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Review Article

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Uncovering the Regulators of CRISPR-Cas Immunity

Abstract

The clustered regularly interspaced short palindromic repeats (CRISPR) system and the CRISPR-associated proteins (Cas) make up an adaptive immune mechanism used by many bacteria and archaea to protect themselves from invading genetic material. Despite the immense evolutionary advantage of the CRISPR-Cas system, it must be meticulously regulated as it has the potential to target endogenous genes and damage the host organism. Identifying the main regulators involved in this process and how they are influenced by distinct conditions are of great clinical interest, since this prokaryotic defense system can be exploited for genome editing and therapy development. This review aims to elucidate the regulation of the CRISPR system in bacterial communities—upon quorum sensing and alginate production in biofilms—and in stressed conditions—upon antibiotic treatment or induction of the Rcs response. Despite the intrinsic contradictions of the results gathered in this review, growth rate is identified as a potential unifying regulator of CRISPR immunity. Overall, the regulation of the CRISPR-Cas system is shown to be multi-dimensional and cross-sectional, to greatly vary amongst lineages, and to be highly sensitive to conditional changes.

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Introduction

One of the reasons why bacteria remain such prominent and persistent invaders of the human body is their ability to quickly adapt and evolve through acquisition of novel genetic material. This can both provide significant evolutionary advantage and threaten bacteria survival. Microorganisms have evolved many mechanisms to protect themselves from parasitic DNA. The clustered regularly interspaced short palindromic repeats (CRISPR) system and its associated proteins (Cas) make up the only known adaptive and heritable defense mechanism in bacteria against bacteriophage invaders and other mobile genetic elements[1].

It was first discovered in *Escherichia coli* in 1987, but it has now been identified in around 50% of bacteria, and growing interest for this system has bloomed a new field of microbial and genetic studies [2,3]. Although the CRISPR-Cas system has been identified in numerous bacteria, its distribution does not align with bacterial phylogenies[4].

It has been predicted that factors such as oxygen prevalence, temperature, and the abundance of viral threats in the environment influence the distribution of this system but the ecological factors involved remain unclear[5]. This is further complicated by the fact that the defense system can readily be transferred through horizontal gene transfer[4].

The CRISPR-Cas system functions as an intracellular patrolling complex that will recognize incoming foreign nucleic acid sequences and induce their degradation. There are two main components to this system: the CRISPR array and the Cas proteins. The former is a genomic locus where a series of identical repeats sequences and unique spacer sequences are alternately distributed downstream of a leader sequence. While the short repeats are intrinsic to the bacteria, the spacers are integrated into the host genome from previously encountered foreign DNA.

During the adaptation phase, the Cas1 and Cas2 proteins will scan foreign DNA that has entered the bacterial cell and capture a protospacer fragment from this invading genetic material. In most bacteria, this capture is dependent on the presence of a protospacer adjacent motif (PAM) which allows the host to differentiate between self and foreign genetic material. The protospacer can then be integrated at the 5' end of the spacer array, adjacent to the leader sequence. For this reason, the CRISPR array represents a physical memory of past infections. Subsequently, in the biogenesis phase, also known as the maturation or expression phase, the spacers are transcribed and processed by host enzymes into CRISPR RNA (crRNA) which will be bound by Cas proteins and adaptor sequences, such as the transactivating noncoding CRISPR RNA (tracrRNA), to generate a mature antiviral complex.

Lastly, during the interference phase, the mature antiviral complex will once again scan the intracellular environment. The crRNA will mediate the recognition of foreign genetic material via sequence complementarity, and the accessory proteins of the mature system will induce targeted sequence damage to protect the bacterial cells **(Figure 1)**. The Cas proteins are required at each step and they carry specialized functions: Csy proteins will generate a multi-protein surveillance complex that is essential for the adaptation phase, whereas the Csm proteins generate complexes that mediate interference.

