RESEARCH PAPER

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CRISPR-Cas systems preferentially target the leading regions of MOB_F conjugative plasmids

Edze R. Westra,[1,‡] Raymond H.J. Staals,[1,‡] Gerrit Gort,[2] Søren Høgh,[1] Sarah Neumann,[1,†] Fernando de la Cruz,[3] Peter C. Fineran[1,4] and Stan J.J. Brouns[1,*]

[1]Laboratory of Microbiology; Department of Agrotechnology and Food Sciences; Wageningen University; Wageningen, The Netherlands; [2]Biometris; Wageningen University and Research Center; Wageningen, The Netherlands; [3]Departamento de Biología Molecular e Instituto de Biomedicina y Biotecnología de Cantabria; Universidad de Cantabria-Consejo Superior de Investigaciones Científicas-SODERCAN; Santander, Spain; [4]Department of Microbiology and Immunology; University of Otago; Dunedin, New Zealand

[†]Current address: Department of Microbiology; Institute for Water and Wetland Research; Faculty of Science; Radboud University Nijmegen; Nijmegen, The Netherlands

[‡]These authors contributed equally to this work.

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Most prokaryotes contain CRISPR-Cas immune systems that provide protection against mobile genetic elements. We have focused on the ability of CRISPR-Cas to block plasmid conjugation, and analyzed the position of target sequences (protospacers) on conjugative plasmids. The analysis reveals that protospacers are non-uniformly distributed over plasmid regions in a pattern that is determined by the plasmid's mobilization type (MOB). While MOB_P plasmids are most frequently targeted in the region entering the recipient cell last (lagging region), MOB_F plasmids are mostly targeted in the region entering the recipient cell first (leading region). To explain this protospacer distribution bias, we propose two mutually non-exclusive hypotheses: (1) spacers are acquired more frequently from either the leading or lagging region depending on the MOB type (2) CRISPR-interference is more efficient when spacers target these preferred regions. To test the latter hypothesis, we analyzed Type I-E CRISPR-interference against MOB_F prototype plasmid F in *Escherichia coli*. Our results show that plasmid conjugation is effectively inhibited, but the level of immunity is not affected by targeting the plasmid in the leading or lagging region. Moreover, CRISPR-immunity levels do not depend on whether the incoming single-stranded plasmid DNA, or the DNA strand synthesized in the recipient is targeted. Our findings indicate that single-stranded DNA may not be a target for Type I-E CRISPR-Cas systems, and suggest that the protospacer distribution bias might be due to spacer acquisition preferences.

Introduction

Bacterial and archaeal genomes have been shaped to a considerable extent by events of horizontal gene transfer (HGT).[1,2] The three main routes of HGT are transformation (DNA uptake from the environment), transduction (virus or phage mediated DNA transfer) and conjugation (plasmid transfer through mating between self-transmissible plasmid containing donor cells and plasmid free recipient cells).[3] Whether or not the invasion provides a fitness gain to the host depends on the nature of the incoming DNA[4,5] and on the genetic background of the host.[6-9] In addition, the impact of a particular HGT event may depend on environmental parameters,[4,10-12] such as the presence or absence of antibiotics, toxic metal ions and nutrients. Upon a change in environment, previously beneficial DNA may no longer provide a selective advantage, and instead can reduce host fitness.[13] In addition to DNA uptake mechanisms, bacteria therefore require systems that either block the entry of alien DNA or can remove such DNA from the cell.

CRISPR-Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated) is a widespread prokaryotic adaptive and heritable immune system that specifically degrades non-self DNA from cells.[14] Three main types of CRISPR-Cas systems are currently recognized,[15] and these show many striking mechanistic and structural dissimilarities (reviewed in refs. 16 and 17). A universal property of CRISPR-Cas systems is that they make use of a genomic CRISPR locus for integration of short sequences derived from invader genomes. The invader sequences (spacers) in a CRISPR array are typically 30 nt each and are separated from each other by host-derived repeating sequences of approximately the same size. The acquisition of new spacer sequences during the CRISPR-adaptation stage provides resistance against genetic elements containing cognate sequences.[18-21] During the expression and interference stages, the CRISPR is transcribed into precursor CRISPR RNA, which is subsequently cleaved in the repeat sequences by a Cas endoribonuclease in Type-I and Type-III systems, and by RNaseIII in

*Correspondence to: Stan J.J. Brouns; Email: stan.brouns@wur.nl
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Type-II systems.[22-25] The resulting processed crRNA is further trimmed from the 3' or 5' end in some cases,[25-27] to yield mature crRNA species. Cas proteins utilize mature crRNAs to bind and cleave nucleic acids containing a complementary sequence.[28-35]

CRISPR systems appear to be active against all forms of invading DNA. A high number of studies have demonstrated that CRISPR systems can provide adaptive immunity against phage infections under laboratory conditions.[18,22,36-42] In addition, CRISPR-Cas systems can mediate plasmid curing and resistance against plasmid transformation.[19,30,42-45] A recent paper indicates that CRISPR systems can be active against conjugative transposons,[46] and two studies reported on CRISPR-mediated resistance against conjugative plasmids.[45,47] In agreement with this, analyses of the CRISPR content of bacteria from environmental samples recovered spacer sequences that match a variety of known viral and plasmid sequences,[48-54] or can even be used for identifying new mobile genetic elements.[55]

Whereas CRISPR-mediated protection against virulent phages provides a clear selective advantage to the host cell, the outcome of targeting conjugative plasmids is expected to be crucially dependent on environmental parameters and accessory genes encoded by the plasmid. Excess baggage theory predicts that when a plasmid does not encode proteins that provide a selective advantage, the presence of the plasmid results in a fitness cost for the host.[13] A number of experimental studies have indeed reported a fitness cost for the host associated with plasmid carriage.[6,9,10,56-59] However, some studies have reported examples where plasmid carriage by a bacterial strain in the absence of selective pressure on plasmid maintenance does not incur a fitness cost to the host, or can even provide a fitness gain under laboratory growth conditions.[9,60,61] It appears likely, however, that a host's fitness cost or benefit for carrying a plasmid is strongly dependent on the environmental conditions (e.g., nutrient availability, temperature, presence of other mobile DNA elements, etc.). For example, a number of phages use pili encoded by conjugative plasmids as receptors for adsorption to the host cell (e.g., refs. 62 and 63). In contrast, certain plasmid-encoded toxin-antitoxin loci provide both plasmid stability and phage-resistance phenotypes, and are maintained in either the presence or absence of viruses.[64] Hence, depending on the conditions, plasmid loss can provide a fitness gain to the host cell. Plasmid fitness on the other hand is determined by its ability to spread both vertically (dependent on copy number,[65] stable partitioning (*par*) genes[66] and multimer resolution systems[67]) and horizontally [dependent on plasmid mobility genes and plasmid size (transduction)] to new hosts. Furthermore, many plasmids carry genes encoding addiction systems[68,69] to avoid segregational loss (reviewed in ref. 70).

Here, we elaborate on the role of CRISPR-Cas systems in targeting conjugative plasmids. By analyzing all spacers that show complementarity toward plasmids containing an origin of transfer (*oriT*, the site that allows for plasmid transfer via conjugation), we demonstrate that protospacers are distributed non-randomly over these conjugative plasmids. While MOB_P plasmids are most frequently targeted in the lagging region (the plasmid region entering the recipient cell last), MOB_F plasmids are mostly targeted in the leading region (the plasmid region entering the recipient cell first). Next, we performed an in-depth analysis of spacers targeting conjugative plasmids belonging to the MOB_F family, which is one of the best-studied conjugative plasmid families. By studying the CRISPR-mediated targeting of conjugative plasmid F by *Escherichia coli*, we experimentally show that the level of CRISPR interference appears to be independent of the target location on the plasmid (leading region vs. lagging region). In addition, CRISPR-immunity levels are similar for recipient cells targeting the incoming single-stranded plasmid DNA, as for recipient cells targeting the DNA strand that is newly synthesized in the recipient. Our findings indicate that single-stranded DNA may not be a target for Type-I-E CRISPR-Cas systems. Furthermore, these data suggest that either spacers are derived more frequently from the leading region of MOB_F plasmids during CRISPR adaptation (possibly due to interrupted mating) or that, for unknown reasons, cells targeting the leading region have a higher Darwinian fitness as compared with cells targeting the lagging region of MOB_F conjugative plasmids.

