Processing-Independent CRISPR RNAs Limit Natural Transformation in *Neisseria meningitidis*

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http://dx.doi.org/10.1016/j.molcel.2013.05.001

SUMMARY

CRISPR interference confers adaptive, sequence-based immunity against viruses and plasmids and is specified by CRISPR RNAs (crRNAs) that are transcribed and processed from spacer-repeat units. Pre-crRNA processing is essential for CRISPR interference in all systems studied thus far. Here, our studies of crRNA biogenesis and CRISPR interference in naturally competent *Neisseria* spp. reveal a unique crRNA maturation pathway in which crRNAs are transcribed from promoters that are embedded within each repeat, yielding crRNA 5′ ends formed by transcription and not by processing. Although crRNA 3′ end formation involves RNase III and *trans*-encoded tracrRNA, as in other type II CRISPR systems, this processing is dispensable for interference. The meningococcal pathway is the most streamlined CRISPR/Cas system characterized to date. Endogenous CRISPR spacers limit natural transformation, which is the primary source of genetic variation that contributes to immune evasion, antibiotic resistance, and virulence in the human pathogen *N. meningitidis*.

INTRODUCTION

Clustered, regularly interspaced, short palindromic repeat (CRISPR) loci confer sequence-based, adaptive immunity against virus infection and plasmid conjugation in bacteria and archaea (Haft et al., 2005; Makarova et al., 2006; Barrangou et al., 2007; Terns and Terns, 2011; Wiedenheft et al., 2012). CRISPRs consist of short repeats separated by similarly sized, nonrepetitive sequences called spacers, which are derived from previously encountered invasive sequences such as viral genomes or plasmids (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). CRISPR loci are transcribed as long CRISPR RNA (crRNA) precursors that are processed into small crRNAs (Brouns et al., 2008; Hale et al., 2008). Pre-crRNA tran-

scription is generally driven by promoters within "leader" sequences outside of the CRISPR array. The crRNAs assemble with CRISPR-associated (Cas) proteins into complexes that cleave complementary "protospacer" sequences within invasive nucleic acids, a phenomenon known as CRISPR interference (Karginov and Hannon, 2010; Marraffini and Sontheimer, 2010; Terns and Terns, 2011; Wiedenheft et al., 2012). The sequence information in crRNAs is used to guide Cas complexes to their targets on viruses and plasmids, leading to their destruction (Barrangou et al., 2007; Brouns et al., 2008; Marraffini and Sontheimer, 2008; Hale et al., 2009; Garneau et al., 2010; Westra et al., 2012). Most CRISPR/Cas systems cleave incoming DNAs (Marraffini and Sontheimer, 2008; Garneau et al., 2010; Westra et al., 2012), though RNA-cleaving systems have also been identified (Hale et al., 2009, 2012; Zhang et al., 2012).

CRISPR/Cas systems have been classified into types I, II, and III based primarily on their *cas* gene complement (Makarova et al., 2011a). Common to all of these three types is that the CRISPR array is transcribed as a multimeric pre-crRNA that is processed into crRNAs that each contain an individual spacer flanked on one or both sides by partial repeat sequences (Bhaya et al., 2011). However, the molecular events underlying processing dramatically differ. Whereas in type I and III systems the processing enzymes are encoded within the CRISPR/*cas* locus, type II systems use the host enzyme RNase III (encoded by the *rnc* gene) and a noncoding RNA called tracrRNA (Deltcheva et al., 2011). In *Streptococcus pyogenes* SF370, an *rnc* mutation abolishes the function of a type II CRISPR/*cas* locus, indicating that pre-crRNA processing is essential (Deltcheva et al., 2011).

The importance of the type II CRISPR/Cas pathway has been dramatically enhanced by its development into a system for RNA-guided DNA cleavage in vitro (Jinek et al., 2012) and genome editing in vivo (Jinek et al., 2013; Cho et al., 2013; Cong et al., 2013; Dicarlo et al., 2013; Hwang et al., 2013; Jiang et al., 2013; Mali et al., 2013). Our ability to further exploit this new technology will depend on a deeper understanding of the underlying molecular mechanisms, and will be increased by the characterization of systems that are as simplified and streamlined as possible. Type II CRISPR/*cas* loci, which are found in bacteria but not archaea, usually contain four *cas*

genes: *cas1*, *cas2*, *cas9*, and either *csn2* (subtype II-A) or *cas4* (subtype II-B) (Makarova et al., 2011b). Cas9 is the effector protein for the interference function of existing spacer sequences in the CRISPR array (Sapranauskas et al., 2011; Gasiunas et al., 2012; Jinek et al., 2012), whereas the other proteins are thought to be involved in the acquisition of new CRISPR spacers. The tracrRNA is essential for crRNA-directed DNA recognition and Cas9-catalyzed DNA cleavage in vitro, even with crRNAs that bypass processing (Jinek et al., 2012). DNA targeting in both type I and type II systems requires not only crRNA/target complementarity but also a protospacer adjacent motif (PAM), which is a short (2–5 nt), conserved sequence just outside of the protospacer (Deveau et al., 2008; Horvath et al., 2008; Mojica et al., 2009; Semenova et al., 2011; Sashital et al., 2012).

Although CRISPR interference was originally defined as a phage defense pathway, CRISPR/Cas systems are now understood to play a broader role in limiting horizontal gene transfer (HGT) (Marraffini and Sontheimer, 2008). The three primary routes of HGT are transformation, conjugation, and phage transduction, and the latter two are well established as being subject to interference by naturally occurring spacers. Artificial means of transformation (e.g., electroporation) can also be blocked by CRISPR interference (Marraffini and Sontheimer, 2008; Deltcheva et al., 2011; Sapranauskas et al., 2011; Semenova et al., 2011), though natural transformation uses a very different DNA uptake process (Chen et al., 2005). An engineered spacer can prevent natural transformation specified by an *S. pyogenes* CRISPR/*cas* locus transplanted into *Streptococcus pneumonia* (Bikard et al., 2012). However, although this artificial system suggests that natural CRISPR/Cas contexts may do likewise, the fundamental question of whether native CRISPR/Cas systems play a role in natural transformation remains to be addressed.

Strains from the genus *Neisseria* serve as paradigms for natural transformation, as they are competent during all phases of their life cycle and use this process for frequent genetic exchange (Hamilton and Dillard, 2006). Although functional CRISPR/Cas systems have not been identified in *Neisseria gonorrhoeae*, some strains of *Neisseria lactamica* and *Neisseria meningitidis* carry apparent type II CRISPR/*cas* loci (Grissa et al., 2007). Meningococci are obligate human commensals that can invade the bloodstream and cerebrospinal fluid (Bratcher et al., 2012), and meningococcal disease is responsible for tens of thousands of deaths per year (Stephens et al., 2007).

Here we characterize the CRISPR pathway in neisseriae and find that it exhibits several unique features, most notably a streamlined functional architecture that includes a previously unknown, processing-independent mode of crRNA biogenesis. Furthermore, naturally occurring spacers match sequences from other *Neisseria* genomes, including a prophage-like meningococcal disease-associated (MDA) island that correlates with invasiveness and pathogenicity (Bille et al., 2005, 2008). We find that a native meningococcal CRISPR/*cas* locus prevents natural transformation of spacer-matched sequences, suggesting that it can limit the horizontal spread of virulence genes.

RESULTS

dRNA-seq Reveals that Each Repeat in the *Neisseria* CRISPR Carries Its Own Promoter

We analyzed all 19 sequenced *Neisseria* genomes available in the NCBI database (fifteen from *N. meningitidis*, three from *N. gonorrhoeae*, and one from *N. lactamica*) using CRISPRFinder (Grissa et al., 2007) or CRISPRi (Rousseau et al., 2009). We identified seven putative type II CRISPR/*cas* loci: six in *N. meningitidis* strains, and one in *N. lactamica* 020-06. All were highly similar and, unlike other type II loci characterized previously (Barrangou et al., 2007; Deltcheva et al., 2011; Magadán et al., 2012), included a set of only three predicted protein-coding genes (*cas9*, *cas1*, and *cas2*) but neither *csn2* nor *cas4*. To examine the expression status of a representative locus, we performed our recently developed dRNA-seq approach (Sharma et al., 2010) on *N. lactamica* 020-06. We prepared two cDNA libraries from total RNA using a strategy that allows us to distinguish between transcripts with either primary or processed 5′ ends: one library is generated from untreated RNA, whereas the other is treated with terminator exonuclease (TEX), which specifically degrades RNAs with 5′-monophosphate ends (including the abundant rRNAs and tRNAs) that are formed by processing. Primary transcripts with 5′-triphosphate ends survive TEX treatment, resulting in their relative enrichment in the TEX+ library.