The three steps described above are ubiquitous to all CRISPR-Cas systems, but there is still great variability amongst the proteins encoded, the specificity of the CRISPR-Cas system, and the mechanisms of protection adopted by this defense mechanism. There are 2 large classes, 6 types, and over 20 subtypes of bacterial CRISPR-Cas systems that are functionally distinct[6]. In this review, we will focus on two model bacterial strains and their corresponding CRISPR-Cas defenses: *Pseudomona aeruginosa* PA14 which carry the Type I-F system and *Serratia* species 39006 which carries Type III-A, Type I-E, and Type I-F CRISPR-Cas systems. Type I and Type III systems are both members of class 1 systems which encode multi-protein effector complexes, but they have additional unique characteristics that distinguish them[7]. Type I systems recognize double-strand DNA which are degraded via Cas3 upon recognition of a PAM and the short neighboring 'seed' sequence[7]. Type III systems target foreign RNA transcripts to activate the Cas10 nuclease and induce non-specific RNA degradation, independently of canonical PAM requirements[7].

Overall, there is a great understanding of the functional requirements for the activity of CRISPR-Cas. However, much remains unclear regarding the regulation of this system in different conditions. Constitutively active

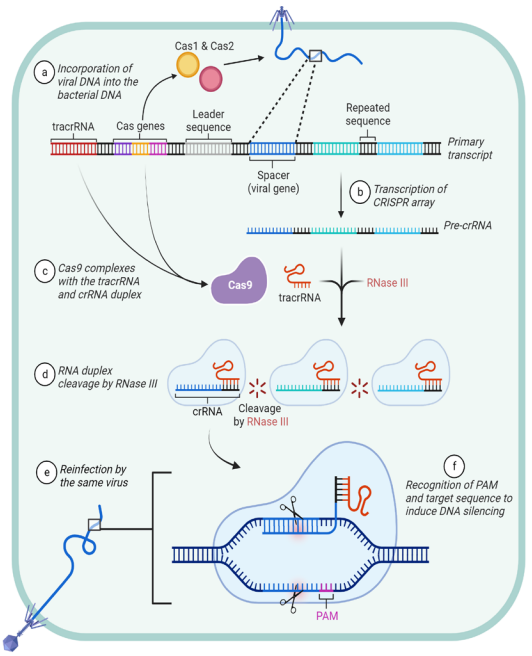

Figure 1[9]. Summary diagram of CRISPR-Cas system. Schematic showing the different steps of the adaptive immune response upon exposure to bacteriophage genetic material. Step A illustrates the adaptation phase. Steps B-D summarize the biogenesis phase, which is also known as the

CRISPR-Cas systems pose a significant evolutionary cost as they predispose the bacteria to self-targeting and to degradation of genetic material[8]. Thus, this defense mechanism must be tightly regulated. Additionally, there is a great interest in understanding the factors controlling the acquisition, biogenesis, and interference phases of the CRISPR-Cas system as this knowledge can be applied to very precise genome editing techniques and phage therapies. Consequently, this review aims to uncover the key regulators of the CRISPR-Cas system, the importance of each sensor, and the regulatory fluctuations that arise upon changes in conditions **(Figure 2)**. To this effect, various intracellular pathways and intercellular signals will be analyzed: quorum sensing, biofilm formation, alginate biosynthesis, and the regulator of capsule synthesis (Rcs) response induced upon antibiotic treatment.

Methodology

Quorum Sensing: the more you seek, the less you know

Quorum sensing (QS) is a system of coordinated chemical signals that allow bacteria to communicate with one-another in order to detect population density, transfer genetic material with proximal cells, modulate cellular functions, control motility and synthesize structural and signaling metabolites[10]. There are three general types of QS systems: the acyl homoserine lactone (AHL) system, the autoinducing peptide (AIP) system, and the autoinducer-2 (AI-2) system[10]. The AHL system is expressed by Gram-negative bacteria exclusively and it functions via the release of signaling molecules with a common homoserine lactone ring[10]. The AIP QS system is found in Gram-positive bacteria and it is characterized by short peptides and a two-component regulatory system[10]. The AI-2 system is distributed amongst both Gram-positive and Gram-negative bacteria, and it confers interspecies communication abilities via a collection of inter-convertible molecules[11].

