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# CRISPR RNA maturation by *trans*-encoded small RNA and host factor RNase III

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**CRISPR/Cas systems constitute a widespread class of immunity systems that protect bacteria and archaea against phages and plasmids, and commonly use repeat/spacer-derived short crRNAs to silence foreign nucleic acids in a sequence-specific manner. Although the maturation of crRNAs represents a key event in CRISPR activation, the responsible endoribonucleases (CasE, Cas6, Csy4) are missing in many CRISPR/Cas subtypes. Here, differential RNA sequencing of the human pathogen *Streptococcus pyogenes* uncovered tracrRNA, a *trans*-encoded small RNA with 24-nucleotide complementarity to the repeat regions of crRNA precursor transcripts. We show that tracrRNA directs the maturation of crRNAs by the activities of the widely conserved endogenous RNase III and the CRISPR-associated Csn1 protein; all these components are essential to protect *S. pyogenes* against prophage-derived DNA. Our study reveals a novel pathway of small guide RNA maturation and the first example of a host factor (RNase III) required for bacterial RNA-mediated immunity against invaders.**

Organisms of all kingdoms of life have evolved RNA-guided immunity mechanisms to protect themselves against genome invaders[1–6]. In bacteria and archaea, CRISPR/Cas (clustered, regularly interspaced short palindromic repeats/CRISPR-associated proteins) constitutes an adaptive RNA-mediated defence system which targets invading phages or plasmids in three steps: (1) adaptation via integration of viral or plasmid DNA-derived spacers into the CRISPR locus, (2) expression of short guide CRISPR RNAs (crRNAs) consisting of unique single repeat-spacer units and (3) interference with the invading cognate foreign genomes by mechanisms that are yet to be fully understood[7–27]. A key event in CRISPR activation is the maturation of the active crRNAs from the CRISPR precursor transcript (pre-crRNA)[28,29]. Three Cas proteins, Cse3 (CasE), Cas6 and Csy4, have been identified as endoribonucleases that cleave within the repeat sequences of pre-crRNA to generate the mature crRNAs[28–31]. However, their homologues are missing in many CRISPR/Cas subtypes, suggesting the existence of alternate crRNA maturation pathways involving other Cas proteins and/or fundamentally different RNA processing events. Here, our study of the human pathogen *Streptococcus pyogenes* uncovered a new pathway of CRISPR activation wherein a *trans*-encoded small RNA, the host endoribonuclease III and the CRISPR-associated Csn1 protein are responsible for the production of the active crRNAs.

## CRISPR/Cas systems in *S. pyogenes*

Our analysis of *S. pyogenes* genome sequences revealed the presence of two CRISPR/Cas loci of two different subtypes, CRISPR01 (system II, Nmeni/CASS4 subtype) and CRISPR02 (system I-C, Dvulg/CASS1 subtype)[32,33], each having a distinct set of repeats and *cas* genes (Fig. 1a, Supplementary Fig. 1 and Supplementary Table 1). Almost all of the associated CRISPR spacers show homology to chromosomal prophage sequences[34–38] (Supplementary Tables 2–5), indicating that the CRISPR/Cas systems of *S. pyogenes* target lysogenic phages.

To examine the *in vivo* expression of CRISPR01 and CRISPR02, we analysed *S. pyogenes* strain SF370 (M1 serotype) by differential RNA sequencing (dRNA-seq)[39]. The most abundantly recovered small RNA species were CRISPR01 crRNAs originating from a ~511-nucleotide pre-crRNA (Fig. 1a; Supplementary Fig. 2a, b and Supplementary Table 6), confirming that the CRISPR01 locus is active. In contrast, the CRISPR02 locus seems not to be expressed (Supplementary Fig. 3).

We detected six crRNAs from CRISPR01 which were 39 to 42 nucleotides in length and probably were processed species, as judged by their depletion in the dRNA-seq library for primary transcripts. The individual crRNAs appeared to result from double cleavage, one within the repeat and the other within the spacer, and carry a 20-nucleotide spacer-derived 5′-guide sequence and a 19–22 nucleotides repeat-derived 3′-sequence (Supplementary Fig. 2a, b). The latter sequence is distinct from the crRNA-tag (8 nucleotides of the upstream repeat sequence) located in 5′ of mature crRNAs produced by the Cse3 (CasE) and Cas6-encoding CRISPR/Cas subtypes of *Escherichia coli*, *Pyrococcus furiosus* and *Staphylococcus epidermidis*[28,31], providing evidence for the diversity of crRNA-tags among CRISPR/Cas systems and perhaps also for the underlying crRNA maturation and immunity mechanisms.