Our global mapping of cDNA reads identified a tracrRNA and small crRNAs as highly abundant classes of transcripts (Figure 1A), which suggests a highly active CRISPR/Cas system. Reads obtained from the tracrRNA locus reveal the existence of two tracrRNA forms (107 nucleotides [nt] and 91 nt) (Figure 1B). The treatment with TEX eliminated the shorter tracrRNA reads, which indicates that they are products of processing as observed in a type II-A CRISPR system (Deltcheva et al., 2011). These sequencing results were confirmed by northern analyses (Figure 1C). In striking contrast, crRNAs were enriched rather than depleted in the TEX-treated libraries (Figure 1D), suggesting that formation of many if not most crRNA 5′ ends is processing independent. TEX treatment did affect the relative amounts of individual crRNAs; for example, crRNAs from spacers 4, 6, and 9 exhibited very similar read counts in the untreated sample but varied over a 5-fold range in the TEX-treated sample. These results suggest that the crRNA pool contains some 5′-monophosphorylated crRNAs, the fraction of which varies from spacer to spacer. Northern blots confirmed the resistance of a population of mature, ~48 nt crRNAs, as well as a subset of longer crRNA precursors, to TEX digestion (Figure 1E). This crRNA profile indicates a different mode of crRNA biogenesis from that reported for other type II systems (Deltcheva et al., 2011). Intriguingly, we noted that the terminal nine nucleotides of each CRISPR repeat exhibit sequence hallmarks of an extended −10 box promoter element (Hook-Barnard and Hinton, 2007) and that the TEX-resistant crRNA 5′ ends map 9–10 nt downstream from the center of each such element (Figure 1D).

crRNA Biogenesis in *Neisseria lactamica* Depends on Single Promoter Elements in Each CRISPR Repeat

The dRNA-seq results and −10 box similarity suggest that in *N. lactamica* 020-06, each CRISPR repeat carries its own
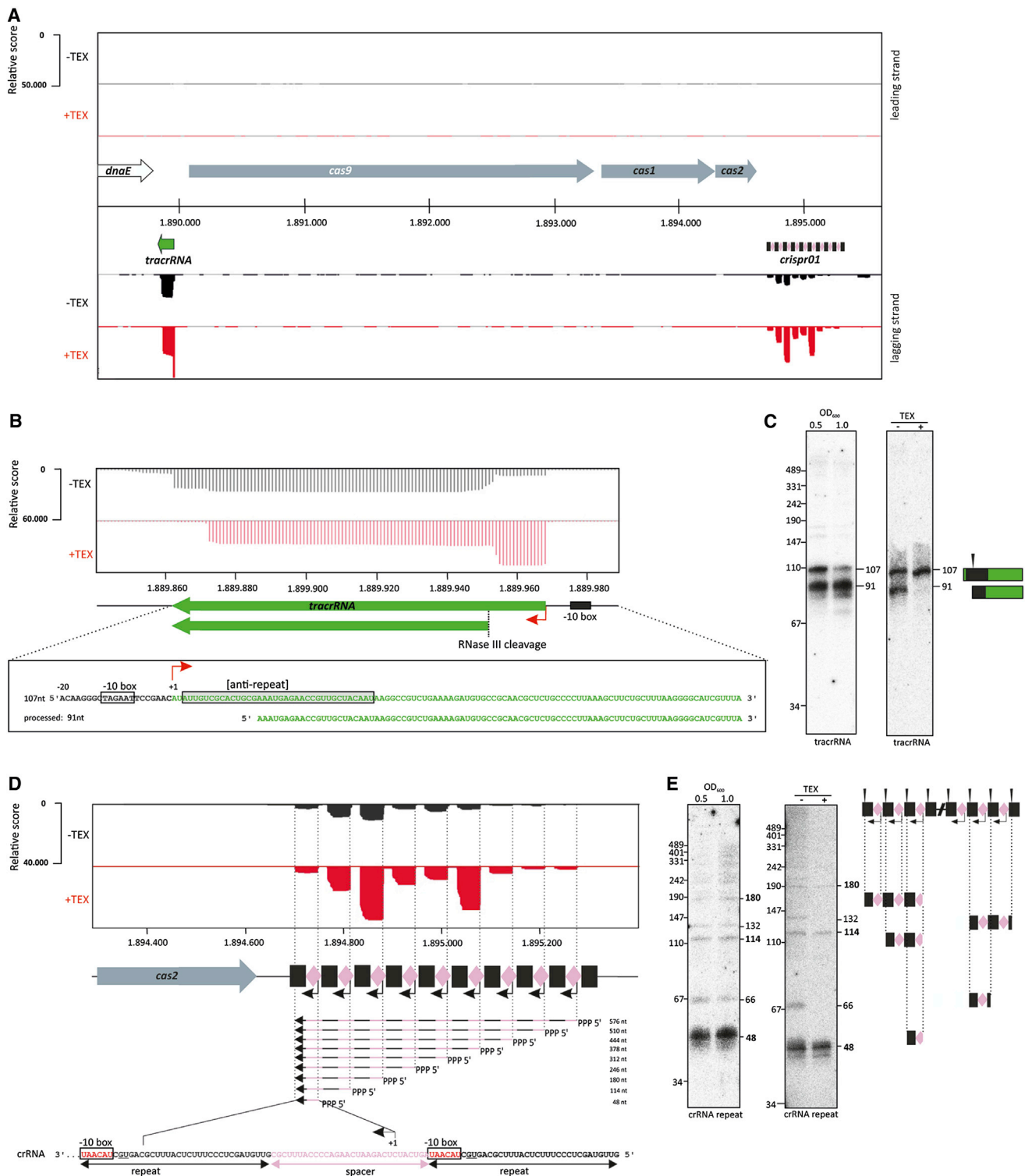
Figure 1. A Newly Identified Mode of crRNA Biogenesis in *Neisseria lactamica*

(A) Differential RNA-seq (dRNA-seq)-based analysis of the minimal CRISPR/Cas system in *N. lactamica* 020-06 reveals expression of tracrRNA and crRNAs. Approximately three million cDNAs from untreated and TEX-treated RNA were sequenced and mapped to the genome. Read counts are plotted here for the CRISPR/*cas* locus. Both strands of all libraries were adjusted to the same scale (maximum of 50,000 for leading strand; minimum of −50,000 for lagging strand) that reflects a relative expression score. The horizontal black bar encompasses the genomic organization of the CRISPR/*cas* locus. Black rectangles, CRISPR repeats; pink diamonds, CRISPR spacers; green arrow, tracrRNA; gray arrows, *cas* genes. The number of reads

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minimal promoter, and that pre-crRNA transcription initiates independently within each spacer. As an initial test of this hypothesis, we designed a series of transcriptional green fluorescent protein (*gfp*) fusion constructs containing either single or multiple CRISPR repeats, introduced these constructs into *E. coli*, and analyzed cellular GFP fluorescence. As shown in Figure 2A, the *gfp* fusion construct with a wild-type CRISPR repeat led to robust cellular fluorescence, whereas a 2 nt substitution in the extended −10 promoter TG motif (Hook-Barnard and Hinton, 2007) reduced *gfp* expression to background levels. Constructs with *gfp* fused to three CRISPR repeat-spacer units increased the fluorescence signal almost 2-fold, consistent with the possibility of increased transcription from the multiple repeats. We obtained similar results using repeat sequences from a type II CRISPR/*cas* locus in *Campylobacter jejunii* NCTC11168 (Figure 2A); this locus, like that of *N. lactamica* 020-06, also contains only *cas9*, *cas1*, and *cas2* and has CRISPR repeats that include an extended −10 box consensus (Dugar et al., 2013). Thus, our *gfp* reporter assays prove that the putative promoter elements in each *N. lactamica* repeat are indeed active in bacterial cells, and are likely also present in some other type II CRISPR/*cas* loci with similar, minimized *cas* gene content (Figure 2B and see Table S1 online). The *Neisseria* and *Campylobacter* systems are also unusual in that the crRNAs and *cas* genes are transcribed in opposite directions, and the CRISPR arrays lack recognizable "leader" sequences with external promoters. Based on these considerations as well as independent phylogenetic analyses (Koonin and Makarova, 2013; Chylinski et al., 2013), we now consider these variant type II CRISPR/Cas loci as members of a distinct and newly defined subtype, type II-C (Figure 2B).

To obtain additional proof that each *N. lactamica* CRISPR repeat carries its own promoter element, we used purified *E. coli* σ^{70} RNA polymerase (RNAP) holoenzyme in in vitro transcription assays with linear DNA templates containing either a wild-type or a mutant repeat. A transcript of the expected length (168 nt) was obtained with the wild-type CRISPR repeat template (and with a control −10/−35 promoter construct), but not with the mutated repeat (Figure 2C and Figure S1). This result demonstrated that the extended −10 motif in the *N. lactamica* CRISPR repeat was recognized even by the heterologous *E. coli* σ^{70} RNAP holoenzyme. These data show that crRNAs in *N. lactamica* exhibit a unique mode of biogenesis involving transcription from extended −10 class promoters located within CRISPR repeats.

RNase III Is Involved in 3′ End Formation of *Neisseria* crRNAs

The observation that crRNA 5′ ends correspond to sites of transcription initiation in *N. lactamica* suggests a reduced dependence on processing relative to other CRISPR systems. To determine whether this reduced dependence extends to crRNA 3′ end formation as well or if 3′ processing still occurs, and to extend our studies to other *Neisseria* strains, we deleted the *rnc* gene (which encodes RNase III) in *N. meningitidis* WUE2594, and then compared the tracrRNA and crRNA populations from this Δ*rnc* mutant with wild-type bacteria by northern analysis. As shown in Figure 2D, the Δ*rnc* strain exhibited a complete loss of the shorter (91 nt), TEX-sensitive tracrRNA, indicating that the 5′ end of this RNA is generated by RNase III-dependent processing as observed previously in a type II-A system (Deltcheva et al., 2011). We also observed dramatic differences in the crRNA population in the Δ*rnc* mutant: the 48 nt population is virtually abolished, and longer pre-crRNAs accumulate. This result strongly suggests that *Neisseria* spp. crRNA 3′ end formation depends upon RNase III rather than direct transcription termination.

