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The CRISPR-Cas immune system and genetic transfers: reaching an equilibrium

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Abstract

Horizontal gene transfer drives the evolution of bacterial genomes, including the adaptation to changing environmental conditions. Exogenous DNA can enter a bacterial cell through transformation (free DNA or plasmids) or through the transfer of mobile genetic elements (MGE) by conjugation (plasmids) and transduction (bacteriophages). Favorable genes can be acquired but non-desirable traits can also be inadvertently acquired through these processes. Bacteria have systems, such as CRISPR-Cas, that can cleave foreign nucleic acid molecules. In this review, we discuss recent advances in understanding CRISPR-Cas system activity against mobile genetic element transfer through transformation and conjugation. We also highlight how CRISPR-Cas systems influence bacterial evolution and how CRISPR-Cas components affect plasmid replication.

24

Introduction

In 1987 Ishino *et al.* (1) sequenced the *Escherichia coli* alkaline phosphatase isozyme conversion gene (*iap*). Downstream of *iap*, they observed an array of short repeats (29 nucleotides) separated by non-repetitive short sequences (spacers) (2). The terms “CRISPR” for Clustered Regularly Interspaced Short Palindromic Repeats and “Cas” for CRISPR-associated genes were first coined by Jansen *et al.* (3) in 2002 to describe the genetic structure of these loci. Increasing availability of genomic sequences in databases allowed Mojica *et al*. (4) to identify CRISPR as specific family of repeats. Now, we know that CRISPR-Cas systems are found in approximately 90% of archaeal and 40% of eubacterial sequenced genomes (5-7). In 2005, three groups independently reported similarities between spacer sequences and foreign mobile genetic elements such as phages and plasmids (8-10). These observations led to several hypotheses including that CRISPR-Cas systems may play a role in immunity and protect archaeal and bacterial cells from invasion by foreign DNA.

The immune mechanism of the CRISPR-Cas systems was experimentally demonstrated for the first time by Barrangou *et al.* in 2007 (11). These authors showed that the lactic acid bacterium *Streptococcus thermophilus* could acquire resistance against a bacteriophage by integrating a genome fragment (the protospacer) from this infectious bacterial virus into its CRISPR locus (spacer). Later studies discovered the expanded role of CRISPRs in preventing horizontal gene transfer through conjugation and transformation (12-14). The CRISPR spacer sequences of archaeal species frequently match those of their own resident viruses or plasmids,

suggesting a regulatory rather than inhibitory role for CRISPR-Cas. Indeed, it has been demonstrated that CRISPR-Cas systems may also play a role in transcriptional regulation (15,16), DNA repair (17), pathogenesis (16,18), modulation of the biofilm production (19,20) and sporulation (21).

CRISPR-Cas organisation

CRISPR loci are genomic DNA clusters consisting of a series of short repeat sequences (typically 24–37 bp) separated by spacer sequences of similar length (Fig. 1) (5). Within a given locus, the length of the repeats and spacers sequences is typically conserved. Spacer sequences correspond mostly to fragments derived from viral genomes or mobile genetic elements (MGEs). They appear to serve as a “genetic memory” of previous nucleic acid invasions and provide the specific CRISPR immunity (8-10). An adenine- and thymine-rich leader region containing 20 to 534 bp, including a transcriptional promoter, is present upstream of the CRISPR locus (3). Moreover, the *cas* genes, essential for the CRISPR-Cas machinery, are encoded in the vicinity of the CRISPR locus (upstream or downstream) (3). Based on the presence of specific signature *cas* genes, CRISPR-Cas systems are divided into three main types (I, II and III). These types are further divided into ten subtypes (I-A, I-B and so on), each of which expresses a different protein complex responsible for the CRISPR-Cas immunity mechanism (22).

Steps of the CRISPR-Cas mechanism

67 CRISPR–Cas systems function in three general steps: 1) adaptation or immunization
68 (involving the acquisition of spacers); 2) biogenesis and maturation of CRISPR RNA (crRNA
69 encoded by the repeat–spacer region); and 3) interference (cleavage of invading nucleic acids)
70 (23). These steps are summarized below (figure 1).

