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The Role of CRISPR-Cas Systems in Virulence of Pathogenic Bacteria

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SUMMARY

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) genes are present in many bacterial and archaeal genomes. Since the discovery of the typical CRISPR loci in the 1980s, well before their physiological role was revealed, their variable sequences have been used as a complementary typing tool in diagnostic, epidemiologic, and evolutionary analyses of prokaryotic strains. The discovery that CRISPR spacers are often identical to sequence fragments of mobile genetic elements was a major breakthrough that eventually led to the elucidation of CRISPR-Cas as an adaptive immunity system. Key elements of this unique prokaryotic defense system are small CRISPR RNAs that guide nucleases to complementary target nucleic acids of invading viruses and plasmids, generally followed by the degradation of the invader. In addition, several recent studies have pointed at direct links of CRISPR-Cas to regulation of a range of stress-related phenomena. An interesting example concerns a pathogenic bacterium that possesses a CRISPR-associated ribonucleoprotein complex that may play a dual role in defense and/or virulence. In this review, we describe recently reported cases of potential involvement of CRISPR-Cas systems in bacterial stress responses in general and bacterial virulence in particular.

INTRODUCTION

In 1987, a repetitive stretch of DNA was detected on the *Escherichia coli* K-12 chromosome, downstream of the alkaline phosphatase isozyme *iap* gene (1). Similarly organized repetitive elements were found on the chromosomes of *Shigella dysenteriae* and *Salmonella enterica* serovar Typhimurium (2). The physiological role of the repetitive DNA was not obvious at that time. In the subsequent decennium, repetitive sequences were frequently detected in the genomes of both bacteria and archaea (3). A typical feature was the fact that the repeats were interspaced by noncoding, nonrepetitive sequences of similar lengths (3). In 2002, Jansen et al. discovered that these repetitive loci were always accompanied by conserved sets of genes encoding nucleic acid processing enzymes, including nuclease or helicase proteins. The latter authors proposed the names clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (Cas) genes/proteins (4), now referred to as CRISPR-Cas.

In 2005, three groups independently observed that some of the interspaced sequences were 100% identical to DNA sequences from viruses and plasmids; it was proposed that CRISPR-Cas could be a novel defense system (5–7). Comparative genomic analyses revealed that the CRISPRs and their associated (*cas*) genes were present in diverse bacterial phylogenetic groups, resulting in the classification of these genes into several protein families (8–10). In 2007, Barrangou et al. provided the first experimental evidence that the CRISPR-Cas system is an adaptive immune system that protects its host against invading viruses (11). A role for CRISPR-Cas in defense was further established in a range of subsequent studies on viral transfections and plasmid transformations (5, 12–20). In 2011, Makarova et al. (21) suggested the classification of the different CRISPR-Cas systems into the following three main types: type I CRISPR-Cas systems, based on the presence of the *cas3* gene; type II CRISPR-Cas systems, based on the presence of the *cas9* gene; and type III CRISPR-Cas systems, based on the presence of the *cas10* gene. This has now become established nomenclature (21).

An overview of the CRISPR-Cas types in species that are covered in this review is provided in Table 1. A selection of well-studied bacteria, their distribution across the human body, and the diversity of *cas* gene expression is summarized in Fig. 1. In general, most strains of the same species appear to contain identical CRISPR-Cas types, with some exceptions (such as the rare

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TABLE 1 CRISPR-Cas types in the species covered in this review[a]

Species	Presence of CRISPR-Cas system[b]										
	Type I						Type II			Type III	
	A	B	C	D	E	F	A	B	C	A	B
Campylobacter concisus/curvus/fetus/hominis/rectus											
Campylobacter jejuni											
Corynebacterium diphtheriae											
Desulfovibrio vulgaris											
Enterococcus faecalis/faecium											
Erwinia amylovora											
Erwinia pyrifoliae											
Erwinia tasmaniensis											
Escherichia albertii/coli						Few					
Francisella novicida/tularensis											
Lactobacillus rhamnosus											
Lactobacillus salivarius											
Legionella pneumophila											
Listeria monocytogenes											
Mycobacterium tuberculosis											
Mycoplasma gallisepticum											
Myxococcus xanthus											
Neisseria cinerea											
Neisseria gonorrhoeae											
Neisseria lactamica											
Neisseria meningitidis											
Neisseria mucosa											
Pasteurella multocida											
Propionibacterium acnes (type II)											
Pseudomonas aeruginosa											
Salmonella enterica											
Shigella dysenteriae											
Staphylococcus aureus											
Streptococcus agalactiae/dysgalactiae/equi											
Streptococcus gallolyticus											
Streptococcus gordonii											
Streptococcus mutans											
Streptococcus pyogenes											
Streptococcus sanguis			Few								
Streptococcus thermophilus											
Sulfolobus solfataricus											
Treponema brennaborense/saccharophilum											
Treponema denticola											
Treponema succinifaciens											
Yersinia pestis											

[a] In general, most strains of the same species were found to contain identical CRISPR-Cas types.
[b] Few, only a few species within the particular genus were found to contain the respective system.

occurrence of the type I-F system in *E. coli* strains and the type I-C system in some *Streptococcus sanguis* strains). At the genus level, the diversity of CRISPR-Cas systems is somewhat larger. One of the most striking examples is in the *Campylobacter* genus: while most species (*Campylobacter concisus*, *Campylobacter curvus*, *Campylobacter fetus*, *Campylobacter hominis*, and *Campylobacter rectus*) have been found to harbor a type I-B system, *Campylobacter jejuni* contains a type II-C system instead. Most bacterial species seem to contain either one or a combination of two CRISPR-Cas types, although some of the *Streptococcus* species harbor all three types of CRISPR-Cas system. *Helicobacter* species and at least two genera belonging to the *Pasteurellaceae* family (*Haemophilus* and *Pasteurella*) harbor either a type II-C system or a virulence-associated protein D (VapD) that exhibits homology to Cas2. In the sequenced genomes of *Mycobacterium* spp., mainly the type III-A system is observed; in some strains, only the VapD protein is detected. In the sequenced *Clostridium* species, type I-B, type II-B, type II-C, and type III-B CRISPR-Cas systems have been detected. In the sequenced genomes of *Bacillus* species, mainly type I-B and type I-C CRISPR-Cas systems have been observed, whereas in *Bacillus cereus*, only a large CRISPR array has been found, reminiscent of a degenerate CRISPR-Cas system. Also apparent is the complete absence of the type I-D system, which so far has been found in genomes of only a few pathogenic bacteria (21), in the species covered in this review.

Whereas type I and type III CRISPR-Cas systems share some common features (21), the type II system is rather unique. Apart from a conserved set of *cas* genes (*cas1*, *cas2*, and *cas9*), three