Results

CRISPR systems are biased toward targeting specific regions of conjugative plasmids. Conjugative plasmid transfer is a multistep process (reviewed in refs. 71 and 72) that requires an *oriT* and a set of transfer proteins encoded by the transfer region of the plasmid. Contact between a plasmid-encoded pilus of a donor cell and the cell surface of a recipient cell leads to a mating signal, pilus retraction and conjugative pore formation.[73] Next, an *oriT* relaxosome complex is formed that causes nicking of one strand of the *oriT*. The relaxosome interacts via the coupling protein with the Type IV secretion system.[74] Starting with the 5' end (leading region), the nicked strand of the plasmid DNA is then transferred from the donor to the recipient cell. After the leading region enters the recipient cell first, conjugation generally proceeds until the entire plasmid is transferred. Mating is completed when the plasmid DNA in the recipient cell is re-circularized and the complementary DNA strand is synthesized.

The well-conserved directionality of conjugative plasmid transfer may have implications for target selection/recognition during CRISPR adaptation and interference, since the timing of DNA entry is different for sequences within the leading region, which enter the cell first, than for sequences within the lagging region, which enter the recipient cell last. To investigate this, we used an in silico approach to check for a correlation between the location of protospacers (leading vs. lagging regions) and the frequency they are being acquired as a spacer by a CRISPR system. Slightly more than 70,000 spacer sequences (of which roughly 65,000 were unique) were obtained from the CRISPRdb[14] and were locally blasted against 3,167 plasmid sequences taken from GenBank. After removing duplicate spacers, self-targeting spacers (e.g., when a CRISPR-locus is located on a megaplasmid), as well as spacers having multiple hits on the same target plasmid, we found that 30% of this subset of spacers in the database match known plasmid sequences. Only protospacers from

plasmid sequences containing an annotated *oriT*-feature (2.3% of all plasmids in the database) were selected for further analysis. To establish the direction of conjugation (and thereby identifying the leading and lagging regions), the *oriT*-containing plasmids were screened for the presence of relaxase genes, which are usually located in the lagging regions of the plasmid, in close proximity of the *oriT*.[75,76] Although the relaxase genes are not located at a fixed distance from the *oriT*, relaxase genes can be used as a marker gene for allocating lagging regions of a plasmid.

This analysis revealed that 375 unique spacers target 39 different conjugative plasmids, yielding a total number of 506 protospacer sequences (**Table 1**), indicating that many spacers match multiple protospacers located on different, possibly related, plasmids. From this data set, the shortest distance from each protospacer to the *oriT* was calculated and expressed as a percentage of the total plasmid size (**Fig. 1A**). Since the *oriT* marks the boundary between leading and lagging regions of the plasmid, distance-scores smaller than 50% are indicative of spacers targeting the leading regions, while distance-scores larger than 50% represent spacers targeting the lagging regions.

To analyze whether the distribution of protospacers on these plasmids was random, we performed a statistical analysis using the Kolmogorov-Smirnov test. This test revealed a statistically significant difference between the observed protospacer distribution and a uniform protospacer distribution ($p = 0.044$). The majority of spacers were found to target the lagging regions of these conjugative plasmids (**Fig. 1A**). Apart from this, we also observed a slight bias toward protospacer occurrence in the leading region at distances between 10 and 20% away from the *oriT*. Moreover, a significant clustering of protospacers ($p < 0.05$) was observed for 8 out of 36 plasmids, as determined by comparisons of the circular distributions of protospacers per plasmid to uniform distributions using Kuiper's tests. If no clustering per plasmid would occur at all, the expected number of significant clustering (at $p \leq 0.05$) would be $0.05*36 = 1.8$. In that case, the probability to find eight or more would be 0.00005. These analyses show that protospacers display significant clustering, and that they are slightly more often located within the lagging regions of conjugative plasmids.

The protospacer distribution bias is MOB family-dependent. Conjugative plasmids can be grouped into six different MOB families, based on the six different families of relaxase genes.[75,77,78] It is envisaged that differences exist between the different MOB family plasmids with respect to the molecular mechanism and kinetics of plasmid transfer. The observed bias toward targeting of the lagging region of conjugative plasmids may therefore be different for different MOB families. To investigate this, the data shown in **Figure 1A** were re-analyzed for each MOB family independently (**Fig. S1**). While two families were not represented in this data set due to the lack of annotated *oriT* (MOB_H and MOB_C), the results show that the targeting of lagging regions is most evident in the MOB_P family ($n = 351$). The MOB_F family ($n = 42$) however, shows a clear bias for targeting the leading regions.

To extend this analysis to conjugative plasmids lacking an annotated *oriT*, a complementary approach was performed using annotated relaxase genes.[75] This analysis is warranted by the fact that relaxase genes are located in the lagging region of conjugative plasmids.[76] The MOB_F family is a well-characterized family of conjugative plasmids[77] that is well-suited for this approach due to two important characteristics. First, the relaxase genes are well annotated (contrary to *oriT*s), allowing many members of this family to be included in our analysis. Second, the direction of transcription of the relaxase gene is (in the vast majority of cases) oriented away from the *oriT*,[76] which can therefore be exploited as a marker to determine the directionality of the *oriT* and, hence, the transition between leading and lagging regions can be predicted.

In this way, 127 different MOB_F-plasmids with known relaxase gene orientations were obtained, and these were used for screening the spacer BLAST-hits database. This revealed a total number of 1,213 protospacers on 70 different MOB_F plasmids, resulting from 815 unique spacers (**Fig. 1B**, **Table 1**). Since the exact position of the *oriT* site could not be determined, the distance-scores were calculated as the shortest distance from each protospacer to the start of the relaxase gene.

Checking for overall distribution of spacer hits over the MOB_F plasmids (analyzing the position relative to the relaxase gene) through the Kolmogorov-Smirnov test, showed a significant deviation from the uniform distribution ($p = 0.0025$). Protospacers are most frequently located approximately ~40% of the plasmid size away from the relaxase gene (**Fig. 1B**). Although the *oriT* is not taken into account in this analysis, based on the previous analysis of MOB_F plasmids containing an annotated *oriT* (**Fig. S1**) it is likely that this region corresponds to the leading region of the plasmid. In addition, significant clustering ($p < 0.05$) of protospacers was observed for 17 out of 68 plasmids, as determined by comparisons of the circular distributions of spacer hits per plasmid to uniform distributions using Kuiper's tests. The frequency of plasmids that show statistically significant clustering (17 out of 68) is substantially more than expected by chance ($p < 0.00001$).

Table 1. Specifications from the bioinformatics analysis of spacers targeting conjugative plasmids

Database	
Total spacers[a]	72,431
- unique	65,574
Total plasmids[b]	3,167
- annotated *oriT* and relaxase	48
- annotated relaxase	127
Conjugative plasmids with annotated *oriT* and relaxase (Fig. 1A)	
BLAST hits	506
Unique spacers	375
Unique plasmids	39
Conjugative MOB_F plasmids with annotated relaxase (Fig. 1B)	
BLAST hits	1,213
Unique spacers	815
Unique plasmids	70

[a]CRISPRdb (crispr.u-psud.fr/crispr). [b]GenBank plasmid database (ftp://ftp.ncbi.nlm.nih.gov/genomes/Plasmids).