71 Spacers in the CRISPR locus are acquired from the DNA of invading plasmids or viruses in
72 a process known as adaptation. New spacers are usually added at the 5'-leader region of the
73 CRISPR locus (11,24,25) and come from defective or fragmented molecules (26). For type I and
74 type II CRISPR–Cas systems, a conserved sequence motif in the vicinity of the protospacer,
75 known as the protospacer-adjacent motif (PAM), is needed for spacer acquisition and
76 interference (23,25,27). The incorporation of spacers in the CRISPR locus implies the action of
77 two Cas proteins, Cas1 and Cas2, and sometime the help of accessory elements specific for
78 some CRISPR-Cas systems (28,29). During this step, a repeat is also duplicated to conserve the
79 genetic organisation of the repeat-spacer region. Despite the "adaptive" mode of CRISPR-Cas
80 systems, the molecular mechanism of this process still remains enigmatic.

81 The CRISPR locus is then transcribed as a long primary precursor CRISPR RNA (pre-crRNA)
82 transcript, which is processed within the repeat sequences to produce a collection of short
83 crRNAs (29). Further trimming can be done at the 3' end or 5' end to complete the maturation
84 of crRNAs (29). This is a process known as crRNA biogenesis and maturation. Recently, an
85 unusual crRNA maturation pathway was discovered in *Neisseria meningitidis*, in which crRNAs
86 are transcribed from promoters embedded within each repeat, thus initiating the crRNA
87 transcription independently within each spacer (13).

In conjunction with a set of Cas proteins, these crRNAs form the core of CRISPR–Cas ribonucleoprotein complexes. These complexes act as a guided-surveillance system and provide immunity against ensuing foreign nucleic acid invasion of DNA or RNA molecules complementary to each specific crRNA. On recognition of a matching target sequence, the plasmid or viral DNA is cleaved in a sequence-specific manner (30,31) known as the interference step. The combination of recognition of an effective PAM (Types I and II) and the base-paring of a “seed” crRNA spacer region of 6-8 nucleotides almost identical to the viral or plasmid targeted protospacer are necessary to induce nucleic acids cleavage (5,29). However, for some systems, the PAM sequence necessary for the acquisition step can diverge from the PAM recognized for the interference step, although they usually overlap considerably (32). Type I and type II CRISPR-Cas systems cleave DNA while type III systems can cleave DNA or RNA (33). To discriminate between “self” vs. “non-self” (foreign) target, the crRNA-Cas complexes base-paired with sequences outside the protospacer (PAM or non-PAM) to avoid autoimmunity (34). In this way, CRISPR-Cas systems recognise and target invading nucleic acids molecules avoiding the destruction of their bacterial own genomes.

CRISPR targets mobile genetic elements

Rapid evolution of bacteria is not just the consequence of rearrangements in the genome. Horizontal gene transfer (HGT) is also an important mechanism for bacterial evolution (35). Large proportions of bacterial genomes contain genes that come from the horizontal exchange of genetic material. This transfer of genetic material can occur by acquiring DNA

110 directly from the environment (transformation) or by incorporating heterologous DNA using
111 mobile genetic elements (MGE) such as plasmids (conjugation and transduction) and
112 bacteriophages (transduction) (Fig. 2). Generally, the outcome of horizontal gene transmission
113 depends on the nature of the incoming DNA and on the genetic background of the host (36-38).
114 Favorable DNA helping to cope with a specific environmental condition may no longer provide a
115 selective advantage to the same bacteria that grow under other conditions and even can reduce
116 the host fitness (36,37,39,40). To control the entry of new genetic material, bacteria possess
117 systems that either block the access to foreign DNA or remove MGE from the cell. Restriction-
118 Modification (R-M) and CRISPR-Cas systems protect bacteria against foreign DNA by cleaving
119 specific DNA sequences (41). Both systems are compatible in the same cells and efficiently
120 protect bacterial genomes against phages and plasmids (42).

121

122 **Incompatibility of plasmids and CRISPR-Cas systems**

123 CRISPR-Cas systems can cleave foreign DNA and was expected to cause plasmid loss or
124 block plasmid acquisition (Fig. 2). This phenomenon was demonstrated experimentally *in vivo*
125 using a CRISPR-Cas system (type II-A) from *S. thermophilus*. When the CRISPR-Cas system
126 acquired spacers from the artificially transformed plasmid, this led to plasmid loss (30).
127 Moreover, some acquired spacers match an antibiotic-resistance gene (present on the plasmid),
128 which could interfere with the dissemination of other plasmids carrying the same antibiotic
129 resistance marker. This antagonism between CRISPR-Cas interference and plasmid maintenance
130 could explain why *S. thermophilus* naturally contains few plasmids as well as other bacteria (30).