The AHL system will be explored here as it is the one held by both *P. aeruginosa* and *Serratia* model strains[12]. In these bacteria, the LasI, RhII, and SmaI autoinducers will signal through their corresponding receptors which results in the expression of QS genes[13] **(Figure 2)**. One downstream target of QS signals is the upregulation of type I-E and type I-F cas genes[13]. Indeed, P. aeruginosa PA14 mutants lacking autoinducer genes have been shown to exhibit attenuated efficacy in all three phases of the CRISPR-Cas system, and complementation restored function to wild-type (WT) levels for all steps of the immune process[13]. This suggests that the engagement of the QS system positively upregulates CRISPR-Cas immunity. This is in agreement with other studies, which have shown that QS-deficient mutants of either bacterial model were found to be less adaptable to invading nucleic acids[12]. However, it is critical to acknowledge the limitations of these findings, as these results were obtained from studying only one of the three QS systems. Thus, it is possible that the regulation of CRISPR-Cas immunity by QS molecules and condition varies in other species that communicate via the AIP or the AI-2 systems. Additionally, the regulation of CRISPR-Cas immunity is likely more complex in bacteria that encode more than one QS system.

Certain groups that have attempted to modulate the expression of *cas* genes via quorum quenching (QQ)—a process of QS disruption—have obtained varying efficacy. Mion et al. measured the expression levels of *cas1*, *cas3*, and *csy1-4* in the presence or absence of the lactonase SsoPox-W263I to test the effects of QQ in the *P. aeruginosa* PA14 laboratory model and in 6 other clinically isolated strains of this species. The lactonase SsoPox-W263I is known to degrade acyl-homoserine lactones which are essential for QS in proteobacteria. Upon treatment with the enzyme, all *cas* & *csy* gene expression was abolished in PA14. However, clinically isolated strains showed variable results with some having a decreased cas and csy gene expression, while other showed no change or an increase in gene expression[12]. This demonstrates that regulation of the CRISPR-Cas system can vary greatly between strains of a single species despite significant genetic homology. Additionally, these results highlight that laboratory studies with model organisms in controlled settings do not necessarily translate to clinical cases. This is particularly relevant as there are strong initiatives that aim to treat certain diseases through the genome editing power of the CRISPR-Cas system.

Other groups have studied the modulation of CRISPR-Cas using QS inhibitors. It has been reported that the chemical baicalein can increase survival of phage-sensitive bacteria such as *P. aeruginosa*. This QS inhibitor has been found to reduce DMS3vir phage absorption, delay lysis of bacterial culture, and favor the action of CRISPR immunity[14]. This sharply opposes previously published data, which proposes that QS inhibitors limit