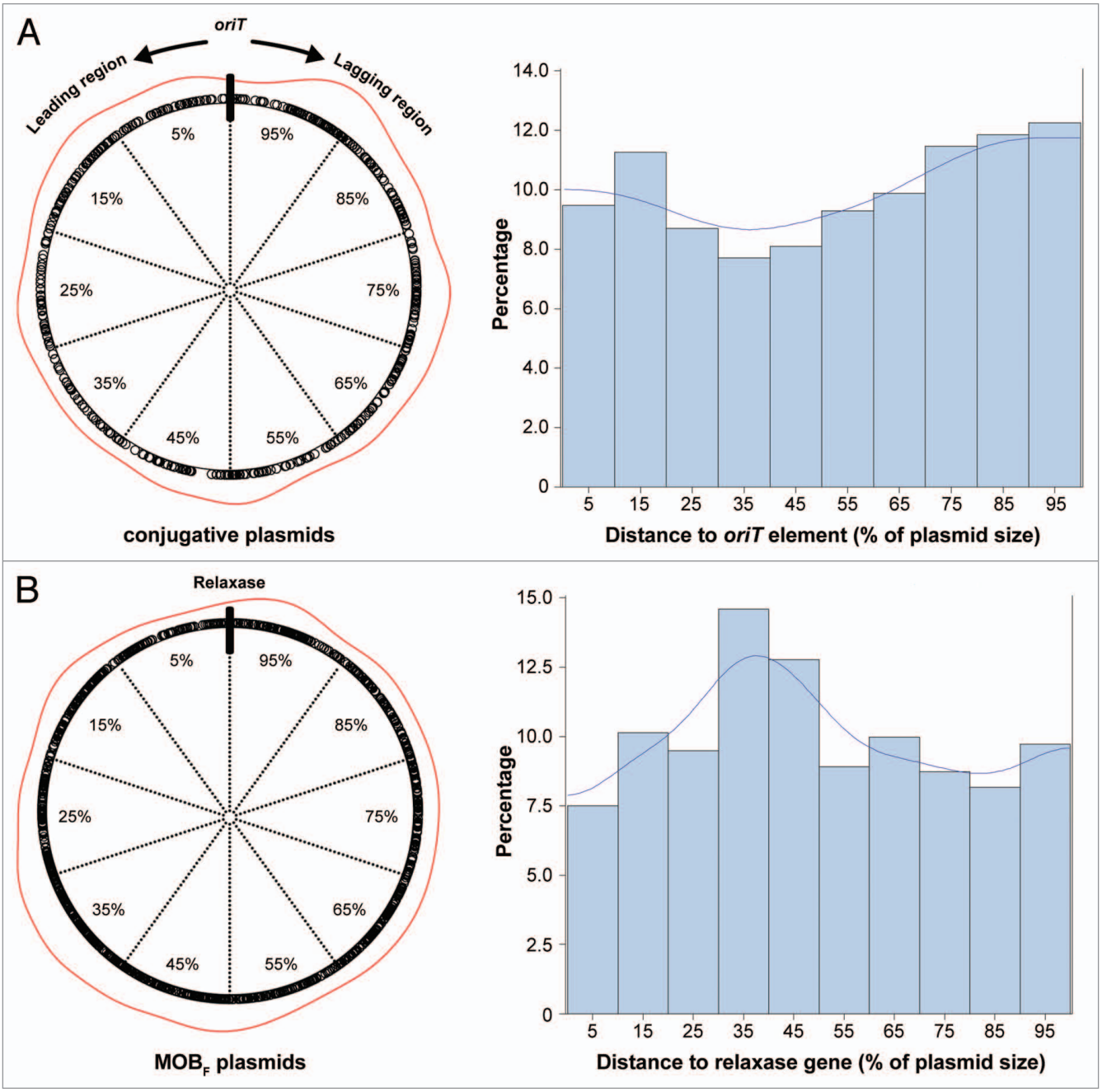


Figure 1. Spacers from CRISPRdb targeting conjugative plasmids. (**A**) Conjugative plasmids, of which the *oriT* site and the relaxase gene could be identified, were screened for homology with spacers from the CRISPRdb. After establishing the leading and lagging regions of the plasmid, by taking into account the location of the relaxase relative to the *oriT* site, the distance of each spacer hit from the *oriT* site is expressed as a percentage of the total plasmid size. These values are depicted as open circles on the plasmid map (left). The red line indicates the protospacer density at the respective position. The protospacer distribution is also shown in a histogram (right). To this end, the plasmid is divided into 10% segments (i.e., plasmid fragments corresponding to 10% of the plasmid size). When equally distributed, each 10% segment would carry 10% of all protospacers. The actual percentages of protospacers present in each 10% segment are indicated by the blue bars. (**B**) A similar analysis as in (**A**) was performed, but using only the MOB$_F$ family of conjugative plasmids, and using the relaxase gene start position to calculate distances of the spacer hits.

CRISPR targeting of conjugative plasmid F predominantly occurs within the leading region. To experimentally investigate the functional importance of the enriched targeting of MOB$_F$ conjugative plasmids within the leading region, we selected plasmid F as an exemplary case. The approximately 100 kb conjugative plasmid F (**Fig. 2A**) was discovered over 60 y ago as a sex factor in *E. coli* K12,[79] and has been well-studied over the past decades. It encodes the CcdAB toxin/anti-toxin system (encoded roughly at position 46.5 k of plasmid F) to prevent plasmid loss: the stable CcdB toxin targets the *E. coli* gyrase in the absence of the short-lived CcdA anti-toxin (which therefore is absent in plasmid-cured cells) (reviewed in ref. 80). Plasmid F

belongs to the MOB_{F1} subfamily of conjugative plasmids, which includes many members belonging to the Inc.F, Inc.N, Inc.W and Inc.P9 incompatibility groups.[77] Most plasmids belonging to the MOB_{F1} subfamily are hosted by Gamma-Proteobacteria.[77]

The large size of plasmid F, together with the existence of F-specific phages, indicate that, depending on the conditions, plasmid F can be a fitness cost to the host. In accordance with this, in silico analysis reveals that CRISPRdb[14] contains 17 spacers that match the plasmid F sequence (e-value < 0.05) (**Fig. 2D**). Of these spacers, eight are found in CRISPR loci of *E. coli* strains ED1, LF82, UM146 and O83:H1 NRG857C, whereas the other nine spacers are found in CRISPR loci of the following Gamma-Proteobacteria: *Klebsiella variicola* At-22, *Klebsiella oxytoca* E718, *Pectobacterium carotovorum* PC1 (a.k.a. *Erwinia carotovora*) (all belonging to the Enterobacteriacaea) and *Cellvibrio japonicus* Ueda107, and in CRISPR loci of Firmicutes (*Caldicellulosiruptor bescii* DSM 6725, *Caldicellulosiruptor lactoaceticus* 6A), Ignavibacteria (Ignavibacterium album JCM16511) and Archaea (*Thermococcus onnurineus* NA1 and *Desulfurococcus kamchatkensis* 1221n). In accordance with its classification in the MOB_F subfamily, most spacers (12 out of 17) were found in species belonging to the class of Gamma-Proteobacteria. F-like plasmids have a narrow host range, which is restricted to enteric bacteria (e.g., Escherichia, Klebsiella and Erwinia spp).[81,82] The observation that non-enteric species contain spacers that give a significant BLAST hit with plasmid F may arise from the possibility that these strains host related plasmids, or, alternatively, these strains may have experienced invasion by plasmid F, despite the fact that they cannot host this plasmid, as described before.[83,84] In agreement with the previous analysis, the majority (12 out of 17) of the protospacers are located within 50% (of the plasmid size) from the *oriT* (**Fig. 2A and B**), containing the leading region. The majority of protospacers shows clustering approximately 40% upstream of the relaxase gene *traI* (**Fig. 2C**), similar to the general trend that was observed for the entire MOB_F family (**Fig. 1B**). This further substantiates the finding that MOB_F family conjugative plasmids are mainly targeted within the leading region.

