucleic acid defense, perhaps based on niche and environmental cues. Alternatively, the presence of multiple systems may be linked to non-canonical functions, whereby some systems are preferentially used for nucleic acid defense and others to regulate bacterial physiology, or multiple systems facilitate different non-canonical functions. We hypothesize that clues to these interactions lie at the envelope, and that by studying the non-canonical functions of CRISPR-Cas systems from this perspective,

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we will gain insight into the evolution of both commensal and pathogenic bacteria to defend against their own pathogens and survive within their diverse replicative niches.

## Acknowledgments

Due to the rapidly expanding field, we have undoubtedly omitted some relevant studies. We apologize in advance to those authors whose work we did not cite.

**Financial support and sponsorship**: DSW is supported by NIH grant R01-AI110701 and a Burroughs Wellcome Fund Investigator in the Pathogenesis of Infectious Disease award.

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**Key points**

- CRISPR-Cas systems play roles in bacterial gene regulation.
- Regulatory roles of CRISPR-Cas systems are linked to processes occurring at the bacterial envelope.
- The ability to respond to envelope stress may have driven the involvement of CRISPR-Cas systems in gene regulation

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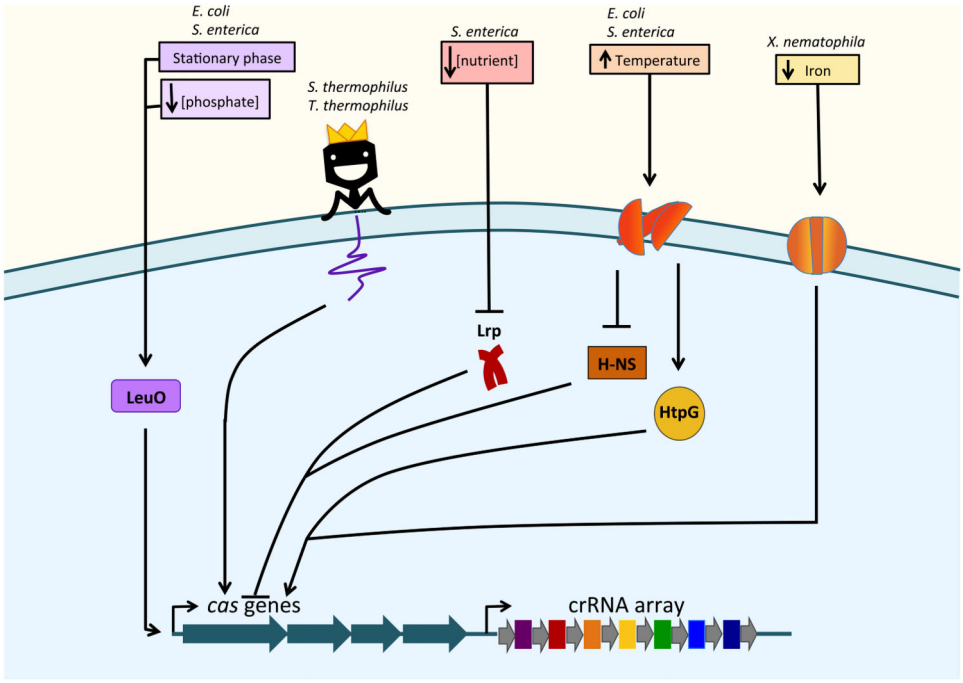


**Figure 1. Activation of CRISPR-Cas systems in response to environmental changes**

CRISPR-Cas systems can be activated in response to the broader environmental stressors of nutrient starvation, stationary phase growth, and iron limitation. Likewise, CRISPR-Cas systems can be activated directly in response to envelope stressors, such as phage infection and high temperature. These examples highlight the influence of the extracellular environment on the regulation of CRISPR-Cas systems.

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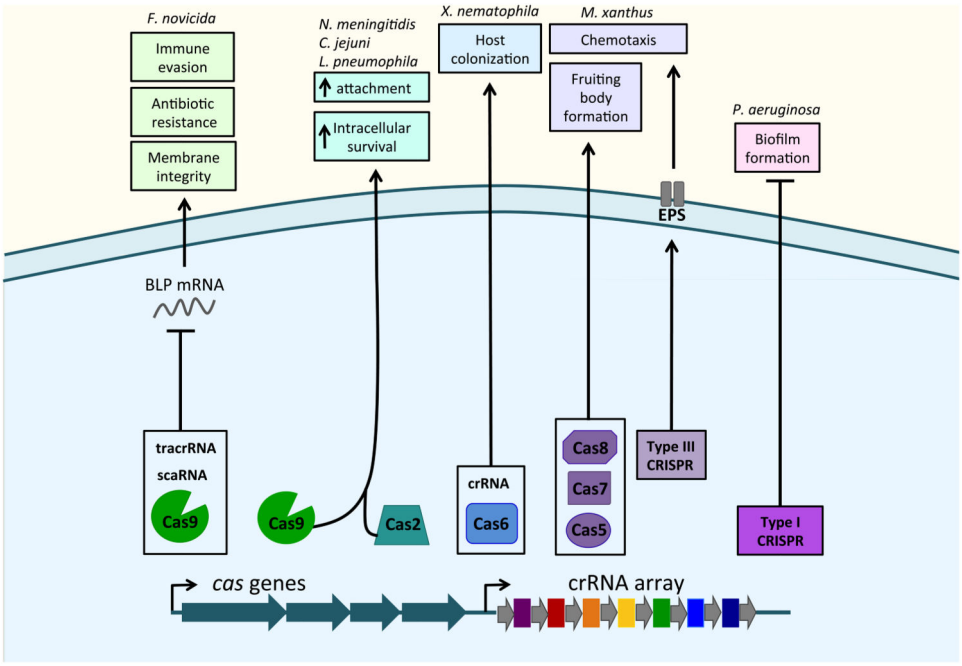


**Figure 2. CRISPR-Cas mediated physiological changes**

CRISPR-Cas systems influence bacterial physiology, altering population behavior and host-microbe interactions through events that are centered at the envelope. In *Francisellanovicida*, Cas9, tracrRNA and scaRNA form a complex that represses a bacterial lipoprotein mRNA (BLP). Repression of the BLP increases membrane integrity, conferring resistance to membrane targeting antibiotics and enabling evasion of the host immune system, increasing virulence. Cas9 from *Neisseria meningitidis* and Cas2 from *Legionella pneumophila* type II systems increase host-cell attachment and intracellular survival. In *Xenorhabdus nematophila*, Cas6 and a CRISPRRNA (crRNA) of the type I-E system are required for host colonization. In *Myxococcusxanthus*, the type III CRISPR-Cas system regulates exopolysacchride production (EPS) to enable chemotaxis, while negatively effecting fruiting body formation. Conversely, Cas5, Cas7, and Cas8 of its type III CRISPR-Cas system are necessary for fruiting body formation and sporulation. Finally, in *Pseudomonas aeruginosa*, all interference components of the Type I CRISPR system are required for biofilm formation and swarming motility. These examples provide a framework for understanding the alternative functions of CRISPR-Cas systems from interactions at the prokaryotic envelope.