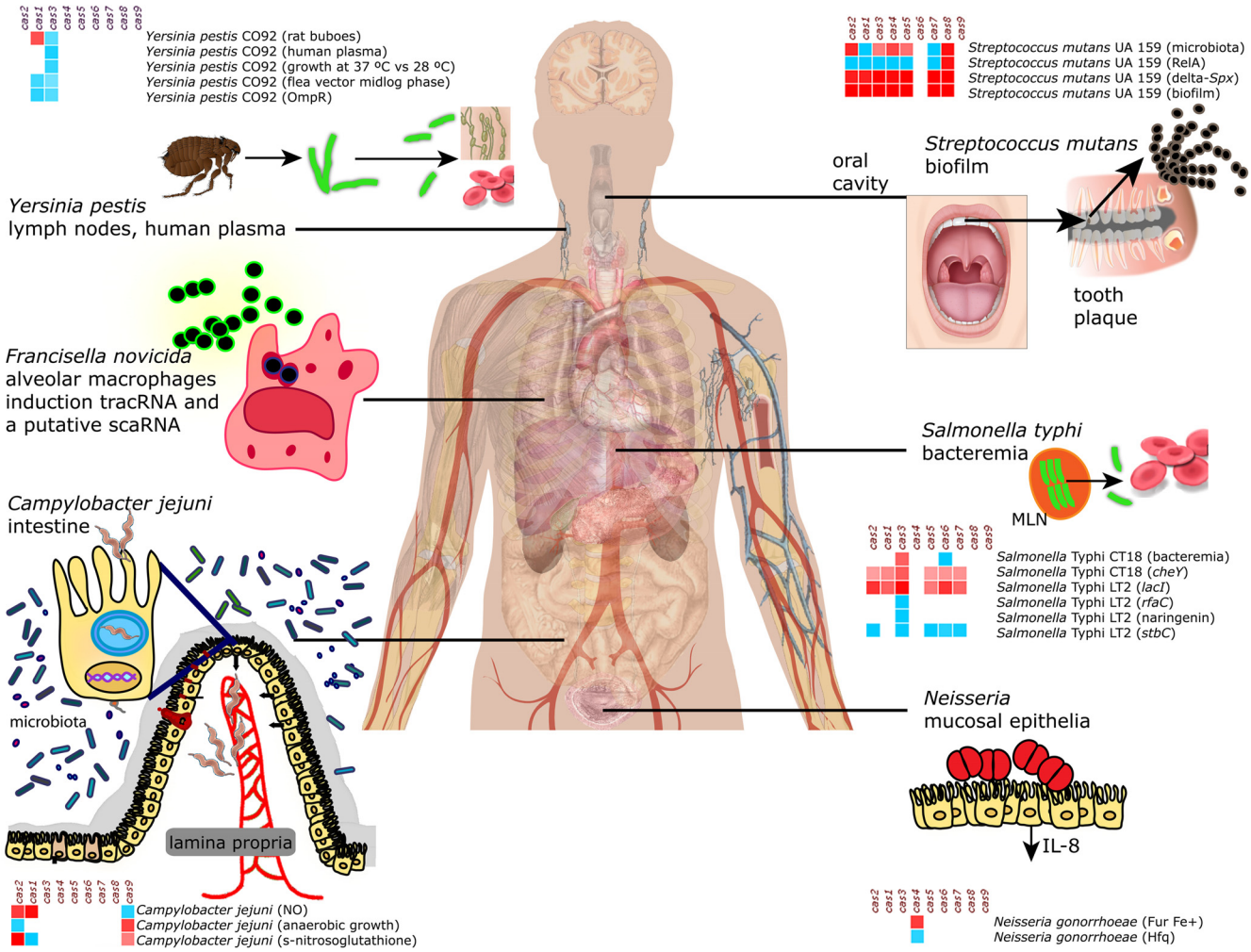

FIG 1 Overview of expression of *cas* genes in human-associated bacteria that occupy different host niches. The heat maps indicate which *cas* genes are induced (shades of red) or repressed (shades of blue) during bacterial responses to changes in the environment. Details are given in the main text. The overview shows that modulation of *cas* gene expression occurs in diverse Gram-positive and Gram-negative bacteria that together occupy very diverse niches throughout the human body. For *F. novicida*, adaptation of gene expression in macrophages depends on Cas9, tracRNA, and possibly also scaRNA, which together inhibit expression of an immunogenic lipoprotein (shown in green) (22). All bacteria depicted in this figure possess Cas9, and scaRNA production has been predicted for *F. novicida*, *C. jejuni*, *L. monocytogenes*, and *N. meningitidis* (22), suggesting that a role of Cas9 in regulation of bacterial gene expression may be more widespread.

variant subtypes have been recognized: type II-A, with an additional *csn2* gene; type II-B, with an additional *cas4* gene; and type II-C, with no additional gene (21) (Fig. 2). The usual type II CRISPR-Cas system genomic arrangement is that the *cas* operons are adjacent to the CRISPR array, together with a DNA sequence encoding a *trans*-activating CRISPR RNA (tracrRNA). tracrRNA is partly complementary to the repeat part of the immature CRISPR-encoded RNA (crRNA): Watson-Crick base pairing results in an RNA duplex that, while associated with Cas9, is further processed by RNase III (20). Interestingly, type II CRISPR-Cas systems occur only in bacteria, not in archaea. Moreover, type II CRISPR-Cas systems are overrepresented in bacteria that use vertebrates as a host, including a wide variety of pathogens (8, 20, 22). Here we review recent insights into the role of CRISPR-Cas systems in the virulence of mainly pathogenic bacteria, but we also describe recently reported links between CRISPR-Cas and general stress responses in nonpathogenic bacteria.

CRISPR-Cas AS A TYPING TOOL

Major discoveries in CRISPR-Cas research, including the elucidation of its role in bacterial virulence, are highlighted in a timeline in Fig. 3. Initially, before an understanding of their physiological role, CRISPR-Cas systems were found to be a useful tool for typing bacterial diversity (23). In *Mycobacterium tuberculosis* (type III-A), for instance, CRISPR variability has been a gold standard for routine genotyping purposes and to study the epidemiology of *M. tuberculosis* (24–27). However, limitations exist for the use of CRISPR typing in evolutionary studies, because it is impossible to study which or how many evolutionary events are responsible for the loss of a cluster of neighboring spacers from the CRISPR (27). In 2003, CRISPR-Cas typing of *C. jejuni* was explored for the first time to reveal phylogenetic relationships between strains in population biology and epidemiology studies (28). For *C. jejuni* (type II-C), CRISPR typing alone appeared not to be useful, since the spacers were too diverse. However, a combination of CRISPR typ-

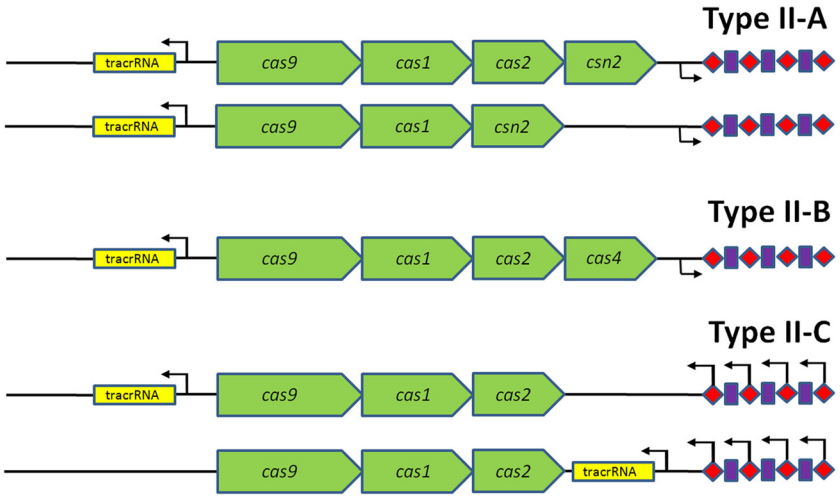

FIG 2 Overview of the three type II CRISPR-Cas subtypes. All three subtypes share a conserved set of *cas* genes: *cas1*, *cas2*, and *cas9*. Type II-A has an additional *csn2* gene, and type II-B has an additional *cas4* gene (21). Type II-C does not feature an additional *cas* gene beyond the three conserved *cas* genes (122). All subtypes feature a small *trans*-encoded RNA called *trans*-activating CRISPR RNA (tracrRNA); type II-C displays variation in the location of tracrRNA. *cas9*, *cas1*, and *cas2* are indicated with green arrows, and tracrRNA is shown with yellow boxes. Transcription start sites are shown as black arrows upstream of the repeats (red diamonds) and spacers (purple squares) in type II-A (e.g., in *Streptococcus* spp.) and -B (e.g., in *Legionella pneumophila*) CRISPR loci, or within each spacer in the case of the minimal type II-C CRISPR systems of *Neisseria meningitidis* (upper) and *Campylobacter jejuni* (lower) (122).