The Type I-E CRISPR-Cas system of *E. coli* K12 provides immunity against conjugative plasmid F. Next, we analyzed whether the *E. coli* K12 Type I-E CRISPR system provides protection against conjugative invasion by plasmid F. The Type I-E system consists of eight *cas* genes (*cas3*, *cse1*, *cse2*, *cas7*, *cas5*, *cas6e*, *cas1* and *cas2*) and CRISPRs with type-2 repeats (**Fig. 3A**).[85] Expression of the *cse1* to *cas2* operon is repressed by H-NS[86] and can be activated by LeuO,[38] or by the BaeSR two-component pathway.[44,87] To test for resistance against conjugation, we made use of the plasmid F-derived pOX38-Tc, which corresponds to the largest HindIII fragment of plasmid F, and contains a tetracycline resistance marker (**Fig. 3B**).

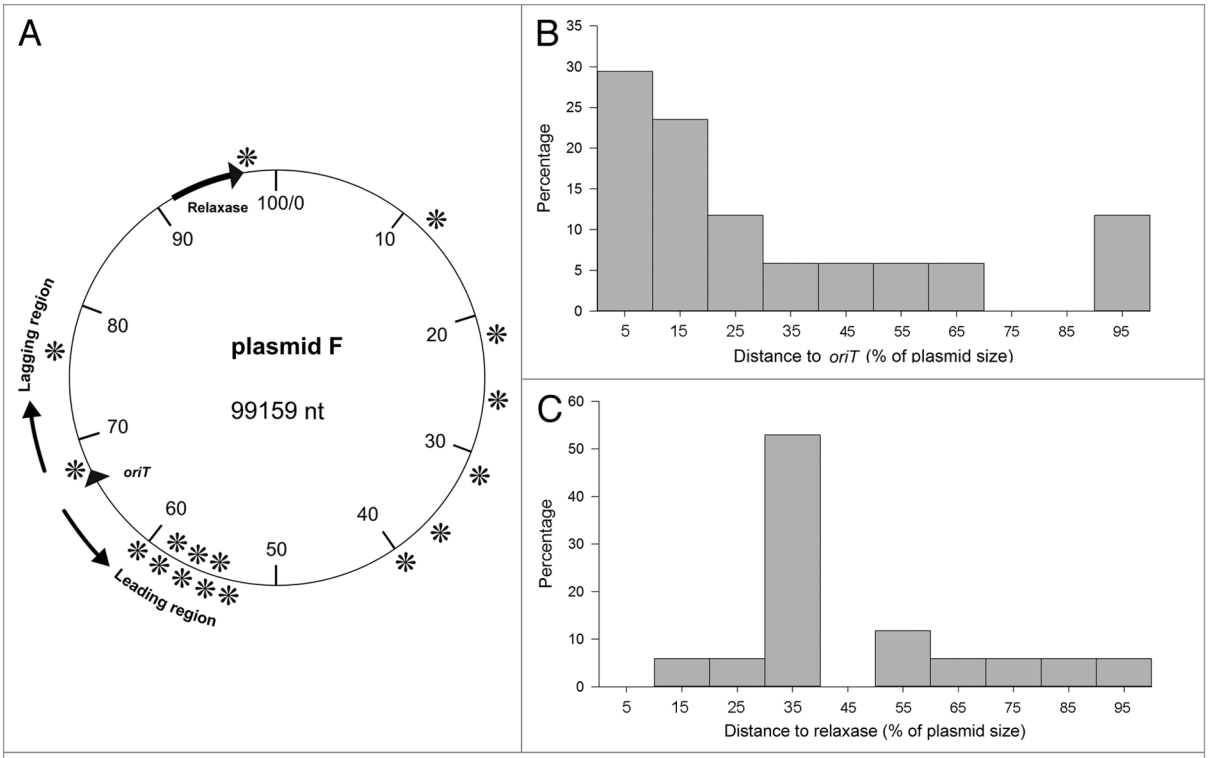
Two synthetic CRISPRs were used to test for resistance against plasmid invasion. CRISPR-J4 contains four identical spacers that target a sequence of phage Lambda, and serves as a negative control (**Fig. 3C**). CRISPR-F contains five different spacers that target the pOX38-Tc plasmid. Four of the spacers are randomly distributed over the plasmid backbone, whereas a fifth spacer targets the tetracycline resistance gene (**Fig. 3B and D**).

Wild-type *E. coli* K12 (recipient), transformed with plasmids containing either CRISPR-J4 or CRISPR-F, shows conjugation efficiencies of ~5×10^{-4} transconjugants/donor with MC4100/pOX38-Tc as donor (**Fig. 3E**). The lack of CRISPR-dependent resistance to plasmid conjugation in *E. coli* K12 is in agreement with the previously reported silencing of *cas* gene expression in this strain by H-NS.[38,86] When *E. coli* K12Δ*hns* transformed with CRISPR-F served as a recipient strain, a ~50-fold CRISPR-dependent reduction in conjugation efficiency was observed as compared with the same strain transformed with CRISPR-J4 (**Fig. 3E**). Conjugation efficiencies in the K12Δ*hns* strains (~2×10^{-5} transconjugants/donor) appear to be ~100-fold lower than in the wild-type cells, which is likely due to growth inhibition caused by *hns* deletion.[88]

When the same conjugation experiments were performed using *E. coli* BL21(DE3) transformed with plasmids encoding *cas* genes and CRISPR as a recipient strain (and MC4100/pOX38-Tc as donor), high-level CRISPR-interference was observed, leading to a 104-fold reduction in conjugation efficiency (as compared with recipient cells carrying a non-targeting CRISPR) when *cas* gene and CRISPR expression were not induced, while conjugation was entirely abrogated when expression of *cas* genes and CRISPR in the same strain was induced with 1 mM IPTG (**Fig. 3F**). These data demonstrate that *E. coli* K12 and BL21(DE3) strains are efficiently protected against conjugative invasion by plasmid pOX38-Tc.

Resistance levels conferred by Type I-E CRISPR-Cas is independent of the DNA strand and the region of plasmid F that is being targeted. The experimental system of CRISPR-mediated resistance in *E. coli* K12Δ*hns* against plasmid pOX38-Tc that is described above allows for testing hypotheses that may explain the observed bias toward preferential targeting of leading sequences of MOB_F conjugative plasmids under conditions that do not make use of overexpression of CRISPR and *cas* genes. A possible reason for the observed bias could be that cells containing spacers targeting the leading region of a MOB_F conjugative plasmid are more resistant to conjugation than cells targeting the lagging region. To investigate this, four synthetic CRISPRs were designed, two of which contain a single spacer that targets the leading region of pOX38-Tc, while the other two CRISPRs target the lagging region of pOX38-Tc (**Fig. 4A**). All four CRISPRs target the incoming strand of pOX38-Tc. The CRISPRs targeting the leading sequence were named CRISPR-F-IE1, CRISPR-F-IE2 [incoming early 1 and 2 (i.e., targeting the leading region of the incoming strand)]. The CRISPRs targeting the lagging region were named CRISPR-F-IL1, CRISPR-F-IL2 (incoming late 1 and 2) (**Fig. 4A**).