131

132 *Escherichia coli* produces a global transcriptional repressor H-NS that represses
133 transcription of the two CRISPR-Cas type I-E systems encoded in its genome (43,44). A
134 prolonged incubation time (around 1-2 weeks) of *E. coli* K12 Δ*hns* under non-selective
135 conditions resulted in loss of a high copy plasmid concomitant with acquisition of new plasmid-
136 targeting spacers in the CRISPR loci (25). The transformation efficiency (by heat shock) of the
137 cured strain that has acquired new spacers targeting the plasmid dropped significantly (25).
138 Others have used these PIMs (Plasmid Interfering Mutants) to artificially transform them with
139 an unrelated plasmid (45). Some spacers in the CRISPR loci match partly with this latter plasmid,
140 but no spacer was 100% identical to the plasmid sequence. When the CRISPR possesses a
141 plasmid-targeting spacer containing a sequence mismatch, it "primes" the strain to acquire new
142 spacers from the invading plasmid, causing plasmid loss after two days of incubation (45). As
143 demonstrated previously, CRISPR loci can acquire new spacers "naively" from the invading DNA,
144 but at low frequency. An intact spacer sequence results in interference, while mutation in the
145 protospacer can abolish the CRISPR interfering activity. However, for some systems, when the
146 CRISPR already contain a spacer that matches the invading DNA with a protospacer mismatch in
147 its seed sequence and/or mutation in the PAM, the CRISPR-Cas machinery is used to accelerate
148 the spacer acquisition of the targeted foreign DNA element in a phenomenon called "priming"
149 (45-47). In *E. coli*, the new spacers acquired by priming come from the same strand as the first
150 spacer acquired (25,45-47) while in other organisms, this strand bias have not been observed
151 (48,49).
152

153 In a similar way, when the CRISPR-Cas I-F systems of *Pectobacterium atrosepticum*
154 contain spacer targeting an engineered plasmid (with the corresponding protospacer and a
155 functional PAM), they cause plasmid loss after few days of culturing (49). Moreover, the
156 presence of this spacer primes the systems to incorporate new spacers into the CRISPR loci that
157 match the plasmid, while no detectable spacer acquisition occurs with the control plasmid
158 (without the protospacer sequence). These newly acquired spacers provide bacterial immunity
159 against the transformation and the conjugation of similar plasmids. Surprisingly, one strain has
160 added as many as 9 new plasmid-targeting spacers in the two functional CRISPR loci of *P.*
161 *atrosepticum* (CRISPR1 and CRISPR2). Interestingly, the more spacers a strain possesses against
162 a plasmid, the more resistant the strain is to transformation and conjugation (49) probably
163 caused by a higher surveillance done by the CRISPR-Cas complexes, the cut at multiple sites
164 within the plasmid DNA and the difficulty to mutate several protospacers for escapees.
165
166 The *Staphylococcus aureus* strain USA300 contains three plasmids including the plasmid
167 pUSA02 that confers resistance to the tetracycline antibiotic. A phagemid construction with
168 Cas9, tracrRNA and a repeat-spacer unit of *S. pyogenes* was designed to target the plasmid
169 pUSA02 and delivered into the cells by transduction using the phage pBD121 (50). Plasmid loss
170 was achieved in 99.99% of cells resulting in restoration of sensitivity to tetracycline. Moreover,
171 cells that have been pre-treated with the engineered CRISPR system targeting pUSA02 could not
172 acquire the plasmid by transduction compare to the control without CRISPR immunisation (50).
173

174 An antagonistic correlation between the presence of CRISPR-Cas systems and the
175 multidrug-resistant phenotype of *Enterococcus* strains have been observed by Palmer and
176 Gilmore (51). In these opportunistic pathogenic enterococci, the principal method of genome
177 adaptation is *de novo* horizontal uptake of genes. The acquisition of new MGEs such as plasmids
178 and transposons is frequently associated with the transfer of antibiotic resistance genes. They
179 observed that many antibiotic-resistant enterococcal strains lack CRISPR-Cas systems.
180 Conversely, bacteria with CRISPR possess spacers in their loci that match MGE sequences,
181 suggesting that CRISPR-Cas systems regulate the flux of these elements (51). Thus, the extensive
182 use of antibiotics appears to have enriched for enterococcal strains with lower defences (no
183 functional CRISPR-Cas systems) against MGE. However, this correlation between the presence
184 of CRISPR-Cas activity and the antibiotic resistance phenotype or the presence of plasmids does
185 not seem to apply for *E. coli* strains from clinical or animal samples (52). Some non-pathogenic
186 group B *E. coli* carry additional CRISPR arrays (CRISPR3 and CRISPR4) with a significant
187 abundance of spacers targeting plasmids compared to CRISPR1 and CRISPR2, which contain
188 more phage-related spacers (53). Experiments demonstrating the link between the presence of
189 plasmids and CRISPR activity should be done to confirm these bio-informatics results.
190
191 Bacteriophages escaping CRISPR-Cas systems have usually mutations in the targeted
192 protospacer or PAM via point mutations or deletion (27). In contrast, when a CRISPR locus
193 contains a spacer targeting a plasmid, especially if the targeted gene induces a strong selective
194 advantage to the bacteria such as virulence factors or antibiotic resistance genes, it often results
195 in the inactivation of CRISPR-Cas system. This inactivation is caused by major modifications in