ing with amplified fragment length polymorphisms (AFLP) and multilocus sequence typing (MLST) techniques enhanced the discriminatory power, enabling subtyping of *C. jejuni* isolates (28). The CRISPR typing techniques for *C. jejuni* were further optimized, resulting in a technique called CRISPR high-resolution melt analysis (29). Six years later, exploration of sequence variation in the *C. jejuni* CRISPR-Cas locus established that (non)synonymous polymorphisms in the *cas* genes were linked to the pres-

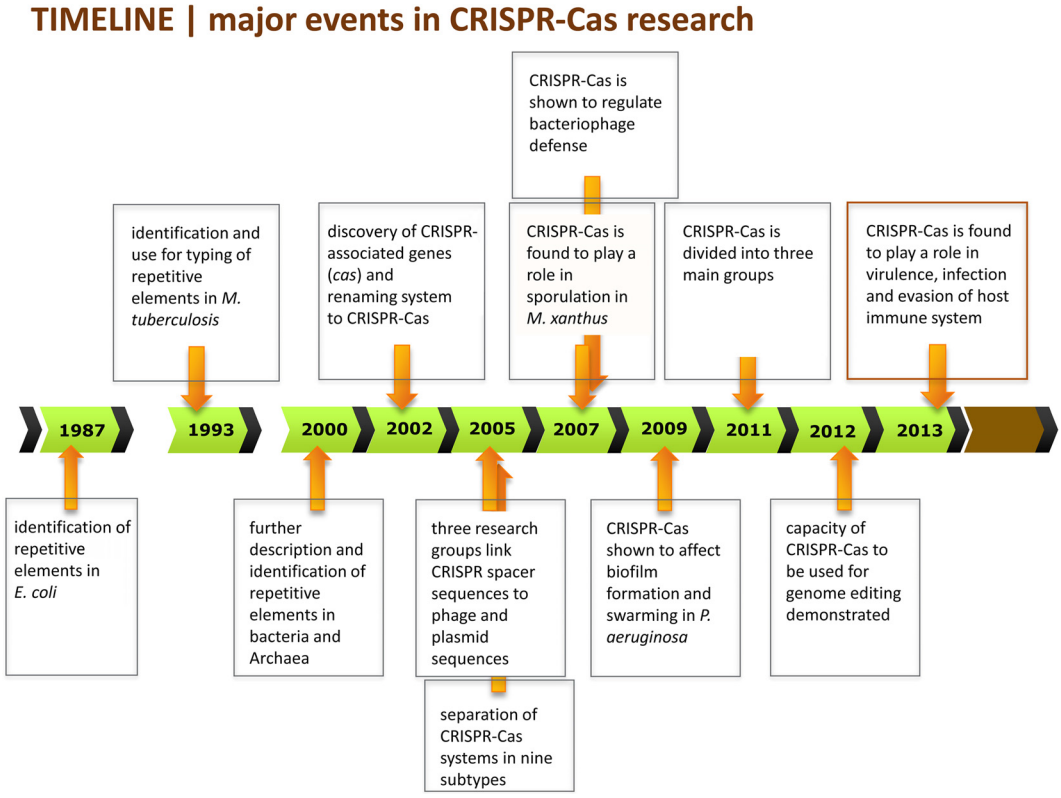

FIG 3 Overview of the most important discoveries in CRISPR-Cas research. The original papers describing the major findings are discussed and cited in the main text.

ence of a gene found specifically in clinical *C. jejuni* isolates retrieved from Guillain-Barré syndrome (GBS) patients (30, 31). Louwen et al. thus established that not only the spacer variation of the CRISPR array but also single nucleotide polymorphisms (SNPs) of the *cas* genes in *C. jejuni* are useful for typing purposes (30). Likewise, in *Corynebacterium diphtheriae*, the CRISPR-Cas systems (type I-E and type II-C) were found to be useful for typing purposes (32, 33). Remarkably, spoligotyping, a technique making use of sequence information contained within the CRISPR spacers, was found to provide enhanced discriminatory power in *C. diphtheriae* compared to pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), and AFLP analyses (33). Furthermore, spoligotyping had discriminatory power for subtyping of *Legionella pneumophila* strains and enabled the identification of environmental sources that caused clinical outbreaks (34).

As described above for the type II CRISPR-Cas system, the type I CRISPR-Cas system was found to be useful for typing of *Yersinia pestis* (type I-F) and *Salmonella* (types I-E and I-F) strains (35, 36). In *Y. pestis*, CRISPR typing was able to identify the origin of the ancestor strains that caused the black plague (36). Whereas it had been assumed that the black plague originated from Mongolia, CRISPR typing suggested that this region harbored a different, less virulent *Yersinia* species (*Yersinia microtus* clade) (37). A separate study had linked geographical sources to outbreaks of *Y. pestis* isolates based on sequence variation within the CRISPR array (38). From this study, it was concluded that the more virulent *Y. pestis* strains could have originated from China and Tajikistan (38). Interestingly, 59% of the 131 studied spacers harbored strong identity to a protospacer in a *Y. pestis* prophage (38), unfortunately without details addressing the correlation between the CRISPR genotype and virulence.

In *Salmonella*, established techniques such as combining MLST with virulence gene polymorphisms were not able to distinguish between individual outbreak strains, rendering subtyping of *Salmonella* isolates causing food-related infections during outbreaks impossible (39). The use of *Salmonella* CRISPR genotypes, either alone (39) or in combination with gene polymorphisms present in virulence-associated genes (35, 40), has strongly improved the ability to separate *Salmonella* strain collections into individual outbreak isolates. Thus, elevated acquisition and turnover of CRISPR spacers of type I-F and I-E CRISPR-Cas systems in *Y. pestis* and *Salmonella* spp., respectively, do allow for high-resolution typing. Indeed, a recent study of *Salmonella enterica* established that CRISPR typing in combination with a method exploiting the hypervariability of the virulence genes, CRISPR-MVLST, was useful for subtyping purposes (41).

In several other bacterial species, typing by making use of CRISPR array polymorphisms has shown some promise in delineating subgroups with biologically relevant characteristics. For the plant pathogen *Erwinia amylovora*, (type I-E) CRISPR typing enabled the separation of this bacterium into three major groups that reflected their geographic origins (42).

For *Propionibacterium acnes*, CRISPR typing revealed three lineages that had also been identified in previous typing studies (43). Remarkably, for this bacterium, there was a profound difference in virulence between the first lineage and the other two. Lineage I was associated with infections of sebaceous glands of the human skin and inflammatory acne, whereas lineages II and III caused more severe deep tissue infections (43). Lineage I was found to contain integrated phages and plasmid DNA, but it lacks a CRISPR-Cas system and harbors only the *cas2* and *cas1* genes (44). Lineage II has a complete type I-E CRISPR-Cas system with at least eight *cas* genes, and lineage III has four *cas* genes, possibly reflecting a degenerate type I-E system (44). The CRISPR-Cas-bearing lineages II and III are more invasive (44), which makes them interesting candidates for generating *cas* gene knockouts for each *P. acnes* lineage and investigating if the differential presence of CRISPR-Cas genes is a major genomic factor explaining their virulence. In 2012, Marinelli et al. revealed that some *P. acnes* strains harboring a complete type I-E CRISPR-Cas system comprising spacers with 100% identity against specific bacteriophage protospacers were resistant to these bacteriophages (45). This study also revealed that strains belonging to the more invasive lineages, but lacking such spacers or a complete CRISPR-Cas system, were lysed by these bacteriophages, suggesting that bacteriophage therapy might eventually be used to treat acne (45).