Conjugation efficiencies (transconjugants/donor) of pOX38-Tc from MC4100 donor cells to *E. coli* K12Δ*hns* transformed with any of these four synthetic CRISPRs are shown in **Figure 4C**. Some spacer sequences are more effective in protecting against plasmid transfer than other spacers: CRISPR-F-IE1, CRISPR-F-IL1 provide high levels of resistance (~100-fold reduction in conjugation efficiencies), whereas CRISPR-F-IE2 provides a



D

Cellvibrio japonicus Ueda107 CRISPR 4 spacer 33

```
Query  3      GTGGTGGCCGTTGGCCTGGTGGCGTGGGCA  32
              ||| || |||||||| |||||| |||| |||
Sbjct  98590  GTGCTGACCGTTGGTCTGGTGTCGTGTGCA  98619
```

Thermococcus onnurineus NA1 CRISPR 1 spacer 3

```
Query  3      AAACATTTCCATCCACCTCCATCTTTTTGC  32
              ||| |||||| | ||| ||||| |||||||
Sbjct  40140  AAAAATTTCCTTGCACATCCATTTTTTTGC  40111
```

Escherichia coli ED1a CRISPR 2 spacer 17

```
Query  1      CTGAACGTTGAAGAGTGCGA  20
              ||||||||||||||||||||
Sbjct  56779  CTGAACGTTGAAGAGTGCGA  56798
```

Escherichia coli ED1a CRISPR 2 spacer 6

```
Query  1      TGGCGCGCCTGGAGGACATCCCGGAAGACCAGC  33
              |||||||||||||||||||||||||||||||||
Sbjct  55239  TGGCGCGCCTGGAGGACATCCCGGAAGACCAGC  55271
```

Escherichia coli ED1a CRISPR 2 spacer 7

```
Query  1      GGTAACGGGTCAGGCCGACCGTGGAATACCGC  32
              ||||||||||||||||||||||||||||||||
Sbjct  25936  GGTAACGGGTCAGGCCGACCGTGGAATACCGC  25905
```

Escherichia coli ED1a CRISPR 2 spacer 8

```
Query  1      ATAAAGCGATCGACGCGGTTCCAGCCATAGAA  32
              ||||||||||||||||||||||||||||||||
Sbjct  54407  ATAAAGCGATCGACGCGGTTCCAGCCATAGAA  54376
```

Escherichia coli ED1a CRISPR 2 spacer 9

```
Query  6      CATCACAAACGGGAAGGGTAACGGGCGA  33
              ||||||||||| ||||||||||||||||
Sbjct  57958  CATCACAAACAGGAAGGGTAACGGGCGA  57931
```

Escherichia coli ED1a CRISPR 3 spacer 8

```
Query  1      GTTCCGTATACCAGTGATATGCAGAAGGAACA  32
              ||||||||||||||||||||||||||||||||
Sbjct  53454  GTTCCGTATACCAGTGATATGCAGAAGGAACA  53423
```

Desulfurococcus kamchatkensis 1221n CRISPR 1 spacer 80

```
Query  4      AGAGAAACTAAAAGAGATGAAGCTCGAGAAGGTGATC  40
              ||||||| | ||| | ||||| |||||||| || |||
Sbjct  12488  AGAGAAAGTTAAAAATATGAAATTCGAGAATGTTATC  12452
```

Caldicellulosiruptor bescii DSM 6725 CRISPR 4 spacer 18

```
Query  14     TCTTTCTCTCTCTTGCATCATCAC  37
              || ||||||||||| ||||||||||
Sbjct  31642  TCCTTCTCTCTCTGGCATCATCAC  31619
```

Pectobacterium carotovorum carotovorum PC1 CRISPR 4 spacer 2

```
Query  14     CTCTTCATCTTCCTCATCTTCTTCCTC  40
              |||||| ||||| || ||||||||||||
Sbjct  58030  CTCTTCCTCTTCTTCCTCTTCTTCCTC  58056
```

Klebsiella variicola At-22 CRISPR 2 spacer 23

```
Query  1      TCGACGATGTTCTGCGTGATGGTGATGTATGC  32
              ||||||||||||| |||||||||| || ||
Sbjct  75947  TCGACGATGTTCTGTGTGATGGTGATATAGGC  75916
```

Caldicellulosiruptor lactoaceticus 6A CRISPR 5 spacer 4

```
Query  8      AATAAAAACATCCACTATAACATTAA  33
              |||||||||||| ||| || |||||
Sbjct  67336  AATAAAAACATCCTCTACAAGATTAA  67311
```

Ignavibacterium album JCM 16511 CRISPR 2 spacer 76

```
Query  10     AAACATATCTAACATCCTTTTA  31
              ||||||||||||||| ||||||
Sbjct  22477  AAACATATCTAACATACTTTTA  22498
```

Escherichia coli UM146 CRISPR 3 spacer 3

```
Query  1      TGTGGCGCTGATGCGTCTGGGCGTCTTTGTAC  32
              |||||||||||||||||||||||| |||||||
Sbjct  37558  TGTGGCGCTGATGCGTCTGGGCGTTTTTGTAC  37527
```

Escherichia coli O83:H1 str. NRG 857C CRISPR 2 spacer 9

```
Query  1      CTGAACGTTGAAGAGTGCGACCGTCTCTCCTT  32
              ||||||||||||||||||||||||||||||||
Sbjct  56779  CTGAACGTTGAAGAGTGCGACCGTCTCTCCTT  56810
```