the CRISPR-Cas locus such as the deletion or significant modification of the spacer, inactivation of a gene coding for an essential Cas protein or deletion of the complete CRISPR-Cas locus (54-58). Therefore, bacteria possess CRISPR-Cas systems that protect the cells from invading DNA, however, they must control CRISPR activity to permit incorporation of novel genes in their genomes.

CRISPR-Cas systems interfere with transformation

Bacterial HGT involving plasmid exchange happens through one of two processes: transformation and conjugation. Transformation is the acquisition of free DNA molecules from the surrounding environment. Natural transformation occurs when bacterial cells are in a physiological state of competence. This time-limited state usually happens in response to specific environmental conditions or stresses such as altered growth conditions, limited nutrient access, cell density (monitored by quorum sensing) or starvation (35). Artificial transformation of weakened bacteria using electroporation or thermal shock is also a commonly used tool in laboratories around the world to introduce DNA fragments into bacteria.

Evolutionary study of competent vs. non-competent strains of *Aggregatibacter actinomycetemcomitans* revealed that competent strains contain more CRISPR-Cas systems than non-competent ones (59). Moreover, the remnant CRISPR-Cas systems found in the non-competent strains often contain mutations or deletions in *cas* genes. The genetic diversity between both types of strains varies: competent strains have larger genomes with frequent rearrangements, while non-competent strains have stable genomes with more extra-

218 chromosomal elements such as prophages and plasmids. This peculiarity is reflected in the
219 spacer's content of the CRISPR loci. Spacers from competent strains more often target plasmids
220 or phages sequences while spacers from non-competent cells more frequently match
221 *A. actinomycetemcomitans* genomes (59).
222
223 To determine if the CRISPR-Cas system inhibits the acquisition of DNA by natural
224 transformation in *Streptococcus pneumoniae*, *in vitro* and *in vivo* (in mice) assays were
225 performed (54). No functional CRISPR-Cas system has been identified so far in *S. pneumoniae*
226 strains for which the genome is available. Thus, the authors engineered the non-capsulated
227 pneumococcal strain R6 to integrate the type II CRISPR system of *Streptococcus pyogenes* into
228 its chromosome. The CRISPR locus was also engineered to add specific spacer sequences
229 derived from prophage DNA or from the capsule biosynthesis gene (54). When a DNA molecule
230 targeted by a CRISPR spacer was introduced into the bacteria by transformation *in vitro*, using
231 the natural competency of *S. pneumoniae*, no transformants were obtained, indicating that the
232 CRISPR-Cas system blocks the transformation or that the bacteria die because the transferred
233 DNA molecule is destroy and the antibiotic used as selection marker is not detoxified. Then, the
234 same authors tried to transfer a CRISPR-Cas locus containing a spacer targeting the
235 chromosome of the recipient cell into bacteria without a CRISPR-Cas system (54). Similarly, no
236 antibiotic-resistant transformant was obtained with a strain carrying a functional CRISPR-Cas
237 system and containing a chromosomal-targeting spacer, reinforcing the idea that an active type
238 II CRISPR-Cas system and its target cannot co-exist in the same cell. Finally, they co-infected
239 mice with non-capsulated and capsulated strains, each containing or missing the CRISPR-Cas