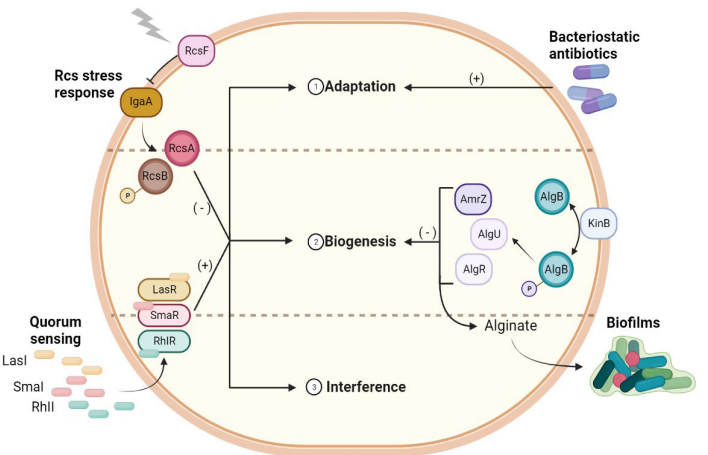

Figure 2[15]. The effects of various cellular signaling pathways on CRISPR-Cas regulation. The Rcs stress response pathway has been shown to downregulate all three phases of the CRISPR-Cas immune system, whereas quorum sensing upregulates these same phases. Some of the autoinducers (LasI & RhlI) directly induce these effects, while others (SmaI) are repressors of the CRISPR-Cas system that are inhibited by AHL molecules upon quorum sensing[1]. Bacteriostatic antibiotics positively regulate the adaptation phase, and the bacterial alginate synthesis pathway negatively regulates the biogenesis phase. The KinB membrane protein can act either as a phosphorylase in acute virulence conditions, or as a phosphatase in chronic virulent conditions[2].

CRISPR-based immunity. Upon further analysis, these effects were attributed to the fact that the DMS3vir phage receptor—the type-4 pili—is regulated by QS genes[14]. Inhibiting QS proportionally downregulated the phage receptor which reduced viral intracellular propagation and provided the host cells with sufficient time to mount an immune response[14]. Thus, when evaluating the effects of QS systems on immunity, we must also take into consideration the downstream genes affected, as they have the potential to alter sensitivity to invaders through indirect actions on infection parameters.

There are clear contradictions between the effects of quorum sensing, quorum quenching, and quorum sensing-inhibitors on the regulation of CRISPR-Cas. This becomes further convoluted when multiple variables are investigated simultaneously. For example, to assess the relationship between temperature changes and QS on the rate of spacer acquisition in the CRISPR-Cas system, Høyland-Kroghsbo et al. constructed a *P. aeruginosa* PA14 strain lacking autoinducer genes, and then measured adaptation rates at various temperatures in bacteria that were supplemented or deprived of autoinducers. As quorum sensing molecules are upregulated at high temperatures and are known to activate the CRISPR-Cas system, it was hypothesized that a strong and effective adaptive immune response would be induced at high temperatures in bacteria. However, the relationship between temperature and QS seems to be slightly more complex. Overall, adaptation rates were highest at low temperatures. Supplementation of mutants with autoinducers had no effect at 15°C, indicating that QS does not affect the rate of spacer acquisition at low temperatures. On the other hand, supplementation significantly increased new spacer acquisition at 30°C and 23°C, but only moderately at 37°C. To rationalize these results, the research group hypothesized that low temperature stabilized CRISPR-Cas complexes and crRNA binding to foreign sequences[13]. This has recently gained further relevance as reports emerge of the temperature-dependent binding and release of Cas proteins to target DNA[16]. Regardless, these results demonstrate that even slight changes in conditions such as temperature can alter the potency of sensors in modulating CRISPR-Cas adaptation.

Biofilm formation and alginate synthesis: Strength in numbers

One of the main functions of QS is to induce microbial biofilms and increase virulence in high-density bacterial populations[10]. In certain favorable environments, bacteria are able to communicate with each other and adhere to surfaces to build a highly structured and complex three-dimensional community that is much harder to eradicate than populations of single cells[10]. This is a multi-step process that involves the synthesis of numerous polymeric extracellular substances such as the alginate polysaccharide. Alginate production has been associated with adherence enhancement, increased persistence, and protection from the extracellular environment[17]. The biosynthesis of alginate is controlled by the KinB-AlgB two-component system[18]. Upon inactivation of the cognate histidine kinase KinB, phosphorylated AlgB will accumulate in the cell and signal through various intermediate protein such as algR, algU, and amrZ to upregulate alginate synthesis genes[18] **(Figure 2)**.