Klebsiella oxytoca E718 CRISPR 2 spacer 3

```
Query  1      GCAGCCTGAACAGCAAGGGTGCGCTCTGAAGA  32
              || ||||| || || || |||||||||| ||
Sbjct  60868  GCTGCCTGGACTGCCAGCGTGCGCTCTGAGGA  60837
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Figure 2. Spacers from CRISPRdb targeting plasmid F. (**A**) Map of plasmid F indicating the size of the plasmid and the location of the origin of transfer (*oriT*), the relaxase gene, the leading region (which enters the recipient cell first) and the transfer region (which encodes the genes essential for plasmid transfer). Asterisks indicate the approximate positions of the protospacers listed in (**D**). (**B**) Similar analysis as presented in **Figure 1A**, where the distance of each spacer hit on plasmid F from the *oriT* or (**C**) from the relaxase gene is calculated and expressed as a percentage of the total plasmid size. (**D**) Alignments of spacer sequences ("Query," top sequences) and the corresponding plasmid F sequences ("Sbjct", bottom sequences). The species, CRISPR and spacer are indicated above each alignment, following the nomenclature used by the CRISPRdb. The numbers adjacent to the alignment indicate the position of the spacer and protospacer sequence, respectively.

lower level of resistance (15-fold reduction in conjugation efficiencies). Interestingly, the level of immunity conferred by the CRISPR-F variants appears to be independent of the plasmid target region (i.e., leading vs. lagging regions). Since no differences in immunity levels were found, the observed protospacer distribution bias is unlikely to be due to differences at the level of CRISPR-interference.

During plasmid conjugation, single-stranded plasmid DNA is transferred by a Type IV secretion system from the donor to the recipient cell, and synthesis of the cDNA strand is generally believed to occur after re-circularization of the conjugative plasmid in the recipient cell.[89] At present, it is unknown whether Type-I-E CRISPR-Cas systems target single-stranded DNA. To test whether differences exist in CRISPR immunity levels that are associated with the DNA strand that is being targeted, four additional synthetic CRISPRs were constructed that target the synthesized strand of pOX38-Tc. Of these four CRISPRs, two target the leading region and two target the lagging region of pOX38-Tc (**Fig. 4B**). These CRISPRs were named CRISPR-F-SE1 and CRISPR-F-SE2 (synthesized early 1 and 2) and CRISPR-F-SL1 and CRISPR-F-SL2 (synthesized late 1 and 2) (**Fig. 4B**).

CRISPR immunity levels appear to be independent of the plasmid strand that is being targeted, as determined by measuring the conjugation efficiencies (transconjugants/donor) of pOX38-Tc from MC4100 donor cells to *E. coli* K12Δ*hns* carrying the CRISPR-F variants (**Fig. 4D**). Again, some variation exists in the levels of immunity. Since the AT-content of the spacers and the PAM sequences flanking the protospacers are identical in all cases, this suggests that additional factors play a role in determining the level of interference conferred by a spacer sequence. Altogether, it seems that the level of resistance is independent of whether the leading or the lagging region is being targeted by a CRISPR spacer and whether the incoming or synthesized strand is being targeted. These data indicate that single-stranded DNA may not be a target for Type I-E CRISPR-Cas systems, and suggest that the protospacer distribution bias might be due to spacer acquisition preferences.

Discussion

CRISPR/Cas systems are highly versatile immune systems that can block invading DNA to provide immunity against phage infections, prevent transfer of conjugative plasmids or transposons, and can remove resident DNA such as plasmids. The spacer content of CRISPRs in natural populations appears to be highly dynamic, with frequent spacer acquisition and spacer loss.[46,48-51,90-93] The adaptive properties of CRISPR-Cas make them uniquely capable of modulating the mobilome of a species.[46] Indeed, these systems are well suited to remove a given DNA element, such as a conjugative plasmid, when such an element is associated with a fitness cost. If environmental conditions change such that the DNA element provides a selective advantage, natural selection will favor clones that have lost or mutated the corresponding spacer sequence.

In this study, we have analyzed the role of CRISPR-Cas in providing resistance against conjugative plasmids. Screening for spacers matching conjugative plasmid sequences revealed that their cognate target sequences are not randomly dispersed along the plasmid DNA sequence. Instead, our analysis showed that spacers preferentially target the lagging region, while a smaller trend for targeting the leading region could also be observed (**Fig. 1A**). The different conjugative plasmids included in this analysis were selected on the basis of an annotated *oriT* site, which might not be representative for the whole population of conjugative plasmids. Indeed, the data set was enriched for three particular classes of conjugative plasmids: MOB_P (n = 351, 69%), MOB_F (n = 42, 8%) and MOB_Q (n = 33, 6.5%) (**Fig. S1**). Interestingly, MOB_P-class plasmids were found to be targeted in the lagging regions, while MOB_F-class plasmids are more often targeted in the leading regions. The rationale for this is currently unclear.

Since the total number of MOB_F plasmids containing an annotated *oriT* was rather low (n = 42), we conducted a complementary approach by screening all known MOB_F plasmids for the presence of a relaxase gene and using its start position to calculate relative distances to the target sequences. The orientation of plasmid transfer was established by the direction of transcription of the relaxase gene that is pointing away from the *oriT*.[76] In agreement with the first analysis, this approach demonstrated that spacer sequences targeting MOB_F family conjugative plasmids are significantly enriched for sequences that likely correspond to the leading region of these plasmids (**Fig. 1B**). The results clearly demonstrate that CRISPR-Cas-targeted regions on conjugative plasmids are not randomly dispersed, which is reinforced by the finding that protospacers on the same plasmid are significantly clustered.