DIFFERENT EFFICIENCIES OF CRISPR-Cas TYPING

In enterococci, the presence or absence of CRISPR-Cas provides the ability to distinguish antibiotic-resistant species, with a wide variety of plasmids carrying antibiotic resistance genes, from less resistant species (46). van Schaik et al. sequenced seven *Enterococcus faecium* strains, including four clinical isolates and three fecal isolates. It was observed that all seven isolates contained a type II-A *cas* operon that did not include a *cas1* gene. Since *cas1* encodes a nuclease that is involved in spacer acquisition (47), this probably implies that in these isolates the CRISPR system had lost its potential for CRISPR adaptation (48). On the other hand, it cannot be ruled out that CRISPR interference is still effective despite the loss of *cas1*. Note that increased antibiotic resistance as well as the uptake of phages and pathogenicity islands by this bacterium was found to be associated with deletions of *cas* genes (48). Indeed, in a recent Canadian study, the emergence of ampicillin-resistant *E. faecium* isolates was associated with a total absence of CRISPR sequences (49). An anticorrelation between the presence/absence of a CRISPR-Cas system and the absence/presence of mobile elements providing antibiotic resistance was also observed in methicillin-resistant *Staphylococcus aureus* (MRSA) isolate ST779 (50). This isolate had acquired a composite island (CI) element (including methicillin resistance genes) that had integrated into the genome. In addition, this strain was found to harbor a type II-C CRISPR-Cas system, possibly resulting from a different integration event (50). Both the resistance genes and the type II-C CRISPR-Cas system were suggested to originate from coagulase-negative staphylococci (CoNS) (50), which are commensal bacteria that lack the ability to clot blood by the enzyme coagulase, a staphylococcal virulence factor. Links between the presence/absence of CRISPR systems and horizontal transmission of antibiotic resistance and virulence genes from CoNS to *S. aureus* are thought to be more prevalent than previously considered (51).

For enterohemorrhagic *E. coli* (EHEC) bacteria, (type I-E/I-F) CRISPR polymorphisms were found to correlate with the presence of two EHEC virulence genes, *stx* and *eae*, encoding the phage-delivered Shiga toxin and the intimin virulence factor, respectively (52). Interestingly, the CRISPR polymorphisms were found to provide a more specific typing profile than the established techniques, which were based on *stx* and *eae* gene polymorphisms alone or together with O:H serotypes (52). This suggests that a significant correlation exists between CRISPR genotypes

and an isolate's virulence. Potential causality between CRISPR spacers and virulence is discussed below. In other *E. coli* strains, it is questionable whether the CRISPR is useful for diagnostic, epidemiology, or evolutionary studies. In some *E. coli* strains, it is observed that the CRISPR arrays not only are relatively small but also appear to have remained unaltered for evolutionarily relevant periods (hundreds of thousands of years), which argues against a role as an active bacterial immune system in these strains (53). This observation was corroborated by a study where the CRISPR-Cas system in *E. coli* was not found to provide strong resistance against the spread of antibiotic resistance plasmids (54). Although the *E. coli* CRISPR-Cas system has often been reported as static and small, a recent study suggests that this might be beneficial for separating commensal fecal *E. coli* isolates from more pathogenic variants (55). The observation that short or absent CRISPRs correspond with increased pathogenicity is in line with a recent study in *C. jejuni*, in which strains causing severe gastroenteritis and postinfectious complications also harbored short CRISPRs or completely lacked the CRISPR array (30).

Genome sequencing of a highly virulent *Streptococcus pyogenes* strain with a high transformation efficiency revealed the presence of a type II-A CRISPR-Cas system (56). In a subsequent study, 13 *S. pyogenes* strains were sequenced. Two distinct CRISPR loci with relatively small numbers of spacers compared to those in other streptococci were detected; five strains harbored a CRISPR-Cas system with *cas* gene deletions, and these strains contained larger numbers of prophages than the other isolates, which harbored a typical type II-A CRISPR-Cas system (57). Spacer analysis revealed that in four isolates, one spacer targeted a protospacer on its own chromosome, i.e., in a prophage-carried gene (57). Nozawa et al. concluded that the limited presence and activity of CRISPR-Cas systems in *S. pyogenes* have allowed the introduction of virulence genes by phages into *S. pyogenes*, thereby contributing to the strain-specific pathogenicity that is characteristic of this species (57).

There is some experimental evidence pointing at an interplay between CRISPR-Cas systems, mobile genetic elements, and host range. An association between a reduction of antibacteriophage CRISPR activity and bacterial virulence was found in *Mycoplasma gallisepticum* bacteria that can infect several bird species (58); these bacteria harbor a type II-A CRISPR-Cas system. During a host switch from poultry to a songbird, a strong reduction in CRISPR spacer diversity and a loss of all of the type II *cas* genes were observed (58). The authors concluded that the extremely rapid evolution of the bacterial genomes, including the CRISPR degradation following the host shift, pointed to an involvement of mobile genetic elements.

We can conclude that CRISPR typing, either alone or sometimes in combination with other markers, has been used successfully for strain typing. In some cases, CRISPR typing has enabled the characterization and identification of outbreak strains at the serotype and genomic subgroup levels in epidemiological or evolutionary contexts. For instance, variability in CRISPR repeat numbers and *cas* gene presence has allowed for clustering of clinical enterococcal isolates into subgroups of highly and lowly antibiotic-resistant isolates and for grouping of *C. jejuni* strains into isolates that induce either postinfectious complications or merely gastroenteritis. Phages and plasmids potentially play important roles in bacterial virulence, e.g., as delivery vehicles of antibiotic resistance and virulence genes (59, 60). Therefore, it is not surprising that grouping of bacterial isolates based on variations in antiphage CRISPR-Cas systems may yield groups of isolates that differ with respect to clinically relevant virulence features. In some peculiar cases, the persistent sequence conservation of the CRISPR-Cas system as observed in *E. coli* or the profound alterations in this system as observed in *Legionella* and *C. jejuni* can negatively affect the discriminatory power of variation in the CRISPR element for techniques such as spoligotyping. Remarkably, in contrast to the established CRISPR typing techniques, a strongly reduced or absent CRISPR in *E. coli* and *C. jejuni* enhanced the discriminatory power between pathogens and less virulent or commensal isolates belonging to the same species, respectively, whereas in enterococci, absence of the CRISPR array was associated with increased antibiotic resistance. In conclusion, the discriminatory power of CRISPR-Cas systems can be extremely high in diagnostic, epidemiology, evolutionary, virulence, and antibiotic resistance studies when they are used alone or in combination with other typing techniques, including MLST and PFGE.

FUNCTIONAL DIVERSITY OF CRISPR-Cas SYSTEMS IN BACTERIAL PATHOGENS

The increase in availability of sequenced bacterial, bacteriophage, and plasmid genomes has provided more detailed insights into CRISPR-Cas variation, as well as in the mobile elements that are targeted by CRISPR systems. In 2005, Mojica et al. suggested that on an evolutionary time scale, the pathogenicity of natural prokaryotic populations is largely controlled by bacteriophages and conjugative plasmids and that CRISPR spacers targeting these mobile elements might therefore affect bacterial evolution, including pathogenicity and virulence (5). As mentioned above, CRISPR activity may interfere with the uptake of bacteriophage DNA carrying virulence genes, including toxin and antibiotic resistance genes (51).