Repeat/Spacer Organization and Potential Targets of *Neisseria* Type II-C CRISPR Loci

Having defined unique features of CRISPR/Cas systems in neisseriae, we turned our attention toward functional analyses, beginning with an examination of CRISPR organization and spacer content. Of the 103 spacers found in the seven CRISPR-positive genomes (Figure 3A and Table S2), one is 29 nt long, while all others are 30 nt. All seven CRISPRs have the same 36 nt repeat consensus, with only a few repeats that deviate from this consensus (Figure 3A and Table S3). Intriguingly, the polarity of spacer conservation is opposite to that generally observed in other CRISPR loci. Conserved spacers that are shared among multiple strains in *Neisseria* spp. (color coded in Figure 3A) are enriched at the upstream end of the array (relative to the direction of transcription) but are far less common at the downstream end. In contrast, other CRISPRs described thus far have the most recently derived and therefore the least conserved spacers at the upstream end, i.e., proximal to the

obtained for tracrRNA and crRNAs is in the range of 40,000–50,000 each, which is comparable to the range we observe for other high-abundance classes of RNAs.

(B) (Top) dRNA-seq data were mapped onto the genomic region corresponding to the *tracrRNA* gene. Expression scores at each position were adjusted to the same relative scale. (Bottom) Sequence of the full-length 107 nt form and processed 91 nt form of tracrRNA. The red arrow indicates the primary tracrRNA transcription start site (TSS) based on enrichment in the TEX+ libraries. Boxes denote the extended −10 promoter element and the anti-CRISPR-repeat region of the tracrRNA.

(C) Northern analysis of *N. lactamica* 020-06 tracrRNA. OD_{600} (left panel) and TEX treatment (right panel) are denoted at the top of each lane. Processed and unprocessed tracrRNAs are schematized on the right, with the antirepeat region in black and the RNase III processing site indicated with an arrowhead.

(D) (Top) dRNA-seq data were mapped onto the genomic region corresponding to the CRISPR array. Expression scores at each position were adjusted to the same relative scale. (Middle) The primary TSS of each CRISPR spacer-repeat unit (based on enrichment in the TEX+ libraries) is indicated by a black arrow. Primary CRISPR transcripts of different lengths with likely 5′ triphosphates are indicated by black/pink arrows. (Bottom) The sequence of spacer 4 and its flanking CRISPR repeats, with the putative extended −10 box (consensus sequence 5′-tgnTACAAT-3′) in each single repeat highlighted in red.

(E) Northern analysis of *N. lactamica* 020-06 crRNA, using a probe complementary to the CRISPR repeat. OD_{600} (left panel) and TEX treatment (right panel) is denoted at the top of each lane. Candidate monomeric, dimeric, and trimeric crRNAs are schematized on the right, each of a predicted size consistent with bands observed on the blots.
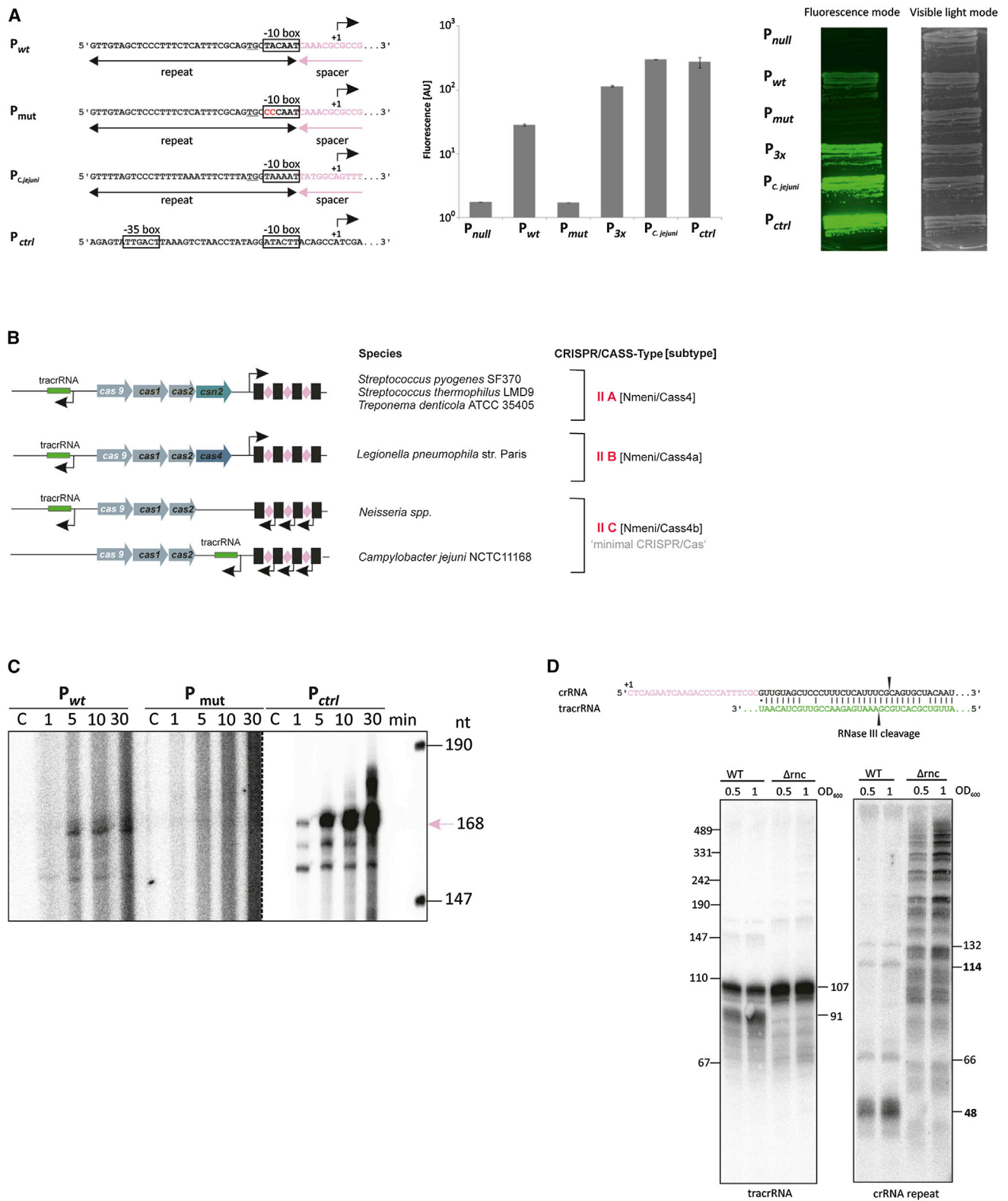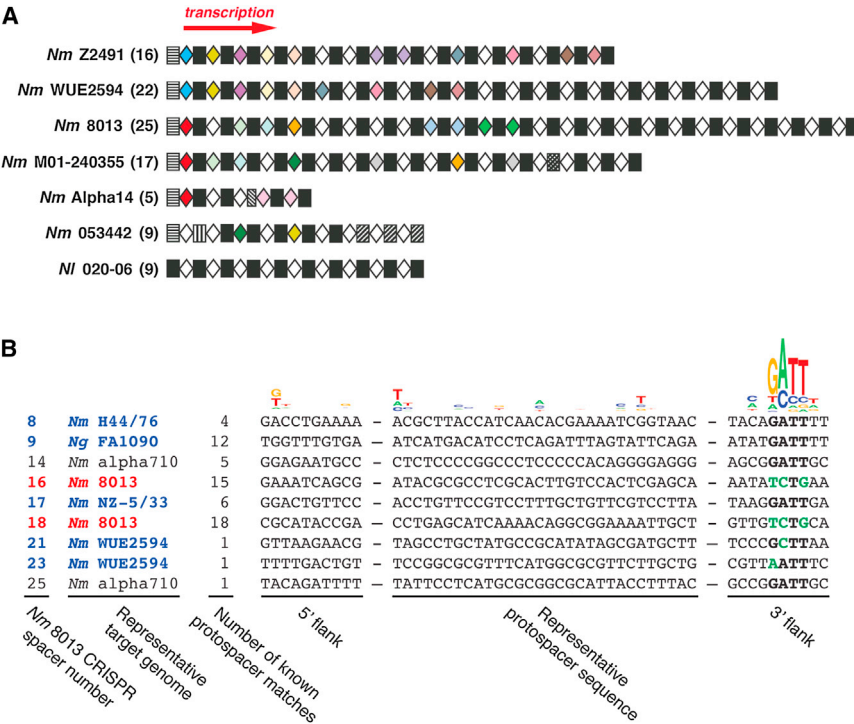
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A

B

Figure 3. CRISPR Organization in *Neisseria*

(A) Schematic representation of CRISPR loci from seven different *Neisseria* strains. Strain names are indicated (*Nm*, *N. meningitidis*; *Nl*, *N. lactamica*), with the total number of spacers in each strain shown in parentheses. The red arrow indicates the direction of crRNA transcription. Repeats and spacers are shown as rectangles and diamonds, respectively. Unique spacers are in white, and nonunique spacers are color coded. Black rectangles indicate repeats that match the consensus, whereas patterned rectangles are variant repeats (see Tables S2 and S3).