To measure the effects of the alginate biosynthesis pathway on CRISPR-Cas immunity, Borges et al. measured the levels of Cas and Csy protein expression in WT, *ΔkinB*, *ΔalgR*, and *ΔalgU* strains of *P. aeruginosa* after growth in liquid culture. They found that in the ΔkinB strain the levels of Cas and Csy proteins decreased relative to WT, whereas this expression was greatly increased in *ΔalgR* and *ΔalgU* strains. In other words, it was found that KinB promotes CRISPR-Cas protein expression, whereas AlgR and AlgU repress it. Thus, alginate biosynthesis inhibits CRISPR-Cas protein expression[19]. This directly opposes proposed trends about bacterial adaptive immunity in high-density populations and in biofilms. It has been previously suggested that since biofilm communities are more susceptible to phage infections due to high cell density and slow growth, these bacteria would exhibit exceptionally high levels of adaptive immunity [13,19]. However, the alginate synthesis pathway, which is induced during biofilm formation, inhibits CRISPR systems. This discrepancy could be partially rationalized by the fact that alginate is not absolutely required for the formation of nonmucoid biofilms in *P. aeruginosa*[17]. Still, with very few studies comparing CRISPR-Cas activity in free-growing bacteria and biofilm communities, the effects of biofilm-related factors on the immune system's regulation remains controversial. The research group tried to tackle this question and they showed in subsequent experiments that the effects of alginate synthesis molecules on adaptive immunity seem to partially depend on the organization of the bacterial community. It was reported that the downstream signaling molecule AmrZ can only control CRISPR-Cas immunity during surface-association and has no effect during planktonic growth. The group hypothesized that his state-dependent regulation could be an attempt at minimizing self-toxicity in bacteria during lifestyle transition[18]. Additionally, there is some data that suggests the possibility of regulatory feedback loops between CRISPR-Cas systems and biofilm-associated genes, as studies of the *Salmonella enterica* species have revealed that mutation of the cas3 gene resulted in reduced biofilm formation and virulence[20]. Overall, the regulation of the CRISSPR-Cas system in biofilms has not been completely characterized, but the research presented above demonstrates that networks of proteins can have distinct opposing or additive effects on bacterial immunity.

Antibiotics and the Rcs response: Stress less and live longer

As previously mentioned, viruses are not the only source of foreign genetic elements that bacteria are exposed to—they also frequently encounter plasmids that encode a variety of properties that can confer selective advantages. Antibiotic resistance genes are typically transferred between species via plasmids, thus at times it can be advantageous for the bacteria to repress its immune defenses. There are two general classes of antibiotics distinguished by their mechanisms of action and their effect on bacterial metabolism. Bacteriostatic antibiotics target a protein that is indispensable for cellular replication which results in growth arrest. Bactericidal antibiotics interfere with a process required for cell survival and subsequently cause cell death.

To evaluate how immunity evolves in bacterial populations when exposed to antibiotics, Dimitriu et al. infected *P. aeruginosa* PA14 strain with phage DMS3vir in nutrient-rich media supplemented with sub-inhibitory concentrations of 4 bacteriostatic and 4 bactericidal antibiotics. Bactericidal antibiotics exerted minimal effects on CRISPR-Cas immunity, whereas a significant proportion of the population upregulated this defense mechanism upon treatment with bacteriostatic antibiotics. To determine which phase of the CRISPR-Cas immunity was enhanced upon bacteriostatic antibiotic treatment, short-term infection assays in the presence or absence of antibiotics were carried out. None of the antibiotics caused an increase in Cas protein abundance. However, it was found that bacteriostatic antibiotics increased the rate of spacer acquisition, whereas bactericidal antibiotics had no effect. To test if the increased spacer acquisition was due to increased DNA damage, the research group quantified the expression of the DNA-repair SOS stress response. There was no correlation between SOS induction and the evolution of CRISPR immunity. Alternatively, an interesting pattern emerged between bacteriostatic antibiotics and slow growth. It was found that bacteria treated with bacteriostatic antibiotics released less phages upon infection, compared to cells treated with bactericidal antibiotics. Since the replication and spread of phages depends on host machinery, the reduced phage production was attributed to slower growth rate. Thus, bacteriostatic antibiotics increase CRISPR immunity via slowed cellular growth rather than stresses induced by the drug on the bacteria[21] **(Figure 2)**.