## tracrRNA directs pre-crRNA processing

Strikingly, dRNA-seq also detected abundant RNA species transcribed 210 nucleotides upstream, on the opposite strand of the CRISPR01-associated genes and the leader-repeat-spacer array (Fig. 1a, b, Supplementary Fig. 4a, b and Supplementary Table 6); we refer to these abundant transcripts as tracrRNA (*trans*-activating CRISPR RNA). Northern blot probing detected four tracrRNA forms with approximate lengths of 171, 89, 75 and 65 nucleotides, all of which were present throughout growth, notwithstanding a slightly decreased abundance of the longer transcripts in late stationary phase (Fig. 1c and Supplementary Fig. 4b–d). According to our dRNA-seq data, the 171- and 89-nucleotide forms corresponded to primary transcripts whereas the

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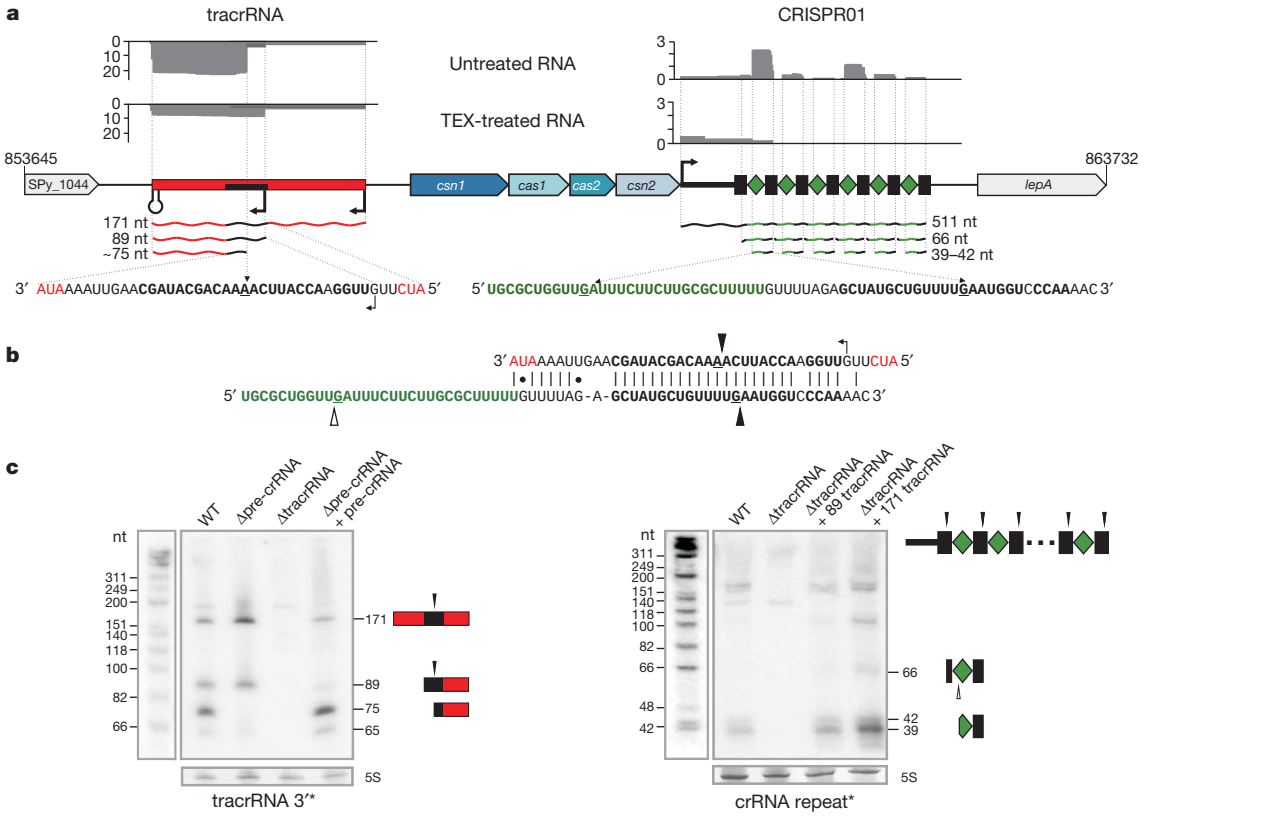