(B) Potential natural targets for *N. meningitidis* 8013 spacers. Nine out of twenty-five spacers match varying numbers of *Neisseria* genomes. For each spacer, its number in the 8013 array, the quantity of known protospacer matches, and a representative target genome (*Nm*, *N. meningitidis*; *Ng*, *N. gonorrhoeae*) are listed. Protospacers and 10 flanking nts (on both sides) from the representative target genomes are aligned. Sequence similarities are indicated at the top, revealing the 5'-GATT-3' PAM consensus 5–8 nt 3' of the protospacer. The WebLogo is derived from the alignment of all *Neisseria*-matched protospacers, not just those shown here that match spacers from the *N. meningitidis* 8013 CRISPR. The PAM regions in the putative targets are in bold, with nonconsensus nucleotides in green. Potential self-targeting spacers are in red, and spacers with possible prophage-like targets are in blue. See also Figure S2.

promoter (Makarova et al., 2011b). This observation suggests that new spacer acquisition in neisseriae, and perhaps in other type II-C loci, occurs at the downstream end. In addition, deviations from the repeat consensus are most common at the upstream end in neisseriae (Table S3), whereas other CRISPRs most frequently exhibit the opposite tendency. Although technical limitations have thus far precluded direct tests of spacer acquisition in neisseriae, several considerations make it likely that these CRISPRs are competent for the adaptive component of the pathway. First, type II-C loci are relatively common, with many more apparent examples among sequenced bacterial genomes than type II-B loci (Chylinski et al., 2013). It is unlikely that type II-C systems would be so widespread if they were unable to provide adaptive protection to their hosts. Second, despite the conservation of the type II-C loci in closely related *Neisseria* strains, there are many differences in spacer content (Figure 3A). This implies that these CRISPR loci are fluid, i.e., can adapt by adding and deleting spacers. Third, reports from other systems indicate that *cas1* and *cas2* can suffice for spacer acquisition (Yosef et al., 2012). It is therefore plausible that *cas1* and *cas2* likewise suffice for adaptation in type II-C systems.

Figure 2. CRISPR Repeats Contain Active Promoters that Form the 5′ Ends of Mature crRNAs, whereas RNase III Processing Forms crRNA 3′ Ends

(A) (Left panel) Promoter-element-containing sequences used for *gfp* fusions. Plasmids included a wild-type (wt) and mutated (mut) *N. lactamica* 020-06 CRISPR repeat (pNH13 and pNH14, respectively), a *C. jejunii* NCTC11168 CRISPR repeat (pNH18), and a positive control promoter from T7 phage A1. The promoterless control *gfp* construct (pAS00046) and a construct with three wild-type *N. lactamica* 020-06 CRISPR repeats (pNH17) are not shown. Promoter elements are indicated by boxes, with mutated sequences colored in red. (Middle panel) Flow-cytometric fluorescence intensity measurements of cells containing the transcriptional *gfp* fusions described in the left panel. Fluorescence values are expressed in arbitrary units (AU). Error bars indicate standard deviation for three independent biological replicates. (Right panel) Fluorescence images of transcriptional *gfp* fusion strains grown on agar plates. The right image was captured in the visible light mode; the left image shows the same plate in fluorescence mode (460 nm excitation, 510 nm emission).

(B) Classification of type II CRISPR/*cas* loci. The genomic organization of representative type II-A or type II-B loci, as defined previously, is given on top. Below are two CRISPR/*cas* loci (including from neisseriae) from the newly defined subtype II-C.

(C) Gel electrophoresis of radiolabeled RNAs from in vitro transcription reactions using linear DNA templates from a subset of the CRISPR repeat-containing sequences given in (A). Full-length run-off transcripts (168 nt) are denoted by the pink arrow. The area to the right of the dotted line was imaged at lower contrast to avoid overexposure. See also Figure S1.

(D) (Top) Base pairing between a primary crRNA and tracrRNA. RNase III cleavage sites inferred from dRNA-seq are indicated by black arrows. (Bottom) Northern analysis of total RNA from *N. meningitidis* WUE2594 and its Δ*rnc* derivative during mid-log and early stationary phase, probed for tracrRNA (left) and crRNAs (right).
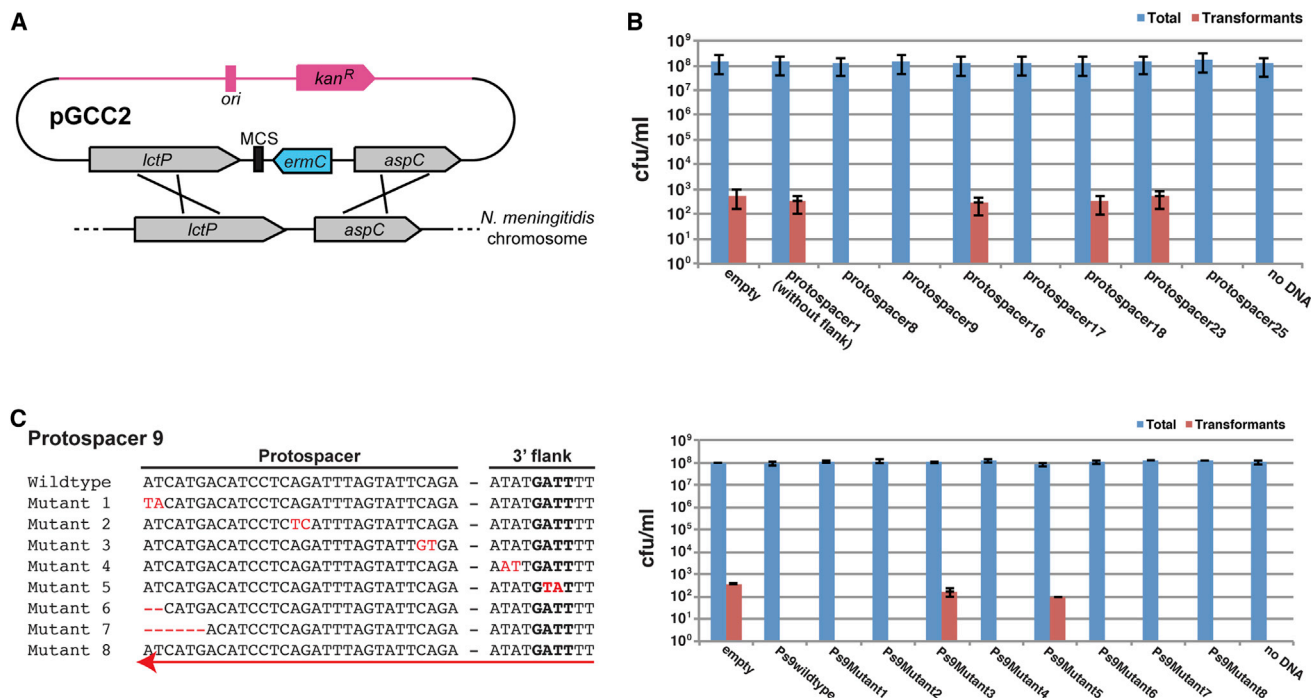
Figure 4. Natural Transformation Is Limited by the Native *Neissiera* CRISPR/Cas System

(A) Schematic representation of integrational vector pGCC2, and recombination between pGCC2 and the meningococcal chromosome (Tobiason and Seifert, 2010). Gray arrows, *lctP* and *aspC* genes; blue arrow, *ermC* gene; pink, regions required for maintenance in *E. coli*; black box, multiple cloning site. Individual elements are not drawn to scale.

(B) pGCC2 derivatives with potential targets for different *N. meningitidis* 8013 spacers (1, 8, 9, 16, 17, 18, 23, and 25) were tested by natural transformation assays using wild-type 8013 as the recipient strain. The data show log-scale plots of colony-forming units (cfu) per ml (mean ± SEM [standard error of the mean] for three independent biological replicates) for total cells (blue bars) and Erm[R] transformants (red bars) from three independent experiments. See also Figure S3.

(C) (Left) Sequences of a series of mutations in the pGCC2 derivative carrying the 350 nt target for spacer 9. Sequence alterations are in red. The red arrow indicates reversed orientation of the target sequence in the plasmid. (Right) pGCC2 constructs containing the spacer 9 target mutations are tested by natural transformation into wild-type *N. meningitidis* 8013. Data are presented as in (B). Error bars represent SEM for three independent biological replicates.