However, different stress responses can exert distinct effects on CRISPR-Cas regulation. When networks of genes that regulate CRISPR-Cas adaptive immunity in *Serratia* were screened, components of the Rcs stress response were identified. The regulator of capsular polysaccharide synthesis (Rcs) is a stress response to a variety of factors such as bactericidal β-lactam antibiotics[22]. Upon interaction between a stressor and the outer membrane lipoprotein RcsF, the inhibitory activity of IgaA on the signal phosphorelay will be lifted and allow downstream proteins RcsA and RcsB to positively regulate *rcs* genes[22] **(Figure 2)**. A mutagenesis analysis found that *ΔigaA* resulted in the greatest fold decrease of *csm* gene expression amongst all regulators screened[22]. Subsequent deletion of the *rcsA* or the *rcsB* genes in the *ΔigaA* mutant background restored csm expression levels by abolishing signaling through the Rcs response. Additionally, IgA mutation abolished CRSIPR-Cas immunity, induced minimal array expansion, and resulted in acquisition of antibiotic resistance genes. Upon deletion of downstream *rcsA* or *rcsB* genes, CRISPR-Cas immunity was restored[22].

Similarly, a genome-wide analysis of Rcs-regulated genes in the plant-pathogenic bacterium *Erwinia amylovora* found that mutation of RcsC resulted in decreased expression of type I-E CRISPR-Cas system[23]. Overall, adaptive immunity is disfavored by the Rcs response upon membrane stress. The induction of the Rcs response results in decreased expression of Cas proteins, adaptation, and interference of both type I and type III CRISPR systems in *Serratia*. Smith et al. suggested that the downregulation of CRISPR-Cas immunity upon antibiotic-induced surface membrane stress might be an evolutionary advantageous technique that can favor genetic sampling and the acquisition of antibiotic-resistance genes[22]. This explanation remains incomplete, as other sensors of cell envelope disruption such as BaeR in *E. coli* have been shown to promote Cas expression[24]. Nevertheless, the experiments studying the Rcs phosphorelay pathway underpin a stress-dependent regulation pattern of the CRISPR-Cas system.

Comparing the available data regarding the effects of antibiotics on CRISPR-Cas regulation reveals a sharp contradiction amongst published results. On one hand, bacteriostatic antibiotics were shown to promote spacer acquisition and the evolution of CRISPR immunity[21]. In contrast, stress responses induced by antibiotic treatment demonstrated an inhibition of the CRISPR-Cas adaptation, interference, and effector proteins expression[22]. These results could be accounted for by the differential metabolic and signaling effects of bacteriostatic and bactericidal antibiotics. Yet, the only definitive takeaway is that distinct molecules such as antibiotics can induce multiple pathways that exert differential effects on CRISPR-Cas regulation.

Growth rate: One variable to unite them all

When interpreting the effects of various sensors on the regulation of CRISPR-Cas immunity, it can be useful to consider the evolutionary competition between bacteria and the viruses that invade them. The dynamics between phages and their hosts can be described as an arms-race where each opponent aims to damage the other. Bacteria must coordinate cell division with spacer acquisition, whereas phages must exploit the host machinery while avoiding bacterial adaptive immunity. Naturally, the growth rate has been suggested as an important factor in controlling the CRISPR-Cas machinery. It is hypothesized that slow bacterial growth rate might enable the bacteria to acquire spacers from the invading phage and integrate the genetic sequence to the spacer array before the phage can exit and lyse the cell[13].