The early comparative analyses of CRISPR spacers revealed sequence homology not only to "nonself" DNA of mobile genetic elements but occasionally also to "self," endogenous chromosomal DNA (5). A multigenome analysis (61) revealed that 1 in every 250 spacers is self-targeting and that such self-targeting occurs in 18% of all CRISPR-bearing organisms. Although the presence of self-spacers has been suggested to allow control of gene expression, the above-mentioned study (61) proposed that (at least in some cases) self-targeting is a form of autoimmunity. The complete lack of conservation of these self-spacers across species, combined with the cooccurrence of degraded repeats near self-targeting spacers, strongly suggests that the acquisition of these spacers is harmful to the stability of the host genome. Indeed, the incorporation of foreign chromosomal fragments with homology against endogenous genes as new CRISPR spacers has been demonstrated to occur frequently in the absence of an active interference system (47). When a complete, active CRISPR-Cas system is present, acquisition of self-targeting spacers is detrimental to genome integrity, and this autoimmunity issue may explain the abundance of degenerated CRISPR systems in prokaryotes with self-targeting spacers. Apart from that, the recent discovery of "CRISPR inhibitors" that reside in certain prophages may also explain the occurrence of self-targeting CRISPR spacers (62). The incidental incorporation of sequences as CRISPR spacers with high identity to endogenous genes thus suggests a role for self-targeting spacers in the regulation of endogenous gene expression

in those cases where this type of autoimmunity is not lethal to the bacterium.

In a study on the virulence of *Enterococcus faecalis* isolates, a mouse urinary tract model (63) was used to analyze two strains: one with and one without a type II-A CRISPR-Cas system (64). Initially, the virulence of the type II CRISPR-Cas-harboring strain appeared to be lower (the 50% lethal dose [LD_{50}] was higher); however, when equal inocula of both strains were used, the CRISPR-Cas-harboring strain induced a more rapid mortality in the mice (64). Histological examinations showed that the CRISPR-Cas-harboring strain had an increased capacity to form biofilms, and as such, it did colonize the organs of the mouse more efficiently than the isolate lacking the system (64). In conclusion, these *Enterococcus* studies suggest that CRISPR-Cas systems in addition to other genomic differences may influence bacterial pathogenicity via two non-mutually exclusive processes: on the one hand, defense by CRISPR-Cas may reduce the potential bacterial virulence when mobile elements could introduce foreign DNA carrying potential virulence factors (toxins or antibiotic resistance genes), whereas on the other hand, control of gene expression by CRISPR-Cas may enhance bacterial virulence, e.g., by promoting host colonization.

Kuenne et al. studied the genome sequences of 16 *Listeria monocytogenes* strains and divided the detected CRISPR-Cas systems into three different loci (65). CRISPR-Cas locus 1 was characterized by a single CRISPR array, CRISPR-Cas locus 2 belonged to type I-B, and CRISPR-Cas locus 3 was classified as type II-A (8, 21, 65). Interestingly, CRISPR-Cas locus 1 had previously been associated with the presence of a tracrRNA suggested to control virulence in *L. monocytogenes* strain 1/2a EGD-e during growth in macrophages (66), but it remained mechanistically unknown how this *trans*-acting noncoding RNA could regulate virulence. The suggestion that an antisense RNA interference system could form the basis for controlling bacterial pathogenicity in this *L. monocytogenes* isolate (66) was close to the actual molecular mechanism identified in a different bacterial species (see below).

To summarize, analyses of genomic sequences of diverse pathogenic bacteria and their virulence features suggest a role of CRISPR-Cas in processes other than defense, e.g., a potential involvement in regulation of endogenous gene expression (5, 67), including that of genes involved in virulence (57, 65). In the aforementioned examples, direct or indirect links between CRISPR-Cas and control of virulence were suggested but were not explored further. Below, we discuss recently reported studies in which more convincing evidence for the involvement of CRISPR-Cas in control of bacterial stress responses, including responses to host immunity, was obtained.

TRANSCRIPTIONAL INDUCTION OF THE CRISPR-Cas SYSTEM UPON STRESS

In *Myxococcus xanthus*, a gene operon, previously named *dev*, represents a type I-B CRISPR-Cas system in which the *cas* genes and CRISPR cassette are activated during stress (68). Expression of this operon contributes to the development of fruiting bodies from which bacterial spores are released (68). A few candidate CRISPR spacers might be involved in the regulation of this sporulation event in *M. xanthus* (68, 69), but no mechanistic connection has been established between these spacers and fruiting body development. An interesting hypothesis put forward was that mutations in one or more of the *cas* genes have led to exaptation of the *M. xanthus* CRISPR-Cas system into a regulatory system that controls the stress-dependent development of fruiting bodies (69).

In *E. coli*, stress on the cell envelope may result in an induction of *cas* gene expression (70). Apart from regulation by H-NS, LeuO, and Rcs/BglJ (71, 72), a two-component signal transduction system named BaeSR has been found to activate the expression of the *E. coli cas* genes (70). Perez-Rodriguez et al. established that the periplasmic expression of a green fluorescent protein (GFP) reporter (fused to the Tat-dependent excretion signal peptide of trimethylamine *N*-oxide reductase [ssTorA]) was diminished in the absence of the chaperone DnaK (70). Random mutagenesis of this *E. coli* DnaK mutant revealed that deletion of the type I-E *cas* operon restored the expression of ssTor-GFP, as did deletion of the BaeSR system (70). Hence, the CRISPR-Cas system appeared to be expressed by BaeSR upon cell envelope stress. Subsequently, upon its expression, the CRISPR-Cas system was found to target ssTorA-encoding sequences via partly complementary spacers, directly affecting protein transport across the bacterial membrane (70). Although the molecular details are not well understood, this study again suggests that the function of CRISPR-Cas goes beyond viral defense and plasmid conjugation.

TRANSCRIPTOME ANALYSIS OF THE *cas* GENES

In several bacterial species, transcriptome studies have revealed that deletion of transcriptional regulators affects *cas* gene expression. For example, in *Y. pestis* CO92, the regulator OmpR was found to control the composition of the outer membrane and to be required for virulence and survival in macrophages (73). Deletion of OmpR in *Y. pestis* CO92 resulted in differential expression of 224 genes, including repression of *cas1* transcription (Fig. 1 and 4) (73). The *cas1* gene was also slightly downregulated during the preadaptation phase, when the *Y. pestis* CO92 strain adapted itself to the environment in fleas that parasitize rats (Fig. 1 and 4). In fleas, which present a low-temperature environment, *Y. pestis* forms a biofilm that promotes transmission when the fleas bite a rat host. Upon transmission to a rat, the temperature shift to 37°C induces production of *Y. pestis* virulence factors that confer resistance to innate immunity responses of the host. When the transcriptome of the *Y. pestis* CO92 wild-type strain that was used in the OmpR study was determined after passage in rats, *cas1* transcription was induced (Fig. 1 and 4). Escape of *Y. pestis* from rat lymph nodes is characterized by systemic spread, leading to fatal sepsis (74). During dispersion, to escape the innate immune system, *Y. pestis* has evolved a strong adaptive response against nitric oxide (NO) (74), which is a free radical that is released in immune cells when bacteria are phagocytosed. NO molecules are highly toxic for bacteria by causing DNA damage (75). Escape from the innate immune response coincided with differential transcription of *cas1* (Fig. 1 and 4), among other gene transcripts (73). It is noteworthy that *Y. pestis* OmpR and Cas1 are involved in the stress response (73), which is reminiscent of the involvement of transcriptional regulators and the CRISPR-Cas systems in the stress responses of *E. coli* and *M. xanthus*.