It should be kept in mind that we did not determine whether the protospacers found by BLAST searches would still support CRISPR interference, as our analysis lacks the ability to take into account biologically important features for the various CRISPR/Cas types such as the presence of a PAM[36,53,94] and seed region of the protospacer.[32,43] In addition to the protospacer distribution analysis presented here, it would also be interesting to investigate whether a bias exists for the strand of the conjugative plasmid that is being targeted. Unfortunately, the direction of transcription of most CRISPR arrays listed in the CRISPRdb is unknown, hence making it impossible to determine which strand of the conjugative plasmid is being targeted. Nevertheless, the distribution of protospacers on conjugative plasmids, even when these protospacers no longer support CRISPR interference due to escape mutations, provides a valuable insight into host defense strategies against conjugative plasmids.

Using plasmid F as an exemplary case, we have investigated the biological basis for the biased protospacer distribution. We propose two explanations that are not mutually exclusive. The first explanation would be a bias occurring at the level of CRISPR adaptation. This hypothesis suggests that sequences from the leading region of MOB_F plasmids are preferentially selected for integration as a novel spacer into a CRISPR array. Such an effect could, for example, be caused by interrupted mating events (i.e., the process where a mating pair is disrupted during conjugational plasmid transfer), which leads to partial transfer of the conjugative plasmid DNA. Hence, only DNA

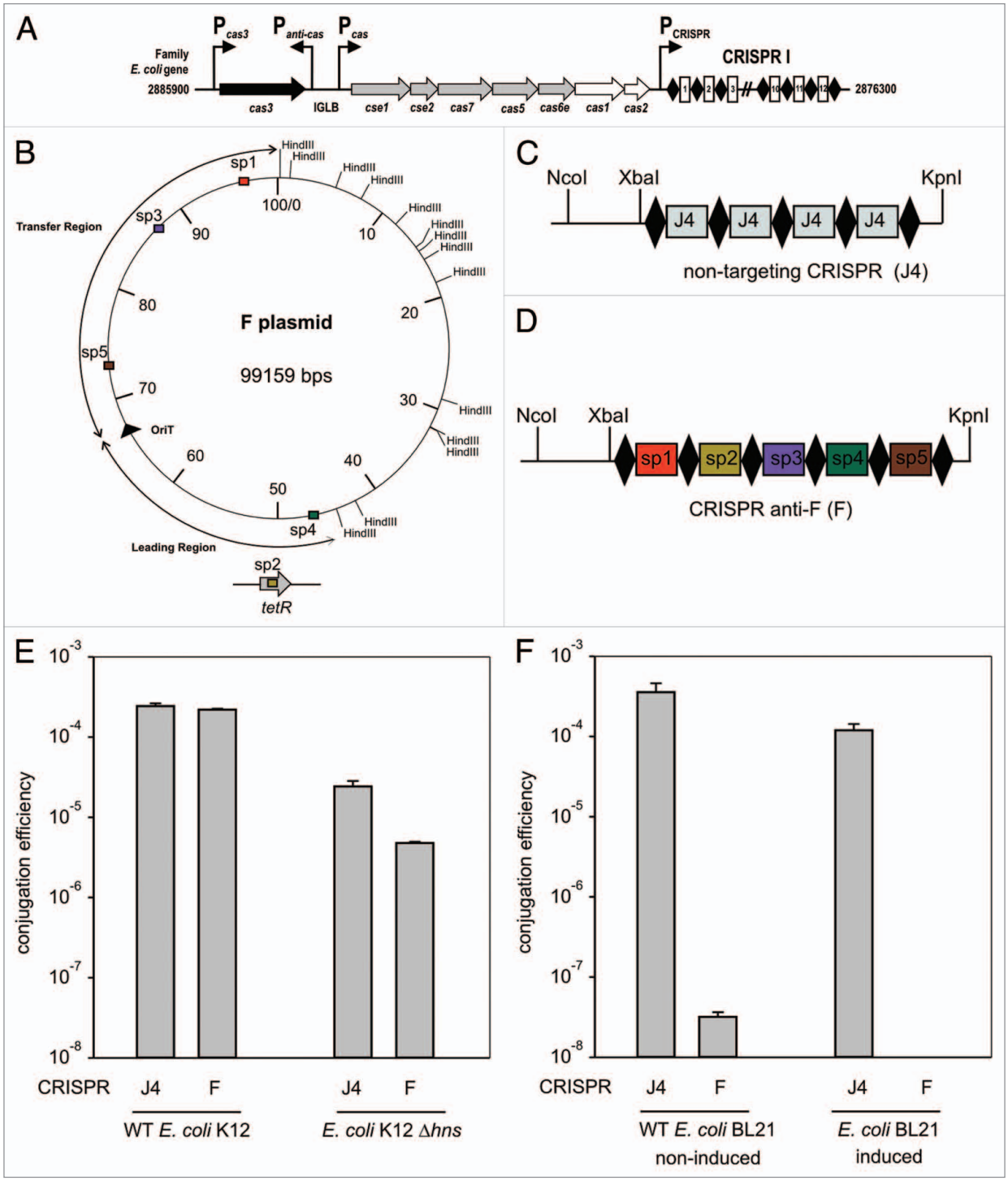

Figure 3. A synthetic anti-pOX CRISPR provides resistance against conjugational plasmid transfer. (**A**) The Type I-E CRISPR-Cas system of *E. coli* contains eight *cas* genes and a downstream CRISPR with type 2 repeats. Promoter elements (P*cas3*, P*anti-cas*, P*cas* and P^{CRISPR}) are indicated by arrows. (**B**) A plasmid map of the F-plasmid with all HindIII sites. The origin of transfer (*oriT*) is indicated by a black triangle, the leading and transfer regions of the plasmid are indicated with arrows. The plasmid F derived pOX38-Tc corresponds to the largest HindIII fragment of plasmid F, and contains a tetracycline resistance marker. The positions of the protospacers targeted by the synthetic anti-pOX CRISPR indicated in (**D**) are indicated by colored boxes and labeled with the name of the spacer that targets the site (i.e., sp1-5). (**C**) Schematic representation of the non-targeting CRISPR J4, which contains four identical spacer sequences targeting the J gene of phage Lambda. (**D**) Schematic representation of the targeting CRISPR-F, which contains five different spacer sequences targeting sequences of pOX38-Tc. The positions of the target sequences are indicated in (**B**) by colored boxes and labeled with the name of the spacer that targets the site. (**E**) Conjugation efficiencies using *E. coli* MC4100 + pOX38-Tc as a donor strain and *E. coli* K12 or *E. coli* K12Δ*hns* transformed with a plasmid carrying either CRISPR-J4 or CRISPR-F as recipient strains. (**F**) Conjugation efficiencies using donor strain as in (**E**) and either induced or uninduced *E. coli* BL21 (DE3) transformed with either CRISPR-J4 or CRISPR-F as recipient strains. Conjugation efficiencies are expressed as transconjugants/donor.

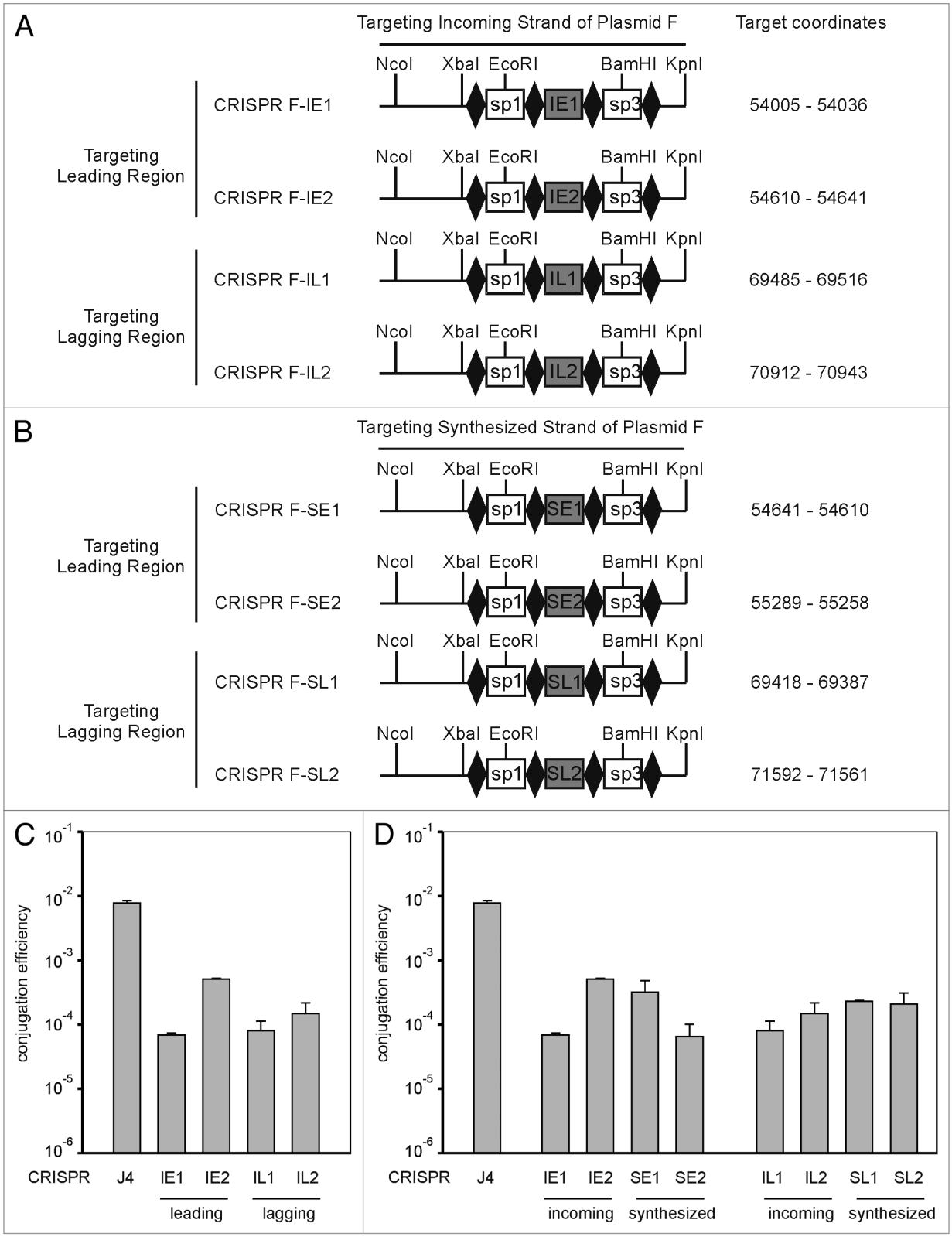

Figure 4. Interference levels of CRISPRs targeting leading regions as compared with CRISPRs targeting non-leading regions. (**A and B**) Synthetic CRISPR constructs targeting plasmid F are shown together with the coordinates of the corresponding protospacer on plasmid F. Four CRISPRs contain a spacer at the second position in the array that targets the leading region of plasmid F (IE1, IE2, SE1, SE2) and four additional CRISPRs target the lagging region of plasmid F (IL1, IL2, SL1, SL2). Of these CRISPRs, four target the incoming strand of plasmid F (IE1, IE2, IL1, IL2) and four CRISPRs target the synthesized strand of plasmid F (SE1, SE2, SL1, SL2). (**C**) Efficiency of conjugation (#transconjugants/#donor) from *E. coli* MC4100 donor cells carrying pOX38-Tc to *E. coli* K12Δ*hns* transformed with any of the CRISPRs shown in (**A**). (**D**) Efficiency of conjugation (#transconjugants/#donor) from *E. coli* MC4100 donor cells carrying pOX38-Tc to *E. coli* K12Δ*hns* transformed with any of the CRISPRs shown in (**B**).

entering the recipient cell first (corresponding to the leading region) would be transferred and subsequently be available for a spacer integration event in the recipient cell. Since the incoming DNA is single stranded during plasmid conjugation, this model would have implications for the mechanism of spacer acquisition (i.e., single-stranded pre-spacers would need to be substrates for spacer acquisition).[16,19,95] We have aimed to examine this hypothesis by following spacer integration under laboratory conditions in response to plasmid F, using both *E. coli* K12Δ*hns* cells and *E. coli* K12Δ*cse1* cells as recipients. Whereas spacer acquisition into the Δ*hns* strain would be subject to selection both at the level of CRISPR adaptation and at the level of CRISPR interference, spacer acquisition into the Δ*cse1* would be subject to selection at the level of CRISPR adaptation only (this strain displays high level expression of the *cas* genes downstream of the integrated kanamycin resistance cassette[39]). Hence, differences between newly acquired spacers by these two strains with respect to the regions that are being targeted would provide insight into the biological basis causing the biased targeting of the leading region of MOB_F plasmids. However, despite numerous attempts, spacer acquisition in response to conjugative plasmid transfer could not be observed (data not shown). This observation contrasts with previously described spacer acquisition during non-mobile plasmid curing by *E. coli* K12Δ*hns*.[19]

An alternative explanation for the biased targeting of the leading region of MOB_F conjugative plasmids would be that this leads to more efficient CRISPR-interference as compared with targeting other regions of the conjugative plasmid. However, using synthetic CRISPRs targeting either the leading or the lagging region of plasmid F, we found no evidence for differences in resistance levels. In addition, we did not observe differences in the effectiveness of CRISPR arrays targeting either the incoming strand directly or the synthesized strand, which might suggest that CRISPR interference takes place on the double-stranded protospacer target only. This is in agreement with the recently determined Cas3 cleavage site in the displaced strand of the Cascade-induced R-loop.[96]