BLASTN searches with the 83 unique spacer sequences for similar sequences in the NCBI database allowed us to identify at least one potential target for 35 (~42%) of them. For simplicity we required either a perfect match or at most a single mismatch in the 10 protospacer nucleotides furthest from the PAM (i.e., well outside of the presumptive "seed" sequence that requires perfect complementarity for interference) (Sapranauskas et al., 2011; Semenova et al., 2011; Wiedenheft et al., 2011; Jinek et al., 2012). Figure 3B shows representative protospacer sequences that match CRISPR spacers from *N. meningitidis* strain 8013; representative protospacers that match spacers from all strains are shown in Figure S2. Protospacer alignments reveal an apparent PAM of 5′-NNNNGATT-3′ (Figure 3B and Figure S2). Of the 325 distinct candidate protospacers that match these 35 CRISPR spacers, all are in *Neisseria* sequences: 248 (76%) in *N. meningitidis* genomes, 69 (21%) in *N. gonorrhoeae* genomes, 6 (2%) in *N. lactamica* plasmids, and 1 each in *N. meningitidis* and *N. flavescens* plasmids (Table S4; also see the Supplemental Information). In some cases (shown in red in Figure 3B and Figure S2), potential protospacers are present in the same genome as the targeting CRISPR spacer, suggesting the potential for autoimmunity (Stern et al., 2010). However, these protospacers all include significant deviations from the PAM consensus,

perhaps explaining the apparent lack of self-targeting that is implied by the persistence of the matching protospacer. Intriguingly, 22 out of 35 CRISPR spacers (shown in blue in Figure 3B and Figure S2) with identifiable targets match at least one potential prophage sequence (Table S5), including the MDA island that is associated with invasiveness and pathogenicity in young adult patients (Bille et al., 2005, 2008).

CRISPR Interference Blocks Natural Transformation in *N. meningitidis*

The preponderance of protospacers in *Neisseria* spp. genomes suggests that the CRISPR/Cas system could interfere with natural transformation. For our functional analyses addressing this possibility, we focused on *N. meningitidis* 8013, primarily because it exhibits the most robust transformation competence in our hands. For transformation assays we used the vector pGCC2, which contains an erythromycin resistance gene (*ermC*) and polylinker inserted into sequences from the gonococcal *lctP*/*aspC* locus (Figure 4A). Upon transformation into *N. meningitidis*, homologous recombination into the meningococcal *lctP*/*aspC* locus leads to *ermC* insertion and erythromycin-resistant (Erm[R]) transformants. We selected potential natural target sequences, including ten or more nucleotides on

both sides of the protospacer, for seven of the spacers of *N. meningitidis* 8013, namely spacers 8, 9, 16, 17, 18, 23, and 25 (Figure 3B and Figure S3). Upon cloning them into pGCC2, the resulting vectors were used in liquid-medium natural transformation assays into wild-type 8013 cells, and transformation frequencies (antibiotic-resistant cfu ml^{-1}/total cfu ml^{-1}) were determined. For comparison, we also cloned a spacer 1 target without any flanking sequences. The results are shown in Figure 4B. Empty pGCC2 exhibited a transformation frequency of 3.9×10^{-6} (Table S6), consistent with previous reports (Rusniok et al., 2009). Plasmids carrying targets for spacers 1, 16, 18, and 23 all exhibited transformation frequencies of $2–4 \times 10^{-6}$ (Table S6), i.e., comparable to that of the empty vector. The cloned protospacers in these plasmids either lack flanking *Neisseria* sequences or have flanking sequences that deviate significantly from the PAM consensus (Figure 3B and Figure S3). In contrast, protospacers 8, 9, 17, and 25, all four of which have flanking sequences that conform to the PAM consensus, consistently failed to yield transformants, indicating that they likely elicited CRISPR interference. Protospacers matching CRISPR spacers 9 and 25 (Figure S3) cloned into a different transformation vector, pYZEJS040 (which confers chloramphenicol resistance), for targeted integration into the distinct capsule locus also yielded no transformants (Figure S4), demonstrating that the observed effect was independent of the vector, the integration locus, and the selectable marker.

To further examine targeting requirements, we generated a series of mutations in the protospacer or flanking sequences of the pGCC2-derived plasmid targeted by spacer 9 (Figure 4C). Substitutions of two consecutive nucleotides within the PAM or the seed sequence of the protospacer yielded plasmids with transformation frequencies comparable to that of the empty vector (mutants 3 and 5; Figure 4C and Table S6), indicating that interference was abolished. In contrast, 2 nt substitutions in a non-PAM flanking region, substitutions in nonseed protospacer positions, small deletions at the PAM-distal end of the spacer, or a wild-type protospacer cloned in the opposite orientation had no effect on interference (mutants 1, 2, 4, 6, 7, and 8). All of these observations are consistent with previously defined characteristics of functional type II CRISPR/Cas systems (Deveau et al., 2008; Sapranauskas et al., 2011; Gasiunas et al., 2012; Jinek et al., 2012), indicating that CRISPR interference is indeed responsible for the observed effects on transformation frequencies. The effects of single-nucleotide mutations in this apparent 4 nt PAM remain to be defined.

Genetic Analysis of the *N. meningitidis* CRISPR/*cas* Locus

In other type II CRISPR/Cas systems, Cas9 is the only Cas protein that is necessary for interference specified by existing spacers (Barrangou et al., 2007; Deltcheva et al., 2011; Sapranauskas et al., 2011; Jinek et al., 2012). To investigate if type II-C CRISPR/Cas systems exhibit the same Cas protein requirements, we introduced transposon insertion mutations in the three *cas* genes—*cas1*, *cas2*, and *cas9*—in *N. meningitidis* 8013 (Figure 5A). We also generated an unmarked, in-frame *cas9* deletion strain to avoid potential polar effects and to generate a guaranteed null allele (Figure 5A). We transformed

wild-type and mutant strains with a pYZEJS040 construct carrying protospacer 25 (as in Figure S4B) and compared their transformation frequencies with those of empty pYZEJS040. As expected, the empty vector readily transformed all strains, with transformation frequencies in the range of $\sim0.5–7 \times 10^{-5}$ (Figure 5B and Table S6). No transformants were observed when the protospacer 25 construct was used with wild-type cells, indicating effective CRISPR interference. Similarly, transposon insertion mutants in *cas1*, *cas2*, or a control irrelevant gene (*cas1::Tn*, *cas2::Tn*, and *1851::Tn*, respectively) exhibited complete interference, consistent with previous results in type II-A systems (Sapranauskas et al., 2011). In contrast, CRISPR function is abolished by both the transposon-induced (*cas9::Tn*) and deletion (Δ*cas9*) mutations in *cas9*. The CRISPR interference defect of both alleles could be complemented with wild-type *cas9* under the control of its native promoter (integrated chromosomally via pGCC2), whereas empty pGCC2 had no effect (Figure 5B).

Previous studies of *cas9* orthologs from *S. thermophilus* and *S. pyogenes* identified two distinct active sites in RuvC-like and HNH nuclease motifs that are important for Cas9 function in vivo and in vitro (Sapranauskas et al., 2011; Gasiunas et al., 2012; Jinek et al., 2012). We engineered alanine mutants in corresponding catalytic residues (D16 in the RuvC domain and H588 in the HNH domain) and tested the abilities of both single mutants to complement the interference defect of the *cas9::Tn* mutant. Both alanine mutants failed to restore interference (Figure 5B). Altogether these analyses demonstrate that the *Neisseria* type II-C CRISPR/Cas system requires *cas9* but not *cas1* or *cas2* for interference of natural transformation, and that the presence of intact RuvC-like and HNH motifs is essential for Cas9 function.

RNase III-Catalyzed Pre-crRNA Processing Is Dispensable for CRISPR Interference in *N. meningitidis*

Two additional loci—*tracrRNA* and *rnc* (the gene encoding RNase III)—have been shown to be essential for crRNA processing and interference in the type II-A system of *S. pyogenes* SF370 (Deltcheva et al., 2011). The unique *Neisseria* biogenesis pathway described above, in which repeat-driven promoters yield crRNAs with unprocessed 5′ ends, raises questions about the roles of tracrRNA and RNase III in this type II-C system. To examine this issue, we generated *N. meningitidis* 8013 derivatives carrying a transposon-induced allele of *rnc*, or a complete deletion of either *rnc* or *tracrRNA* (Figure 5A). These strains were tested in liquid transformation assays as described above, using pYZEJS040 and its protospacer 25-bearing derivative. The results are shown in Figure 5C. The Δ*tracrRNA* strain was completely defective in CRISPR interference, but the defect was restored upon integration of a *tracrRNA* gene with its native promoter in a distinct chromosomal locus. These results are consistent with the strict requirement for *S. pyogenes* tracrRNA for pre-crRNA processing and interference in vivo (Deltcheva et al., 2011), as well as crRNA-directed, Cas9-catalyzed DNA cleavage in vitro (Jinek et al., 2012) and in eukaryotes (Cong et al., 2013).