240 system (with a spacer targeting the capsule biosynthesis gene) to see if the CRISPR system could
241 prevent capsular gene transformation *in vivo* (54). Non-capsulated strains of *S. pneumoniae*
242 can't cause infections, while capsulated strains can avoid the immune system and cause an
243 infection. Non-capsulated strains can naturally acquire a functional capsule gene (*cap3*) to
244 produce a capsule and begin the infection. As expected, the strain without the CRISPR-Cas
245 system acquired the *cap3* gene by HGT, produced a capsule and caused an infection in mice.
246 Conversely, the CRISPR containing cells could not acquire *in vivo* the *cap3* gene and could not
247 cause infection.
248
249 Using the native CRISPR-Cas system of *Neisseria meningitidis* 8013 and the natural
250 competency of this bacterium, Zang and coworkers obtained similar results (13). They cloned
251 different protospacers targeted by the endogenous CRISPR-Cas system of this strain into
252 different plasmids or engineered genomic DNA (gDNA). They then transformed *N. meningitidis*
253 using its natural competence and tested the transformation efficiency using these molecules.
254 When the plasmid or gDNA contained a protospacer and a good PAM sequence, the
255 transformation efficiency was lower (no transformants) than the controls without a protospacer
256 or those containing a potential protospacer with a defective PAM (13). Thus, the CRISPR-Cas
257 system of *N. meningitidis* also interferes with the natural transformation of this bacterium.
258 Moreover, the presence of CRISPR-Cas systems could explain the rare presence of plasmids in
259 this bacterium.
260

261 CRISPR-Cas systems can also inhibit artificial transformation done by electroporation or
262 heat shock (Fig. 2). Transformation efficiency assays are often used to verify CRISPR-Cas activity
263 during the adaptation step (acquisition of new spacers from the transformed plasmid) or
264 interference (capacity to cut the plasmid DNA or eliminate the plasmid). Moreover, these
265 plasmid-invader tests allowed the determination of possible protospacer mutations required to
266 escape CRISPR-Cas systems or the identification of PAM sequences required for CRISPR
267 adaptation/interference steps. These kinds of experiments have been used successfully with
268 many different organisms such as *E. coli* (25,47,60-63), *Haloarcula hispanica* (48), *Haloferax*
269 *volcanii* (56,57,64), *P. atrosepticum* (49), *Sulfolobus spp.* (58,65), *Staphylococcus epidermidis*
270 (14), *Streptococcus agalactiae* (66), *Streptococcus pyogenes* (67), *S. thermophilus* (30) and
271 *Thermococcus kodakarensis* (68). Transformation interference is an important consideration
272 when trying to introduce foreign DNA into industrially relevant cultures. In addition, plasmid
273 stability can be compromised during long-term incubation or when using non-selective media.
274

275 **The role of CRISPR-Cas systems in conjugation interference**

276 Conjugation is the process by which a conjugative plasmid is transferred from a donor to
277 a recipient cell. Conjugative plasmids can be grouped into at least six different MOB families
278 (genes required for the mobilization) based mainly on the comparison of their relaxase genes
279 (69). Since CRISPR-Cas systems defend bacteria against foreign DNA, it is not totally surprising
280 that these systems can interfere with the conjugation process (Fig. 2).
281

282 Westra and coworkers screened public databases to analyse the spacer content of
283 CRISPR loci related to conjugative plasmids (12). They found that targeted protospacers are not
284 randomly distributed on conjugative plasmids: there is a MOB family-dependent bias. Indeed,
285 MOB_P family plasmids are most frequently targeted within the lagging regions (the last plasmid
286 section entering in the recipient bacteria) while the protospacers of MOB_F family are mostly
287 located in the leading region (the first plasmid section entering the cell). Using synthetic
288 constructions, the authors demonstrated that the *E. coli* K12 type I-E CRISPR-Cas system
289 interferes with the conjugation of the plasmid F (MOB_F family) (12). Surprisingly, the level of
290 protection achieved by the CRISPR-Cas system is independent of the protospacer position
291 (leading or lagging) and independent of the DNA strand (parental or newly synthesized strand).
292 Thus, the bias distributions of the protospacers probably depend on the acquisition step of a
293 new spacer in the CRISPR locus or on the first regions that go double stranded as this CRISPR-
294 Cas system target dsDNA.
295
296 Experimental studies demonstrating the conjugation interfering role of CRISPR-Cas
297 systems are not abundant. CRISPR-Cas systems can acquire spacers targeting conjugative
298 plasmids, when the archaeon *Sulfolobus solfataricus* was challenged with the SMV1 virus (70) or
299 when *S. solfataricus* culture was subjected to freeze-thaw stress (71). The *Listeria*
300 *monocytogenes* RliB-CRISPR array can interfere with plasmid conjugation using type I CRISPR-
301 Cas machinery and a PNPase (polynucleotide phosphorylase) (72). Moreover, CRISPR-Cas
302 systems can mediate resistance to conjugative plasmids in staphylococci (14,73) and *P.*
303 *atrosepticum* (49).