E. coli and *S. enterica* are closely related, and both harbor a type I-E CRISPR-Cas system (21, 76). The *E. coli lac* operon is important for maintaining fitness in the presence of lactose, and its expression is regulated by LacI. Both the *lac* operon and the LacI repressor are absent in *Salmonella* spp. (76). Eswarappa et al. addressed why the *lac* operon is absent from *Salmonella* and not from *E. coli*, whereas both *E. coli* and *Salmonella* are exposed to

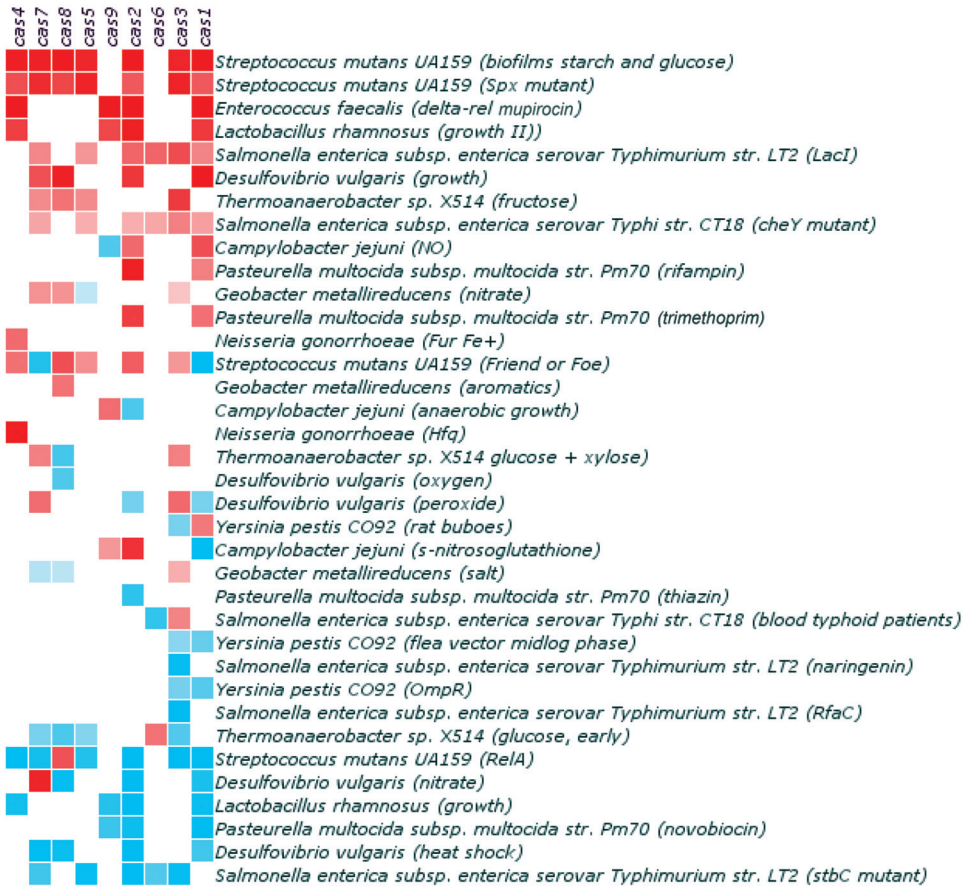

FIG 4 Heat map showing expression of *cas* genes in human-associated bacteria that occupy different host niches. The heat map indicates which *cas* genes are induced (shades of red) or repressed (shades of blue) during bacterial responses to changes in the environment. All gene expression data displayed in this figure have been published, are publicly available in the MicrobesOnline (http://www.microbesonline.org/) and NCBI Entrez (http://www.ncbi.nlm.nih.gov/gene/) gene expression databases, and are further discussed in this review.

lactose in the mammalian gut (76). These authors showed that introducing the LacI repressor into *S. enterica* reduced virulence by affecting the transcription of genes on the SpI-1 and SpI-2 pathogenicity islands, while a lack of LacI enhanced the virulence of this bacterial species via SpI-2 (76). Introduction of the LacI repressor was also concomitant with reduced mouse serum resistance and a significantly induced transcription of the *S. enterica cas* genes (Fig. 1 and 4) (76). In a different study, transcriptomes were obtained for five clinical *S. enterica* serovar Typhi strains that were isolated from the blood circulation system; these transcriptomes were compared to those of *in vitro*-grown *S.* Typhi strains (77). The 331 transcripts that were altered also included multiple *cas* genes (Fig. 1 and 4), suggesting that the *Salmonella* CRISPR-Cas system is involved in infection *in vivo* (77). Also in *C. jejuni*, changes in *cas* gene expression were observed during intestinal passage in a mouse model (78).

E. faecalis is a bacterial species that can cause opportunistic infections of the intestine. The GTP pyrophosphokinase (RelA) of this bacterium, involved in (p)ppGpp biosynthesis during amino acid starvation, was shown to play an important role in stress adaptation and virulence (79). Notably, in *E. faecalis*, the stress response is controlled by the bifunctional synthetase/hydrolase RelA and the monofunctional guanosine pentaphosphate synthetase RelQ, by regulating the production of the effector molecule (p)ppGpp (80). When *E. faecalis relA* mutant and wild-type strains were treated with antibiotics, a strong downregulation of transcription was detected for a wide variety of genes (79), including the *cas* genes. In contrast, in a double *relA relQ* mutant, expression of the *cas* genes was induced (Fig. 4). In addition to *E. faecalis*, expression of RelA has been shown to occur in a context of reduced virulence in *S. enterica* serovar Typhimurium, *M. tuberculosis*, *Vibrio cholerae*, and *L. monocytogenes* (79). How RelA and RelQ control the regulation of *cas* gene expression is unknown; it appears that the *cas* genes function together with the RelAQ system during the stress response and the rapid adaptation to potentially unfavorable changes in the bacterial environment.

In *Streptococcus mutans*, the causative agent of tooth decay, mutations in virulence or global regulatory genes (including genes involved in stress responses) strongly affect gene transcription (81–85), including that of the type II *cas* genes (Fig. 1 and 4). In a protease gene deletion mutant of *S. mutans*, the *cas* genes were differentially transcribed compared to those of wild-type bacteria (Fig. 4). Likewise, deletion of genes from the *spx* operon, which is involved in the regulation of the stress response in survival and virulence features, led to an induction in transcription of the *cas* genes (Fig. 1 and 4). Five *cas* genes belonging to the type I-E and type II-A systems were induced in response to commensal bacteria or microbiota, and two *cas* genes, including *cas1*, were downregu-

lated (Fig. 1 and 4). All of the *cas* genes in *S. mutans* were induced during biofilm formation in the presence of starch or sucrose (Fig. 1 and 4). In short, gene transcription data for *S. mutans* suggest that the *cas* genes of the two different CRISPR-Cas systems present in this bacterial species are activated during stress. Functional analysis of *cas* gene deletion mutants could shed light on the diverse involvement in stress responses of the *cas* genes of *S. mutans*.

Changes in *cas* gene expression in response to stress appear to be a general phenomenon. In *Desulfovibrio vulgaris* (86), *Streptococcus sanguinis* (87), *Pasteurella multocida* (88), *Lactobacillus rhamnosus* (89, 90), and *C. jejuni* (MicrobesOnline database [http://www.microbesonline.org]), it has been demonstrated that *cas* gene transcription is commonly altered in response to changes in growth, bile stress, reactive oxygen species (ROS) and nitrosative stress, antibiotics, and expression of genetic competence (Fig. 1 and 4). In addition, several studies support the idea that regulatory factors involved in stress and virulence, such as LacI in *S. enterica*, OmpR in *Y. pestis*, RelA and RelQ in *S. mutans*, and BaeSR/DnaK in *E. coli*, might somehow interact with the CRISPR-Cas systems of these bacteria. Virulence is a specific stress response of pathogenic bacteria during host infection, resulting in the coordinated expression of genes encoding virulence factors, including host colonization and survival factors. The findings listed above suggest that there might be a more general involvement of CRISPR-Cas in virulence than previously appreciated, although the evidence is circumstantial and descriptive, not providing clues for a molecular mechanism. For the remainder of this review, we discuss findings that have led to the unambiguous demonstration that at least in some bacteria, the CRISPR-Cas system does play an important role in regulating the expression of virulence genes.