Intriguingly, despite the previously demonstrated importance of the tracrRNA as a guide for RNase III-mediated processing,
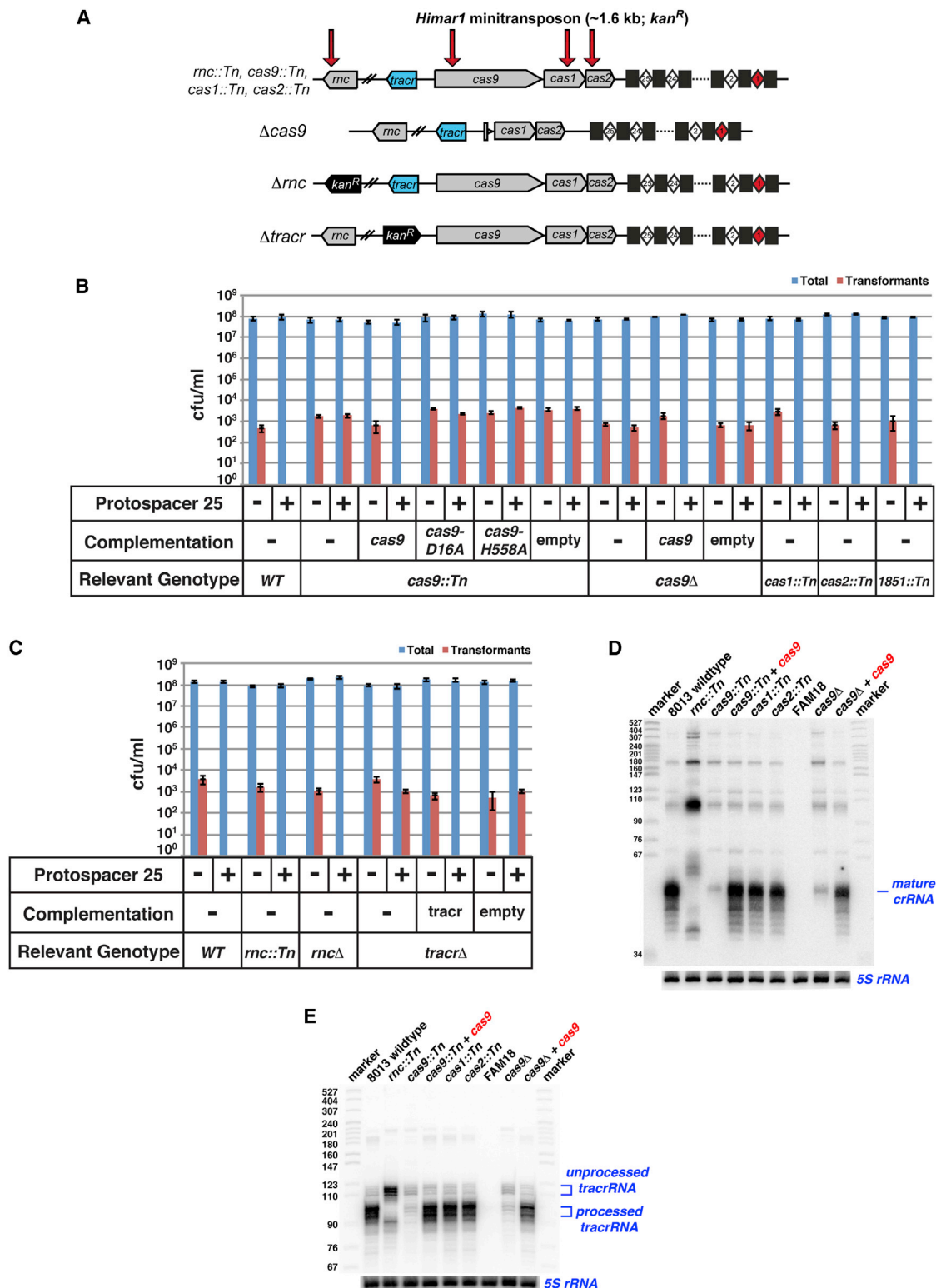
Figure 5. *N. meningitidis* **CRISPR Interference Requires Cas9 and tracrRNA but Is Independent of RNase III-Mediated Processing**

(A) Schematic representations of *N. meningitidis* 8013 mutant strains. Gray arrows, *cas1*, *cas2*, *cas9*, and *rnc* genes; blue arrow, *tracrRNA* gene; black arrow, kanamycin resistance gene; squares and diamonds, CRISPR repeats and spacers (respectively), colored as in Figure 3A. Red arrows indicate transposon insertions in the *rnc::Tn*, *cas9::Tn*, *cas1::Tn*, and *cas2::Tn* mutants.

(legend continued on next page)

we detected no interference defect in either the *rnc::Tn* or Δ*rnc* mutants (Figure 5C). This result is in stark contrast to that observed in the type II-A system in *S. pyogenes* SF370 (Deltcheva et al., 2011). The lack of an interference defect was observed with a vector that is targeted by an internal spacer (spacer 9; Table S6) as well as a terminal spacer (spacer 25; Figure 5C and Table S6). Northern analyses revealed clear processing defects in both crRNA (Figure 5D) and tracrRNA (Figure 5E) for the *N. meningitidis* 8013 *rnc::Tn* mutant, consistent with the results described above with a Δ*rnc* mutant in the WUE2594 strain background (Figure 2D). Mature, 48 nt crRNAs are virtually absent in the *rnc::Tn* mutant, and longer precursors accumulate (Figure 5D and Figure S5). CrRNAs are also strongly depleted in *cas9* mutants, but unlike in the *rnc::Tn* mutant, pre-crRNAs do not accumulate. The latter observation suggests that Cas9 is important for crRNA stability but not processing, or that Cas9 functions in processing and also stabilizes unprocessed precursors. We conclude that the *N. meningitidis* type II-C CRISPR/Cas system, unlike all other CRISPR/Cas systems characterized to date, does not require pre-crRNA processing for interference activity. RNase III-catalyzed pre-crRNA processing occurs within the bacterial cell but is dispensable for interference. The tracrRNA requirement likely reflects its involvement in target DNA binding and cleavage by crRNA-programmed Cas9, as observed in vitro (Jinek et al., 2012).

CRISPR Interference Limits Transformation by *Neisseria* Genomic DNA

Plasmids are rare in *N. meningitidis* (van Passel et al., 2006), and *Neisseria* genomic DNA (gDNA) is thought to be the most frequent substrate for natural transformation (Hamilton and Dillard, 2006). To test whether our results with *E. coli*-isolated plasmids extend to *Neisseria* gDNA, we generated strains carrying a selectable marker tightly linked to a validated target (protospacer 25). We used the *cas9::Tn* strain to enable transformation and integration of both empty pGCC2 (Figure 4A) and protospacer 25-containing pGCC2 into the meningococcal chromosome (Figure 6A). We then isolated gDNA from these strains and used them as donors in liquid-medium transformation assays with wild-type *N. meningitidis* 8013 cells. Transformants (transformation frequency of 1.6×10^{-5}; Table S6) were readily obtained using DNA that lacked protospacer 25 adjacent to the *ermC* marker, whereas no transformants were observed when the protospacer was present (Figure 6B, left panel). Similar results were obtained with gDNA strains carrying the *cat* marker at the capsule locus with and without tightly linked protospacer 25 (Figure 6, Figure S4A, and Table S6), indicating that the interference effect was not marker or locus specific. We conclude that CRISPR interference is effective against the most common natural substrate for transformation in *N. meningitidis*.

DISCUSSION

CRISPR Interference and the Third Major Pillar of Horizontal Gene Transfer

CRISPR/Cas pathways have been revealed as RNA-directed immune systems that protect bacteria and archaea from phage infection and HGT (Karginov and Hannon, 2010; Marraffini and Sontheimer, 2010; Terns and Terns, 2011; Wiedenheft et al., 2012). Several dozen bacterial species are known to be competent for HGT via natural transformation. Of this subset of bacteria, *Neisseria* spp. are unusual in that their transformation competence is constitutive (Hamilton and Dillard, 2006). Only a few phages are known to infect *N. meningitidis*, and although conjugative plasmids are present in some meningococcal isolates (van Passel et al., 2006), transformation is the major mechanism for mobilization of meningococcal chromosomal loci (Moxon and Jansen, 2005). *Neisseria* genomic sequences are preferred substrates for natural transformation, given that DNA uptake is strongly promoted by a short DNA uptake sequence (DUS) that is highly overrepresented in *Neisseria* spp. chromosomes (Budroni et al., 2011). DNA exchange is so frequent that the population structures of most neisseriae are effectively panmictic, with little propensity for the emergence of clonal subpopulations (Smith et al., 1993). Frequent HGT in *N. meningitidis* is thought to promote capsule switching and other forms of antigenic variation, homology-based DNA repair, and other functions (Hamilton and Dillard, 2006). Native CRISPR/Cas systems have previously been shown to prevent phage infection (and, by inference, phage transduction) and conjugation, which constitute two of the primary routes of HGT. Our results reveal a role for a native CRISPR/Cas system in preventing the third main route of HGT, natural transformation. This is consistent with recent reports that CRISPR/Cas systems can target loci that are already established in bacterial or archaeal chromosomes (Edgar and Qimron, 2010; Gudbergsdottir et al., 2011; Jiang et al., 2013), indicating that interference does not depend on the invasive DNA's route of entry. Similarly, an engineered, heterologous CRISPR/Cas system introduced into *Streptococcus pneumoniae* can block natural transformation during active infection in mice (Bikard et al., 2012). We find that a native CRISPR/Cas system in *N. meningitidis* can block the transformation events that can be so important for immune evasion and other critical aspects of invasiveness and pathogenicity. Intriguingly, the ability of native CRISPR systems to block natural transformation would be expected to enable the selection of spacers that discriminate against specific chromosomal loci that negatively affect the fitness of certain strains or under certain conditions.

Although relatively few phages are known to infect *N. meningitidis*, they are not unknown (Kawai et al., 2005).