304 ## Effects of CRISPR-Cas systems on plasmid replication and protein production

305

306 Plasmids are also useful genetic tools for analyzing the activities of the steps of the
307 CRISPR-Cas immunity or to overexpress Cas proteins for characterization. They can also be used
308 to better understand the role of specific Cas proteins. For example, the Cas3 protein (nuclease-
309 helicase) from the type I-E CRISPR-Cas system of *E. coli* can increase the copy number of ColEI-
310 replicon plasmids but has no effect on pRSF1010- or p15A-replicon plasmid yields (74). The Cas3
311 plasmid copy number-increasing activity is RNaseHI dependent and promotes the formation of
312 plasmid concatemers or multimers (74).

313

314 In another study, Maier and coworkers tested the influence of plasmid copy number and
315 the origin of replication on CRISPR-Cas type I-B interference in *Haloferax volcanii* (64).
316 Interestingly, they found that plasmid copy number has no effect on the plasmid transformation
317 rate but the replicon used has an effect. Plasmids with the pHV1 ori were degraded by the
318 CRISPR-Cas system (few transformants) while plasmids with the pHV2 ori were successfully
319 transformed into cells (64). This observation is analogous to the results obtained with the non-
320 random MOB-family conjugative plasmids spacer acquisition in CRISPRs (12). Thus, spacer
321 acquisition from plasmids is potentially dependent on their mode of entry into the cells or their
322 mode of replication. Further experiments are needed to understand the mechanism.

323

324 Perez-Rodrigez *et al*. activated accidentally an *E. coli* CRISPR-Cas system while trying to
325 overexpress the twin-arginine translocation (Tat) protein in cells with a deletion in the *DnaK*

326 chaperone gene (75). This CRISPR activation silences the plasmid bearing the *tat* gene and is
327 dependent on the two-component, extracytoplasmic stress system, BaeSR. Indeed, BaeSR
328 activates transcription of the CRISPR-Cas operon by binding to the promoter upstream of *casA*
329 (75). Henceforth, when trying to overexpress a protein, especially if the protein can induce cell
330 envelope stress, it will be important to test for CRISPR activity if the bacteria inhibit the protein
331 production. Otherwise, the resident CRISPR-Cas systems can be activated and cure the
332 constructed plasmid.
333

334 **Conclusions**

335 Bacteria must adapt to survive in ever-changing environments, including when they face
336 phages or other antimicrobial agents. They need to mutate or acquire new advantageous genes
337 to cope with these harmful conditions. The CRISPR-Cas adaptive immune system of bacteria and
338 archaea can clearly interfere with the transfer of foreign DNA into the cells, including plasmids.
339 This inhibition can also interfere with the maintenance of existing plasmids as observed initially
340 in *S. thermophilus* (30). Future studies are still needed to better understand the spacer
341 acquisition process, including its preference for specific DNA sequences or for the uptake of
342 particular plasmid replicon sequences. Another area of research interest is to understand why
343 only half of the bacteria for which a complete genomic sequence is available do not possess a
344 CRISPR-Cas system. For example, clinical strains appear to contain fewer CRISPR-Cas systems
345 (51,76). Finally, it is largely unknown how so many bacterial strains are able to carry and
346 maintain plasmids in the presence of seemingly functional CRISPR-cas systems.
347