CORRELATION OF CRISPR-Cas WITH BACTERIAL VIRULENCE

Legionella pneumophila strain 130b harbors a type II-B CRISPR-Cas system whose *cas2* and *cas1* genes were induced during intracellular growth in macrophages and aquatic amoebae (91). Analysis of knockout mutants of the type II-B CRISPR-Cas system showed that *cas9*, *cas1*, and *cas4* were not essential for growth in macrophages and aquatic amoebae. In contrast, disruption of *cas2* was found to affect intracellular survival and replication in amoebae (91). This study indicated that the type II CRISPR-Cas system of *L. pneumophila* plays an important role in withstanding stress encountered during intracellular growth in amoebae.

In 2009, Zegans et al. showed that in *Pseudomonas aeruginosa*, CRISPR-Cas was involved in biofilm formation and swarming, which are important characteristics of *P. aeruginosa* virulence (92). These authors observed that infection of *P. aeruginosa* isolate PA14 with the bacteriophage DMS3 blocked biofilm formation and swarming motility (92) and that these changes were dependent on the presence of a type I-F CRISPR-Cas system (92). A follow-up study demonstrated that a specific spacer present in the type I-F CRISPR array was required to inhibit biofilm formation (93). It was hypothesized that transcription of the specific spacer resulted in production of an antisense RNA that was involved in knockdown of the gene involved in *P. aeruginosa* biofilm formation (93). These findings and their associated hypotheses indicated that in addition to involvement in viral defense, CRISPR-Cas systems could indeed be involved in the regulation of expression of virulence genes.

Cas9 AS A REGULATOR OF BACTERIAL VIRULENCE

A wide variety of important pathogens of mammals bear a type II CRISPR-Cas system, including major pathogens such as *L. monocytogenes*, *S. pyogenes*, *Streptococcus agalactiae*, *Neisseria meningitidis*, *C. jejuni*, *Haemophilus influenzae*, and *Helicobacter pylori* (8, 20, 30, 94). These pathogens are able to cause acute or chronic damage to the host (95, 96) or are linked to (postinfectious) complications (97–107).

Which virulence factor(s) could be shared by all these diverse type II CRISPR-Cas-bearing pathogens? Type II CRISPR-Cas systems are represented by the CRISPR-Cas system that was originally found in the *N. meningitidis* isolate Z2491 (21) and later established by Barrangou et al. to be a functional viral defense system in *Streptococcus thermophilus* (11). Next, an important feature of *N. meningitidis* is the ability to express sialylated lipooligosaccharide (LOS) structures on its cell envelope (108). *C. jejuni*, *H. influenzae*, *H. pylori*, and *P. multocida* are also able to sialylate LOS by using species-specific sialyltransferase enzymes (108). *C. diphtheriae*, *L. monocytogenes*, *M. gallisepticum*, *S. pyogenes*, *S. agalactiae*, and *S. mutans* all produce sialidases to remove sialic acid from host glycoproteins in order to uncover host adhesion receptors (108). The enzymatically released sialic acids are then used either as an energy source or as building blocks for incorporation into the bacterial cell envelope (108–113), where they contribute to serum resistance (114). For *N. meningitidis*, *H. influenzae*, and *C. jejuni*, the sialylation of the cell envelope was found to be an important virulence factor that strongly contributed to the ability of these pathogens to adhere to, invade, and translocate across epithelial cells and to evade host immune responses (115–119). Sialylated cell envelopes produced by *C. jejuni* and *H. influenzae* have been linked to the induction of the postinfectious sequela Guillain-Barré syndrome (GBS) (102). In addition to a role in virulence, sialylated LOS produced by Gram-negative bacteria has been proposed to play a role in viral defense (120). Indeed, incorporation of sialic acid into LOS structures present on the cell envelope of *C. jejuni* can protect this bacterium from viral infections (30). Remarkably, deletion of *cas9* in GBS-inducing *C. jejuni* isolates abolished the ability of these isolates to translocate across polarized intestinal epithelial cells, indicating that *C. jejuni* Cas9 not only protects against infection by viruses but also is crucial for virulence (30). An important observation is the fact that *cas9* deletion mutants with sialylated LOS bind more strongly to human serum (30), indicating that Cas9 and sialylated LOS both might play important roles in avoiding immune recognition of *C. jejuni*. As proof of principle, supplementation of *cas9* in an isolate lacking a CRISPR-Cas system led to a significant increase of virulence of this isolate, showing that Cas9 is indeed important for virulence of *C. jejuni* (30). The question remained: how could Cas9 influence virulence? An intriguing answer to this question has now been provided for a different Gram-negative pathogen, i.e., *Francisella novicida*.

In *F. novicida*, overproduction of a specific bacterial lipoprotein (BLP) in the bacterial membrane significantly decreased bacterial survival in host macrophages, and regulation of production of specific BLP strongly contributed to innate immune evasion (121). Sampson et al. demonstrated that predicted antisense base pairing between a 3′ extension of the tracrRNA, in a complex with Cas9, and a complementary BLP mRNA molecule forms a double-stranded RNA (dsRNA) complex that eventually leads to the deg-

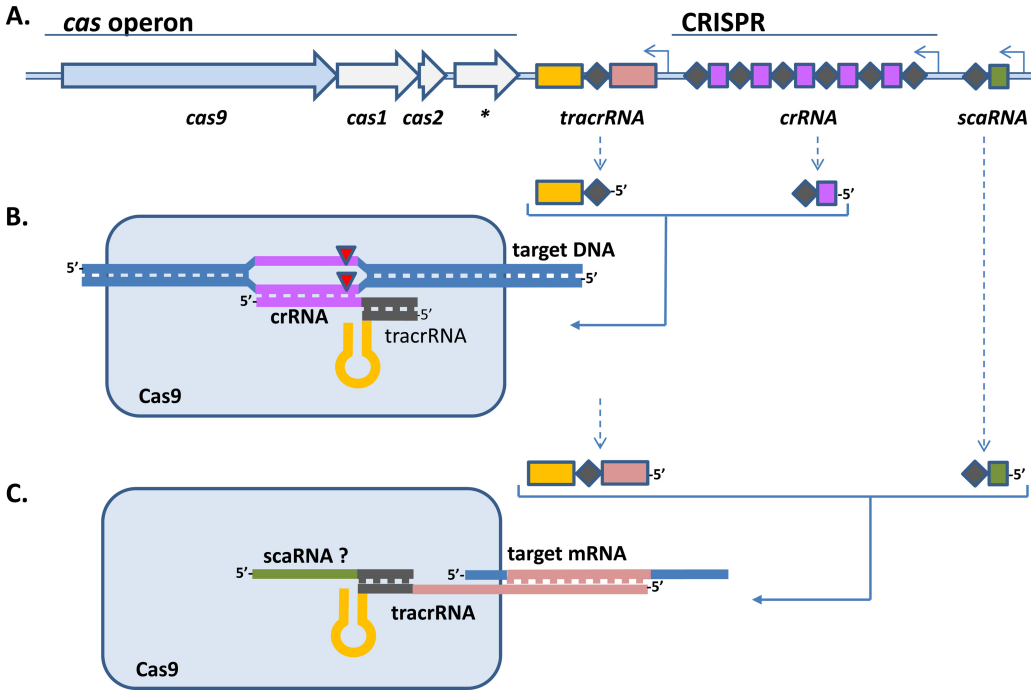

FIG 5 Dual function of type II CRISPR-Cas systems. (A) Genomic locus of type II CRISPR-Cas system. The *cas* operon consists of at least three genes (*cas9*, *cas1*, and *cas2*). A fourth gene (*) is present in type II-A (*csn2*) and II-B (*cas4*) systems but not in type II-C systems (122). Adjacent to the *cas* operon, the CRISPR locus is present (dark purple diamonds indicate repeats, and bright purple squares indicate the spacers), as well as the *trans*-encoded CRISPR RNA (tracrRNA) gene and possibly the recently proposed scaRNA gene (22). The order and orientation of the CRISPR and the genes vary in different genomes. (B) A role in defense against DNAs of invading genetic elements is well established (11), in which processed crRNA and a short version of the tracrRNA (most likely resulting from processing of a longer tracrRNA transcript or transcription from a second promoter; see panel A) eventually are responsible for interaction with target DNA. Eventually, both DNA strands are cleaved at the active sites of Cas9 (red triangles) (20, 129, 130). (C) A distinct role of Cas9 in virulence has been suggested (30), and a molecular basis for how Cas9 can codetermine virulence has been revealed (22): a long version of the tracrRNA shares significant homology with a target transcript, resulting in silencing and probably degradation of this transcript. Involvement of another small CRISPR-associated RNA (scaRNA) has been proposed; if indeed important, this scaRNA may be involved in stabilizing the interaction of the tracrRNA in the Cas9 complex.