(B and C) pYZEJS040 (−) and its protospacer 25-containing derivative (+) were tested by natural transformation assays using *N. meningitidis* 8013 and its mutant derivatives as recipients. Relevant genotypes as well as the presence or absence of pGCC2-mediated *cas9* complementation are given at the bottom. The data show cfu/ml (log scale, mean ± SEM for three independent biological replicates) for total cells (blue bars) and chloramphenicol-resistant transformants (red bars). See also Figure S4.

(D) Total RNA from the indicated strains was subjected to northern analysis using a probe complementary to spacer 25 (top). In the lower panel, the same blot was probed for 5S RNA as a loading control. See also Figure S5.

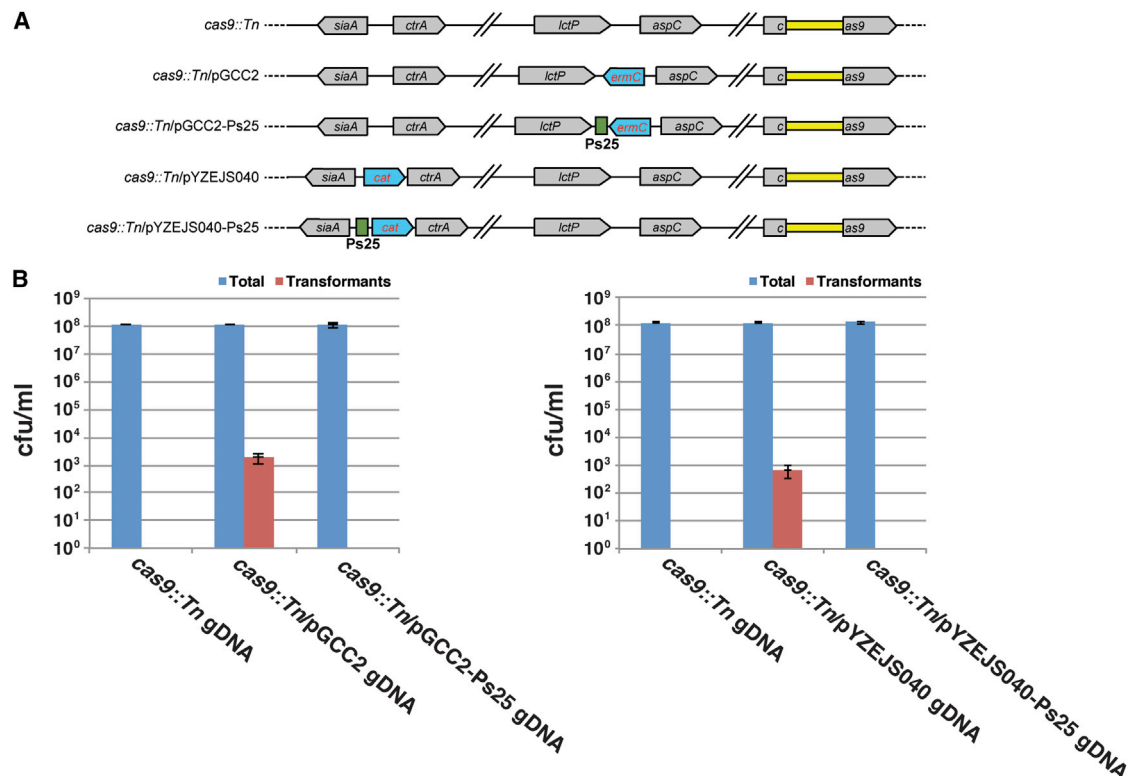(E) As in (D), except that a probe specific for tracrRNA was used.

Figure 6. The *Neisseria* Type II-C CRISPR/Cas System Limits Natural Transformation of Meningococcal Chromosomal DNA

(A) Schematic representation of the *siaA/ctrA* and *lctP/aspC* chromosomal loci in the *cas9::Tn* strain (top; the transposon insertion is in yellow). Below are derivatives of the same *cas9::Tn* strain following transformation with the *ermC*-marked vector pGCC2 with or without a protospacer that matches CRISPR spacer 25, or with the *cat*-marked vector pYZEJS040 with or without a protospacer that matches CRISPR spacer 25.

(B) For the left panel, gDNA from the *ermC*-marked strains shown in (A), as well as the unmarked control strain, was used in transformation assays with wild-type *N. meningitidis* 8013. Natural transformations were performed and presented as in Figure 4B. The right panel shows an analogous experiment using gDNA from the *cat*-marked strains shown in (A), as well as the unmarked control strain. Error bars represent SEM for three independent biological replicates.

Several genomic islands have been identified that resemble phages and could therefore represent prophage sequences (Bille et al., 2005, 2008; Joseph et al., 2011). One such sequence, the MDA island, correlates with invasiveness and pathogenicity in young adults (Bille et al., 2005, 2008). The existence of numerous CRISPR spacers with the potential to target these sequences suggests that CRISPR interference plays a role in shaping prophage content and serves phage defense functions in *N. meningitidis*, as elsewhere. CRISPR interference could limit the spread of prophages via either transformation or infection. Accordingly, CRISPR interference could negatively correlate with meningococcal pathogenicity, as suggested in enterococci (Palmer and Gilmore, 2010) and streptococci (Bikard et al., 2012). Alternatively, meningococcal Cas9 could participate in other regulatory events that contribute to pathogenicity, as suggested very recently (Sampson et al., 2013).

It is noteworthy that many *N. meningitidis* and *N. lactamica* strains encode CRISPR systems, while strains of the closely related *N. gonorrhoeae* with clearly functional CRISPR systems have not been identified. It is believed that these organisms split in relatively recent times (<100,000 years ago), evolutionarily speaking, but exact estimates have been stymied by the large recombination frequencies in these species (Bennett

et al., 2010). It is equally possible that the nasopharyngeal-localized species gained the system after the split, or that *N. gonorrhoeae* lost the CRISPR system after the split. Both pathogens have been suggested not to establish long-lasting clones and tend toward linkage equilibrium (Buckee et al., 2008). It may not be coincidental that *N. meningitidis* carries a CRISPR system and can develop semiclonal lineages (Bart et al., 2001), given that the CRISPR system could provide a short-term barrier to HGT. It is also possible that the coexistence of commensal *Neisseria* species such as *N. lactamica* and *N. meningitidis* in the nasal pharynx (Feil and Spratt, 2001) selects for a system that limits genetic exchange, and intriguingly, some *N. lactamica* and *N. meningitidis* isolates show large amounts of exchange while others show lower signatures of exchange (Hanage et al., 2005; Corander et al., 2012). In contrast, *N. gonorrhoeae* inhabits a niche that is devoid of other closely related bacteria that encode the DUS to allow efficient transformation of their sequences (Vázquez et al., 1993; Cehovin et al., 2013).

Toward a Minimal CRISPR/Cas System

In CRISPR/Cas systems investigated to date, crRNAs are transcribed from an external promoter, generating a multimeric
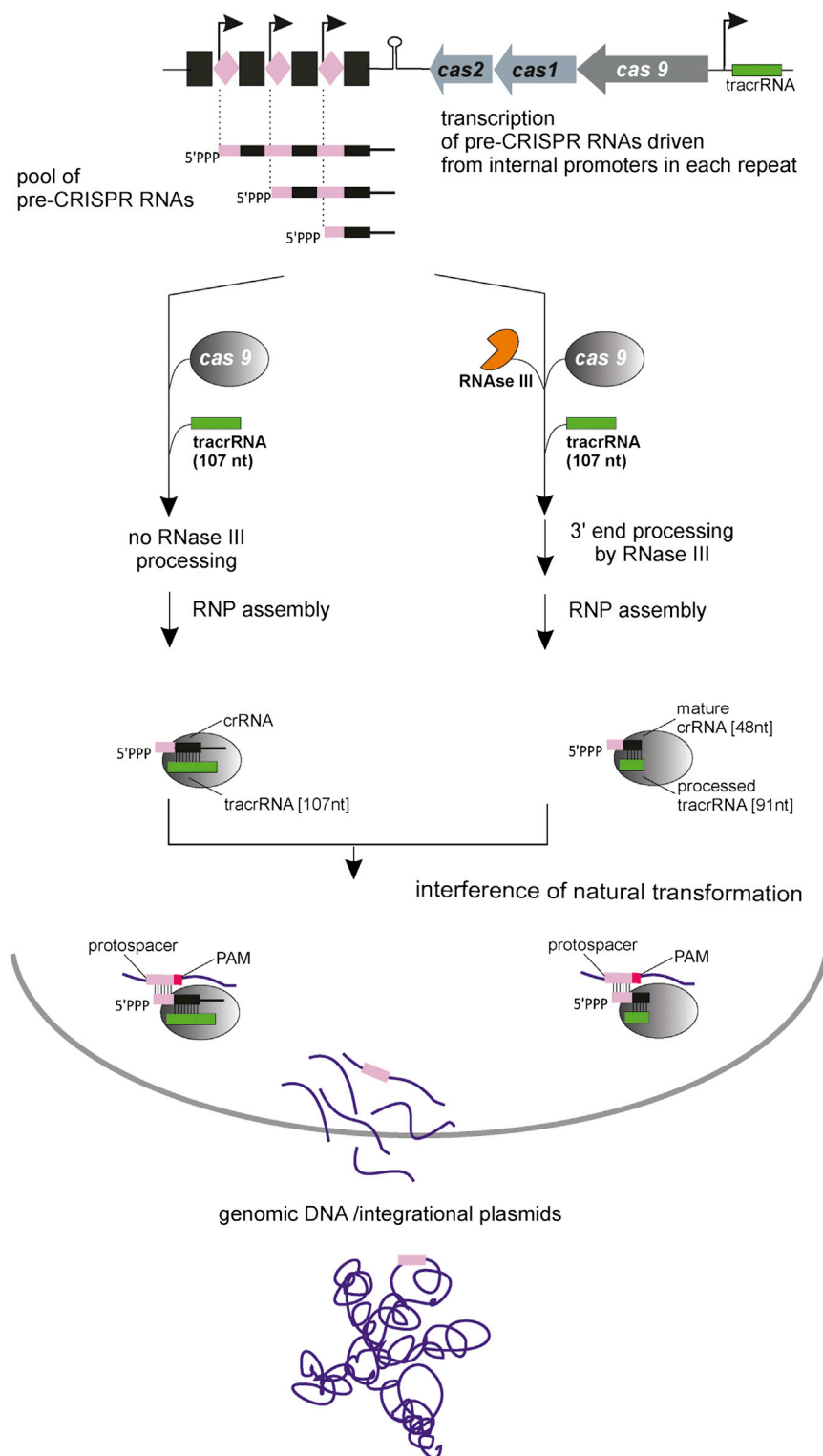
et al., 2011; Hale et al., 2012; Westra et al., 2012). The potential presence of minimal and apparently fortuitous promoter elements has been noted within certain CRISPRs of *Sulfolobus solfataricus* P2, though they are not thought to account for the functional expression of crRNA and in fact appear to be suppressed by the repeat-binding protein Cbp1 (Deng et al., 2012). The results presented here reveal that streamlined CRISPR/Cas systems exist in which pre-crRNA processing is not essential (Figure 7). In CRISPR-containing strains of *N. meningitidis* and *N. lactamica*, as well as other species such as *C. jejuni*, the CRISPR repeats each contain an extended −10 box that drives transcription initiation within the downstream spacer. Thus, many crRNAs contain 5′-triphosphate ends that are not subject to further 5′-processing.