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352

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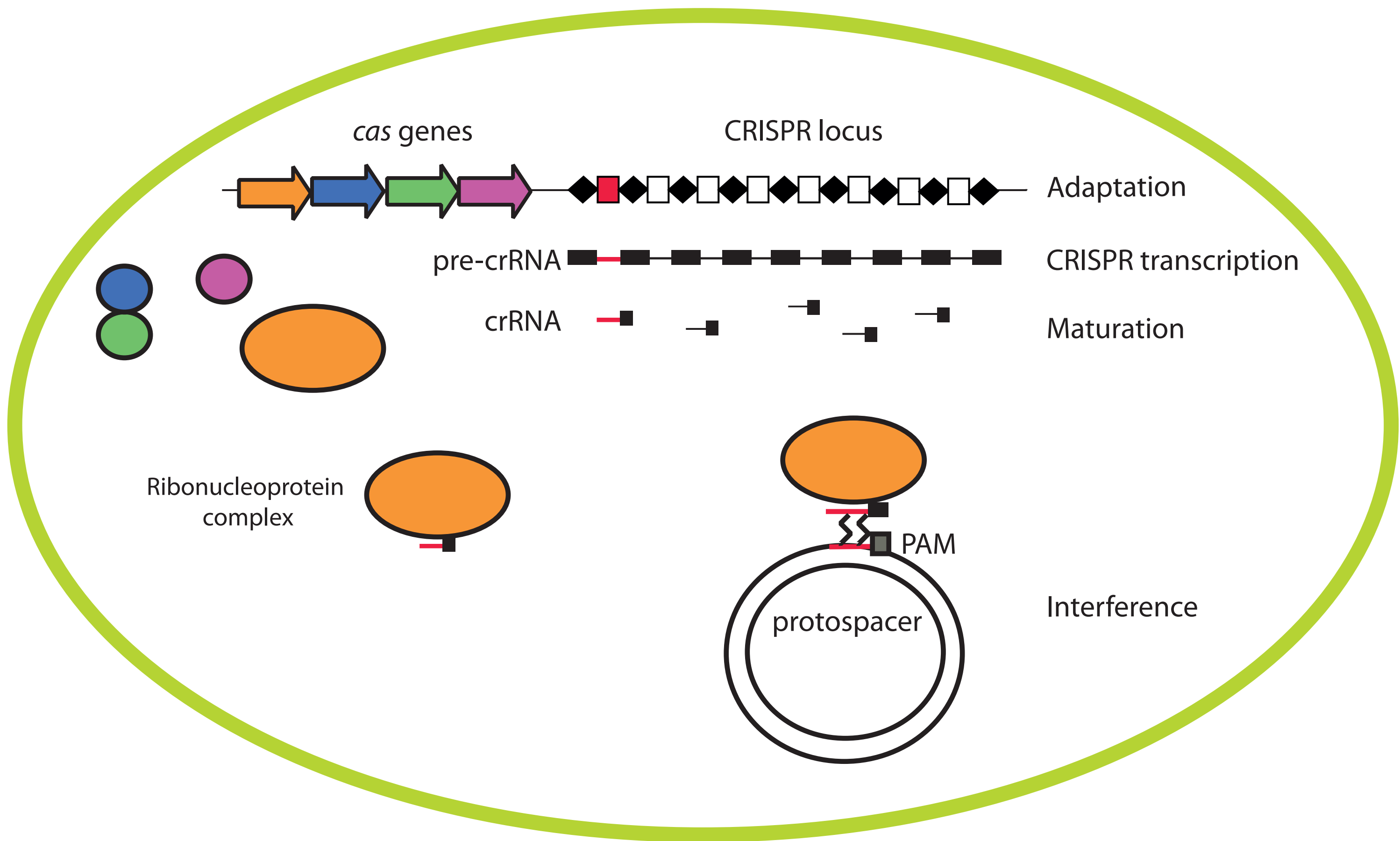
560
561

562 ## Legend of Figures
563
564 **Figure 1.** Genetic organization of a type II CRISPR-Cas system and its general steps of action. The
565 CRISPR locus is composed of repeats (black diamond) interspaced with spacer (red or white
566 rectangle) of similar length. In the vicinity of the CRISPR array, *cas* genes (colored arrows) coded
567 for proteins necessary for the immunity process. During the adaptation step, a repeat and most
568 importantly a new spacer (red rectangle) is acquired in the CRISPR locus usually at the 5' region.
569 Transcription of the CRISPR locus leads to pre-crRNAs that are processed, leading to short
570 crRNAs. These crRNAs are assembled with Cas protein(s) in ribonucleoprotein complexes that
571 act as surveillance guide looking for matching invading sequences. If the crRNA sequence
572 matches a protospacer found on the foreign and invading nucleic acid molecule and if a PAM
573 (grey box) is present next to the protospacer (for types I and II systems), it leads in the cleavage
574 of the invading molecule (interference step).
575
576 **Figure 2.** Probable action of CRISPR-Cas systems against invading plasmids. A) Plasmids entering
577 a bacterial cell via natural transformation (free DNA), conjugation (pilus not represented) and
578 transduction (phagemids). Of note, to date, no CRISPR-Cas system has been identified to cleave
579 ssDNA molecules. However, after their entry in the bacteria, ssDNA will usually be transformed
580 into double stranded DNA molecule (dsDNA) and maintain as plasmid or integrated within the
581 chromosome. If the new dsDNA molecule contains a protospacer matching a crRNA sequence it
582 will be cleaved by the CRISPR-Cas machinery in a sequence specific manner. B) During artificial
583 transformation of bacterium with heat treatments or electroporation, dsDNA will directly enters
584 the cells. Thus, the CRISPR-Cas ribonucleoprotein complexes can target directly these dsDNA