radation of BLP mRNA (22) (Fig. 5). Cas9-mediated degradation of BLP mRNA was shown to be activated after phagocytosis of bacteria by macrophages. tracrRNA-mediated silencing of BLP production led to avoidance of Toll-like receptor 2 (TLR2) signaling and was subsequently associated with intramacrophage survival of *F. novicida* bacteria (22). The importance of Cas9-mediated inhibition of BLP production could also be demonstrated experimentally *in vivo*. *F. novicida* knockout mutants of either *cas9*, tracrRNA, or an additional, putative small RNA containing a CRISPR repeat (scaRNA) were not able to cause lethal infections in a mouse model (22). This landmark paper therefore describes a molecular mechanism by which a CRISPR-Cas system is involved in bacterial pathogenicity, in this case, by repressing production of an immunogenic membrane protein via an antisense RNA-based silencing mechanism that uses two different RNA molecules and the Cas9 protein.

The requirement of different RNA molecules may differ between bacterial species, since in *C. jejuni*, a significant increase of virulence could be achieved by supplementation of *cas9* in a natural strain that lacks CRISPR-Cas (30), an indication that other Cas9-dependent mechanisms that determine virulence may exist. Since *cas9* is present in a wide variety of host-associated pathogenic and commensal bacteria, it is tempting to speculate that type II CRISPR-Cas is important not only in virulence but also in commensalism, as suggested by the presence of tracrRNA at or near CRISPR loci of different commensals (22, 122). In this respect, one might hypothesize that commensal bacteria use the type II CRISPR-Cas system to regulate their immune recognition.

The role of Cas9 in bacterial mRNA degradation could lead to confusion due to the established function of Cas9 as an endonuclease in targeting and cleavage of DNA instead of RNA. This apparent conflict can be reconciled by the observation that it is not Cas9 that digests the target dsRNA but, rather, RNase III (20). According to Deltcheva et al., Cas9 functions only as a stabilizer of dsRNA (20). Other RNases, including RNase A, RNase T1, and RNase H, might also be involved in the digestion of dsRNA molecules upon their stabilization by Cas9.

CONCLUDING REMARKS

Polymorphisms in bacterial CRISPR-Cas systems have been shown to be of use for typing purposes and to study evolution and epidemiology in some bacterial species. In several comparative genomic studies, it was noted that subgroups of bacteria with variant CRISPR-Cas systems also appeared to show substantial differences with respect to virulence. Independent studies of both commensal and pathogenic host-associated bacteria have suggested that there might be a role for type II CRISPR-Cas in virulence, perhaps based on an RNA-based mechanism. Indeed, in the bacterial pathogen *F. novicida*, a ribonucleoprotein complex of Cas9 and a short noncoding tracrRNA was found to suppress production of an immunogenic lipoprotein (BLP) by promoting its mRNA degradation, possibly involving a second short RNA mol-

ecule (scaRNA). Avoidance of BLP production is essential for full virulence and immune evasion of *F. novicida*. Such a mechanism might also be functional in *N. meningitidis*, *C. jejuni*, and other bacterial species harboring type II CRISPR-Cas systems, since in *C. jejuni* and *N. meningitidis*, *cas9* knockout mutants also displayed a loss-of-virulence phenotype as observed for *F. novicida*.

These findings are of interest to those in both academia and companies and to society, since type II CRISPR-Cas-harboring pathogenic species belonging to the genera *Neisseria*, *Campylobacter*, *Streptococcus*, and others exert enormous pressure on the health care system and food industries. Vaccines are available for some of the type II CRISPR-Cas-harboring pathogens, but for most of these pathogens, vaccines still need to be developed or require continuous adaptation due to fast bacterial evolution. Genetic engineering of the type II CRISPR-Cas system might provide the opportunity to obtain suitably attenuated vaccine candidates, as suggested for *F. novicida* (22). Indications for a role of the other two CRISPR-Cas systems in pathogenesis have been reported but require more detailed investigations. Unraveling the mechanisms that lead to control of virulence via CRISPR-Cas systems will certainly provide deeper insight into the genetic regulation of gene expression and the ways that bacteria respond to sudden changes in their environment.

The discovery of CRISPR-type repetitive elements in the 1980s resulted in a fascinating research area of bacterial genetics, with several groundbreaking discoveries covering defense against invasion by genetic elements as well as regulation of gene expression. Potential applications of CRISPR-Cas systems range from protecting bacterial production systems against viral infections to multiplex genome editing of microbial and mammalian cells (123–128), potentially even contributing to future gene therapy approaches. The recent discovery that type II CRISPR-Cas is involved in bacterial pathogenesis is an exciting addition to the upsurge of papers describing CRISPR-Cas-associated functions. A picture now emerges that CRISPR-Cas systems may be involved in controlling virulence of very different pathogens that occupy different niches throughout the human body (Fig. 1). Progressive experience using CRISPR-Cas in epidemiology and evolutionary studies might also shed light on how bacterial epidemics and pandemics developed and evolved over time. In addition, it will be interesting to see if CRISPR-Cas systems codetermine commensal and pathogenic lifestyles and transitions between these two lifestyles in pathobionts. More discoveries are expected in the near future concerning CRISPR-Cas control of bacterial commensalism, pathogenicity, innate immune evasion, and virulence.

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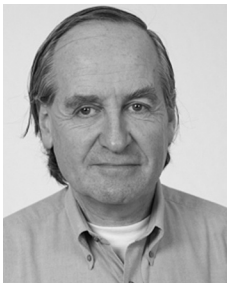
Raymond Staals worked as a Ph.D. student in the department of Biomolecular Chemistry in the Nijmegen Centre for Molecular Life Sciences (NCMLS) at the Radboud University Nijmegen (Netherlands). There, he worked on a multifunctional protein complex which is involved in many stages of human RNA metabolism: the RNA exosome. Over the last 3 years, he has worked as a researcher for the bacterial genetics research group (in the Laboratory of Microbiology, Wageningen University, Netherlands) led by John van der Oost, where he characterized several fundamental aspects of CRISPR-Cas-based defense, with a special focus on the type III-B CRISPR-Cas system of *Thermus thermophilus*.



Hubert Endtz obtained his M.D. from Leiden University in 1989 and his Ph.D. from the same university in 1993. In 1990, he joined Erasmus MC and was Deputy Head of the Department of Medical Microbiology and Head R&D until 2013. He occupies the Chair of Tropical Bacteriology at Erasmus MC. In 2009, this chair was created as part of a strategic collaboration between the International Centre for Diarrheal Research in Bangladesh and Erasmus MC. Since 2013, he has been Scientific Director of the Foundation Merieux in Lyon, France, a family foundation dedicated to fighting infectious diseases in low-income developing countries. His key interests are infectious diseases in low-income developing countries, diarrheal diseases, respiratory diseases, antimicrobial resistance, Guillain-Barré syndrome and other postinfectious neuropathies, and innovative diagnostics.

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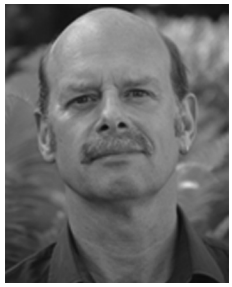

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