Regulator	System type	Mechanism	Step regulated
LasI: LasR RhII: RhIR	I-F	Signaling through quorum sensing signals at high cell densities	Adaptation Biogenesis Interference
SmaI: SmaR	I-E, I-F, III-A	Repressing quorum sensing signals at low cell densities	Adaptation Biogenesis Interference
Temperature	Range undefined	Controls growth rate, stabilizes complexes and binding	Adaptation
AlgR, AlgU	I-F	Transcriptional regulation during planktonic growth	Biogenesis
AmrZ	I-F	Transcriptional regulation during surface-association	Biogenesis
KinB	I-F	Prevents the accumulation of alginate-producing factors in acute virulence; promotes the latter in chronic virulence	Biogenesis
Bacteriostatic antibiotics	I-F	Slow bacterial growth rate	Adaptation
RcsA, RcsB	I-E, I-F, III-A	Signaling through the Rcs stress response pathway upon antimicrobial encounter or membrane alterations	Adaptation Biogenesis Interference
IgaA	I-E, I-F, III-A	Represses the Rcs stress response under normal conditions	Adaptation Biogenesis Interference

Table 1. A summary of regulators involved in CRISPR control.

This framework can be used to explain some of the results described here: low temperature, biofilm growth, and bacteriostatic antibiotic treatment all induce conditions where bacterial growth rate is limited, and CRISPR-Cas adaptation is favored. Similarly, the availability of certain nutrients has been shown to influence the expression of *cas* genes and the overall strength of the immune response. In conditions of iron deprivation or in the presence of suboptimal carbon sources—which decreases bacterial proliferation—there is a heightened expression and activity of the CRISPR-Cas immunity[21,25].

There have been mixed results regarding the activity of the CRISPR-Cas machinery during the different phases of growth. Some groups report high activity of bacterial immunity during the exponential growth phase where bacteria replicate at a fast pace, and repression of the system during the stationary phase where bacteria replicate quite slowly[18]. Other groups have reported that the highest frequency of adaptation occurs during the late-exponential growth phase where bacterial growth rate declines[26].

Using growth rate as the primary determinant for CRISPR-Cas regulation is not an empirically true framework. As previously described, the alginate biosynthetic marker amrZ decreases Csy1 expression upon-surface association but has no effect during planktonic growth[18]. This contradicts the growth-rate theory as alginate is produced during biofilm formation where slow-growing surface-associated bacteria are expected to exhibit high CRISPR-Cas activity. Applying growth rate analysis to predict the expression of the CRISPR-Cas system might not accurately describe the effects of all sensors in all conditions, but it might still be useful to explain the interaction of multiple sensors.

Conclusion

There is a seemingly endless array of signaling pathways that can be examined to identify the sensors of CRISPR-Cas regulation. In this review paper, data was gathered about components from the quorum-sensing system, biofilm formation, bacterial alginate synthesis, antibiotic treatments, and the Rcs stress response **(Table 1)**. These networks are of particular interest given their relevance in clinical settings and therapeutic approaches such as genome editing and phage therapies.

Despite the contradictory nature of the data, it has been demonstrated that regulation is both species-specific and strain-specific, small alterations in external conditions can alter the relative influence of different regulatory pathways, and regulation varies with bacterial community organization, external stressors, and growth conditions.

Overall, the regulation of the CRISPR-Cas system is multi-dimensional and lies at the crossroads of numerous intracellular pathways. Within a single organism, different types of CRISPR-Cas systems can be regulated differently[22]. Considering that these different systems can share components such as conserved Cas proteins, it can become increasingly difficult to understand the effects of various regulators and sensors.

Our understanding of CRISPR-Cas regulation remains far from complete. From the data gathered in this review, we propose a few future avenues of research to fill the gaps. Firstly, the use of clinically isolated strains for molecular studies may be advantageous as these bacteria can behave very differently from the model organisms predominantly utilized thus far.

Secondly, studying bacteria encoding a single type of CRISPR system, concurrently with bacteria encoding multiple types of CRISPR systems would provide critical information about the nuances in regulation. As mentioned, different types of CRISPR systems encode conserved proteins with similar functions which renders data interpretation more convoluted. Adding single-system controls would enable a better understanding of system types co-regulation. Thirdly, when assessing the expression level of *cas* genes under various conditions, it is necessary to test more than a single subject protein, especially if Cas genes are induced by distinct promoters. Finally, future studies should consider the effect of various regulators and sensors through a growth-rate lens, as this might provide a unifying insight into the dynamics controlling CRISPR-Cas.

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