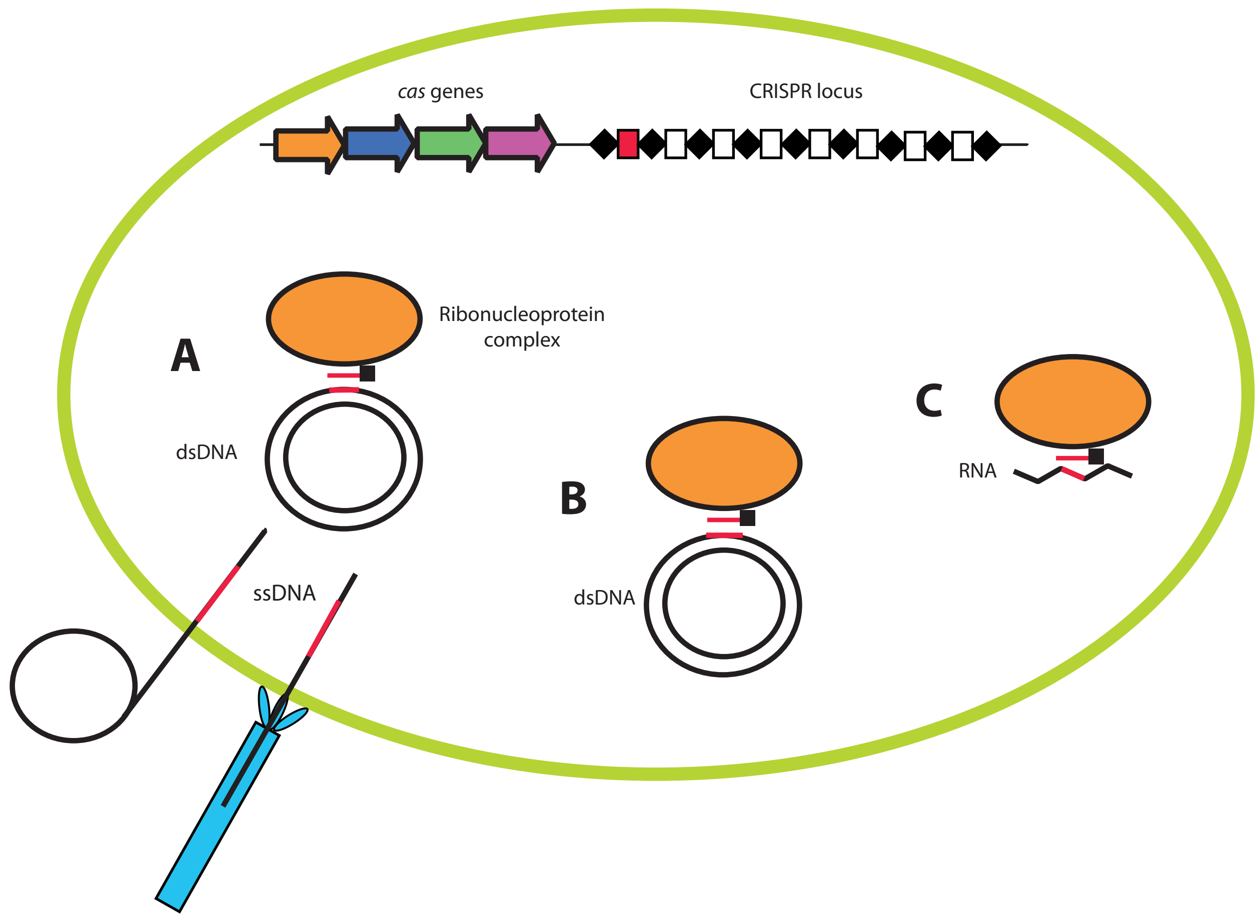
585 molecules to eliminate them. C) Some CRISPR-Cas systems (type III) cleave RNA molecules.

586 After transcription of plasmid genes, these latter will be silenced by the CRISPR-Cas system and

587 after a few round of bacterial replication, these plasmids may be lost.



cas genes
CRISPR locus
Adaptation
pre-crRNA
CRISPR transcription
crRNA
Maturation
Ribonucleoprotein
complex
PAM
protospacer
Interference



cas genes
CRISPR locus
A
Ribonucleoprotein complex
dsDNA
ssDNA
B
dsDNA
C
RNA