One factor that could influence both CRISPR-adaptation and interference is local DNA topology,[30,97] which can be influenced by DNA structuring proteins, such as H-NS.[98-101] This could cause increased exposure of defined regions of the plasmids to the integration and interference machineries. These regions may, for example, be linked to transcriptionally active or inactive regions and may be conserved within MOB plasmid families.

In addition to the analyses presented here, it will be interesting to perform competition experiments between strains carrying different CRISPR-F variants, in order to obtain insight into Darwinian fitness associated with resistance and possible differences between strains due to the plasmid region that is being targeted. One possibility that we consider is that MOB_F plasmid-encoded addiction systems may cause toxic effects when the plasmid is targeted in the lagging region, while this toxic effect is avoided by targeting of the leading region, since the plasmid will be degraded earlier during conjugation. Such effects may not influence resistance levels but could affect Darwinian fitness of the host.

This study has demonstrated that the *E. coli* CRISPR-Cas system effectively protects against conjugative plasmid transfer. Furthermore, we have shown that a biased protospacer distribution exists on conjugative plasmids, which is MOB-family-dependent. Whereas some MOB families are mainly targeted within the leading regions (e.g., MOB_F), others are targeted more frequently within the lagging regions (e.g., MOB_P). Future research is required to explore the biological factors that cause this biased protospacer distribution. The hypotheses that are proposed in this study aim to provide a framework for this exciting field of research.

Materials and Methods

Strains, gene cloning, plasmids and vectors. *E. coli* K12 BW25113, *E. coli* K12Δ*hns* (from the KEIO collection) and *E. coli* BL21 (DE3) (Novagen) strains were used throughout the study as recipient strains. MC4100 carrying pOX38-Tc was used as a donor strain. Plasmids pWUR400 and pWUR397, which encode the Cascade genes and *cas3*, have been described previously,[22,29] and pWUR692, which encodes CRISPR-F, or pWUR691, which encodes CRISPR-J4 were used to express the *cas* genes and CRISPR in BL21(DE3). Synthetic CRISPRs were expressed in *E. coli* K12Δ*hns* by introducing the following plasmids: pWUR692 (CRISPR-F), pWUR691 (CRISPR J4), pWUR693 (CRISPR-F-IE1) pWUR694 (CRISPR-F-IE2) pWUR695 (CRISPR-F-IL1) pWUR696 (CRISPR-F-IL2) pWUR697 (CRISPR-F-SE1) pWUR698 (CRISPR-F-SE2) pWUR699 (CRISPR-F-SL1) pWUR700 (CRISPR-F-SE2). CRISPR-encoding plasmids were generated by subcloning synthetic CRISPRs (GeneArt) in pACYC-duet vectors using the NcoI and KpnI sites. A full description of the plasmids used in this study is provided in **Table S1**.

Bioinformatics analyses. Spacer sequences [taken from the CRISPRdb website (http://crispr.u-psud.fr/crispr/)] and plasmid sequences [taken from the GenBank FTP site (ftp://ftp.ncbi.nlm.nih.gov/genomes/Plasmids/)] were collected on September 26, 2012. Conjugative plasmids sequences were extracted from the initial plasmid database by screening them for the *oriT* feature key or by screening for annotated relaxase genes.[77] In some cases, identification of the relaxase gene was performed by manual inspection of the plasmid's feature table. BLAST (version.2.27) was used to screen for spacers hits in a locally generated BLAST database that was constructed using the above-mentioned plasmids. The BLAST settings included a word-size of 7, with an expectation value threshold of 0.1. The cost to open and extend a gap were set at 5 and 2, respectively, with a +1 reward for nucleotide matches, and a -1 penalty for nucleotide mismatches. Results were imported in Excel for further processing and to calculate the distances.

Statistical analysis. For statistical analysis we used the R program, version 2.14.2, especially the R-package circular.[102] To test for possible clustering of spacer hits within the plasmids, the Kolmogorov-Smirnov test was applied, which compares the distribution of spacer hits over the plasmids (relative to *oriT* in one analysis, and relative to the relaxase gene for the other analysis, as indicated in the text) to the uniform distribution. For this

analysis, the spacer position within a plasmid was specified as a percentage of plasmid size, with small percentages indicating positions close to the reference site (*oriT*/relaxase) in the direction of the leading region of the plasmid. A percentage of 50% indicates a position on the plasmid opposite the reference site and a percentage close to 100% indicates a position close to the reference site, in the lagging region of the plasmid.

To test for clustering of spacers within individual plasmids, we chose a circular approach, so that e.g., spacers on positions 1% and 99%, far apart on the linear scale, were close together on the circular scale. Testing for uniformity of the circular distribution of spacers per plasmid was done applying Kuiper's test. To test whether the number of plasmids with significant clustering was higher than expected by chance, we performed a binomial test.

Conjugation experiments. Conjugation experiments were performed by diluting an overnight culture of the donor strain MC4100 carrying pOX38-Tc 1:50 into fresh LB supplemented with tetracycline (10 μg/ml) and by diluting an overnight recipient strain 1:50z into LB supplemented with the appropriate antibiotics. When donor and recipient cells reached an OD^{600} of 0.6 they were transferred into fresh LB lacking antibiotics: 0.25 ml donor cells and 1 ml recipient cells were added to 5 ml LB. Conjugation was allowed for 4 h at 37°C without shaking. For the experiments where BL21(DE3) served as a recipient strain, 1 mM IPTG was added to the medium during conjugation. Next, cells were plated on LB containing 1.5% agar and appropriate antibiotics [i.e., tetracycline (10 μg/ml) for plating MC4100 + pOX38-Tc donor cells; kanamycin (50 μg/ml) and chloramphenicol (34 μg/ml) for plating recipient Δ*hns* strains carrying CRISPR-encoding pACYC-duet plasmids; kanamycin, streptomycin (50 μg/ml) and chloramphenicol for plating recipient BL21 (DE3) strains carrying CRISPR-encoding pACYC-duet plasmids, Cascade-encoding pCDF1-b and Cas3-encoding pRSF1b; both tetracycline and chloramphenicol to plate transconjugants]. Colony counting allows for calculation of conjugation efficiencies (expressed as #transconjugants/#donor cells).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be found here:
www.landesbioscience.com/journals/rnabiology/article/24202

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