Like other type II CRISPR/Cas systems, neisseriae produce a tracrRNA that apparently anneals to pre-crRNA and enables binding and cleavage by RNase III. This reaction generates crRNA 3′ ends, and *rnc* mutants accumulate multimeric crRNA precursors. However, these *rnc* mutants exhibit no interference defect, indicating that processing is not essential. In addition, while the tracrRNA is essential for interference, its role in

precursor. The pre-crRNA is processed by endonucleolytic cleavage to generate mature crRNAs (Carte et al., 2008; Haurwitz et al., 2010; Gesner et al., 2011), and processing is essential for interference in vivo (Brouns et al., 2008; Deltcheva directing processing is not, since processing is itself dispensable. This provides the first clear indication that the tracrRNA is required for postprocessing events such as target DNA binding and cleavage in bacterial cells, as it is in vitro (Jinek et al., 2012).

Among the three main types of CRISPR/Cas pathways, the type II systems are the simplest ones characterized thus far, as judged by the number of components and essential steps. Both type II-A and type II-B systems include the CRISPR array itself, a tracrRNA, four protein-coding genes encoded within the *cas* locus, and the host factor RNase III (Deltcheva et al., 2011; Makarova et al., 2011b; Magadán et al., 2012; Chylinski et al., 2013). The *Neisseria* systems that we have characterized are even more streamlined: they do not require a separate leader sequence to drive crRNA transcription, they lack one of the four *cas/csn* genes present in type II-A or II-B systems, and they do not require RNase III or crRNA processing. The *Neisseria* systems are among the founding members of a new CRISPR/Cas subtype (type II-C) that is characterized by a smaller number of *cas/csn* genes (Koonin and Makarova, 2013; Chylinski et al., 2013), and in at least some cases by repeat-embedded promoters (Dugar et al., 2013) and processing independence.

Importantly, recent reports have shown that type II CRISPR/Cas systems can be ported into eukaryotic cells and employed for RNA-directed genome editing and genome binding, including multiplexed applications specified by multiple spacers (Jinek et al., 2012, 2013; Cho et al., 2013; Cong et al., 2013; Dicarlo et al., 2013; Hwang et al., 2013; Mali et al., 2013; Qi et al., 2013). The Cas9 effector proteins from neisseriae share the conserved features observed in the *S. pyogenes* and *S. thermophilus* Cas9 enzymes used in these studies (Chylinski et al., 2013). The fewer the functional requirements for the operation of such systems, the greater their versatility and applicability will be. Separately encoded crRNAs and tracrRNAs are more efficient in vivo than single-guide RNAs that combine essential crRNA and tracrRNA domains in the same transcript (Cong et al., 2013). Although endogenous eukaryotic activities can substitute for bacterial RNase III to process tracrRNA/pre-crRNA hybrids in human and mouse cells (Cong et al., 2013), it is not known whether RNase III will be dispensable in other eukaryotic contexts, or indeed in all mammalian cell types. Accordingly, the identification of processing-independent CRISPR/Cas systems could increase the range of applications in eukaryotic genome editing, especially in light of the potential toxicity of bacterial RNase III expression (Pines et al., 1988). Such applications will benefit from further analysis of meningococcal Cas9 activity, including the definition of the presumptive cleavage sites relative to the PAM.

The dispensability of pre-crRNA processing in neisseriae also raises intriguing mechanistic questions. First, can any spacer in a multimeric pre-crRNA engage Cas9 and target DNA cleavage, or is the 5′-terminal spacer the only one that can function in interference? If any spacer can function in target cleavage, can a single multimeric pre-crRNA engage multiple copies of the Cas9 protein and simultaneously direct the interference of multiple targets? Answers to these questions will be valuable in applying the CRISPR machinery as a versatile, adaptable, and scalable tool for multiplex genome editing and genome binding.

EXPERIMENTAL PROCEDURES

Detailed experimental and analytical procedures can be found in the Supplemental Information.

Bacterial Strains, Plasmids, and Oligonucleotides
N. lactamica 020-06, *N. meningitidis* WUE2594 and 8013, and mutant derivatives thereof that were used in this study are listed in the Supplemental Experimental Procedures, as are complete lists of all plasmids and DNA oligonucleotides.

Mutant Strain Construction
All mutants were confirmed by PCR and DNA sequencing. Most mutant strains were generated by transformation with appropriately constructed plasmids. For generation of the *cas9*, *rnc*, and control NMV_1851 transposon-induced alleles in the 8013 strain background, we used gDNA from the corresponding mutant in the NeMeSys collection (Rusniok et al., 2009) to transform 8013. For generation of the Δ*rnc* derivative of 8013, we used gDNA from the WUE2594 Δ*rnc* derivative that was initially made by a plasmid-based approach.

For complementation of *cas9::Tn*, Δ*cas9*, and Δ*tracrRNA* mutants, we cloned wild-type copies of the relevant gene into plasmid pGCC2 and transformed the resulting plasmids into the parental mutant strain.

RNA Extraction and Depletion of Processed RNAs
For 020-06, WUE2594, and its mutant derivatives, total RNA was extracted from frozen cell pellet lysates using the hot-phenol method described previously (Blomberg et al., 1990). For depletion of processed transcripts, equal amounts of total RNA were incubated with Terminator exonuclease (TEX) (Epicenter) or in buffer alone as described (Sharma et al., 2010). For 8013 and its mutant derivatives, total RNAs were extracted from frozen cell pellets using miRNeasy Mini Kit (QIAGEN) with two additional steps: a 10 min initial cell lysis with lysozyme and Proteinase K, and a later on-column DNase digestion step (the RNase-Free DNase Set, QIAGEN).

dRNA-Seq
Libraries for Solexa sequencing (HiSeq) of cDNA were constructed by vertis Biotechnology AG, Germany (http://www.vertis-biotech.com/), as described previously for eukaryotic microRNA (Berezikov et al., 2006) but omitting the RNA size-fractionation step prior to cDNA synthesis. cDNA libraries were sequenced using a HiSeq 2000 machine (Illumina) in single read mode at the Max Planck Genome Centre Cologne (Cologne, Germany). Data analysis was done as described (Chao et al., 2012), with the only exception being that the minimal read length after trimming and clipping was 12 nt instead of 20 nt.

Transcriptional *gfp* Fusions
The inserts used for the construction of the transcriptional *gfp* fusion plasmids were generated with synthetic DNA oligonucleotides. *E. coli* cells were transformed with these plasmids and grown on agar plates for fluorescence imaging. To measure GFP fluorescence, single colonies were grown in LB broth, fixed, and analyzed by flow cytometry.

In Vitro Transcription
Templates for in vitro transcription assays were PCR generated, gel purified 210 bp DNA fragments amplified from pNH13, pNH14, or pNH15. Transcription reactions with σ-saturated *E. coli* RNAP holoenzyme (Epicenter) included α-[^{32}P]-ATP.

Natural Transformation
Natural transformation assays were performed as described (Duffin and Seifert, 2012). Transformation frequencies were reported as antibiotic-resistant cfu/ml divided by total cfu/ml from at least three independent experiments (mean ± SEM).

ACCESSION NUMBERS

The Gene Expression Ominbus (GEO) accession number for the dRNA-Seq data reported in this paper is GSE44582.

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