**Figure 1 | A newly identified tracrRNA is required for crRNA maturation in *S. pyogenes*.** **a**, dRNA-seq reveals expression of tracrRNA and crRNAs. Sequence reads of cDNA libraries derived from untreated and terminator 5′-phosphate-dependent exonuclease (TEX)-treated total RNA are shown. Vertical axis, relative amounts of sequenced cDNAs. The absence of ~75 nucleotides (nt) tracrRNA form and 39–42 nt crRNA fragments in the TEX-treated cDNA library indicates that they are generated by processing. Genomic organization of tracrRNA and CRISPR01/Cas (*csn1-cas1-cas2-csn2*) loci. Transcriptional start sites and a terminator are indicated. (Left) tracrRNA (red) is encoded on the minus strand and detected as 171, 89 and ~75 nt tracrRNA species. Black rectangle, 36-nt sequence stretch complementary to CRISPR01 repeat. (Right) pre-crRNA is encoded on the plus strand. Rectangles, CRISPR01 repeats; diamonds, CRISPR01 spacers; 511, 66 and 39–42 nt, pre-crRNA and processed crRNAs. **b**, Base-pairing of tracrRNA with a CRISPR01 repeat is represented. Cleavages observed by dRNA-seq and leading to the formation of short overhangs at the 3′ ends of the processed RNAs are indicated by black arrows. Open arrow, cleavage in the spacer sequence. **c**, tracrRNA and pre-crRNA are co-processed *in vivo*. Northern blot analysis of *S. pyogenes* total RNA: strains and probes are indicated (Supplementary Figs 2 and 4). Left panel: processing of tracrRNA into the ~75-nt form is abolished in Δpre-crRNA and re-established upon complementation with pre-crRNA. Right panel: processing of pre-crRNA into mature crRNA forms (39–42 nt) is abrogated in ΔtracrRNA. *Trans*-complementation of ΔtracrRNA with 171 or 89 nt tracrRNA restores the processing.

shorter ~75-nucleotide species resulted from processing of those longer tracrRNAs (Fig. 1a and Supplementary Fig. 4b).

Remarkably, both the 171- and 89-nucleotide tracrRNAs contain a 25-nucleotide stretch with almost perfect (one mismatch) complementarity to all repeats of CRISPR01 (Fig. 1b and Supplementary Fig. 5), predicting their potential base-pairing with pre-crRNA. Moreover, the tracrRNA and pre-crRNA processing sites detected by dRNA-seq fell in the putative RNA duplex region, indicative of co-processing of the two RNAs upon pairing. In support of this prediction, tracrRNA processing into the ~75-nucleotide form was absent in a Δpre-crRNA mutant. Conversely, we did not detect mature crRNAs in a ΔtracrRNA strain, indicating that tracrRNA is essential for the processing of pre-crRNA (Fig. 1c, Supplementary Figs 2c and 4c). *Trans*-complementation with the long tracrRNA species restored pre-crRNA processing in ΔtracrRNA bacteria, and showed that the 89-nucleotide form of tracrRNA suffices for co-processing (Fig. 1c and Supplementary Fig. 2c). Together, these findings reveal a novel function of a bacterial non-coding RNA such that a *trans*-encoded small RNA (tracrRNA) directs the maturation of another non-coding RNA (pre-crRNA) to yield the active species (crRNAs).

## crRNA maturation requires RNase III and Csn1

According to our dRNA-seq data, the co-processed tracrRNA and pre-crRNA carry short 3′ overhangs reminiscent of cleavage by the endoribonuclease RNase III[22,40–44] or the related eukaryotic Dicer and Drosha enzymes[1,4–6,45]. Because none of the Cas proteins of CRISPR01 contains an RNase III-like motif, we hypothesized that the endogenous RNase III—a general RNA processing factor[40,42,46] encoded by the conserved *rnc* gene of the host—was recruited to cleave tracrRNA and pre-crRNA upon base pairing. In support of our prediction, tracrRNA and pre-crRNA co-processing was abrogated in a Δ*rnc* mutant of *S. pyogenes* (Fig. 2), yet restored by *trans*-complementation of RNase III expression (Supplementary Fig. 6).

To demonstrate directly that the paired RNAs are substrates of this nuclease, tracrRNA and pre-crRNA were synthesized, annealed *in vitro* and incubated with *E. coli* RNase III. Whereas neither of the two RNAs alone was cut by the nuclease, their annealing promoted the expected singular RNase III cleavage in either RNA (Fig. 3a–c). Consistent with their shared complementarity to CRISPR01 repeats, both the 171-nucleotide and 89-nucleotide tracrRNAs promoted RNase III cleavage of pre-crRNA within the repeat to produce intermediate crRNA species, and both were converted to the ~75-nucleotide tracrRNA species in the process (Fig. 3b, c). Mutations in the complementary regions of tracrRNA or pre-crRNA hindered co-processing with the respective wild-type RNA partner, yet RNase III cleavage was fully restored when the compensatory tracrRNA and crRNA mutants were combined (Supplementary Fig. 7), corroborating that RNA duplex formation underlies the observed processing. Others have

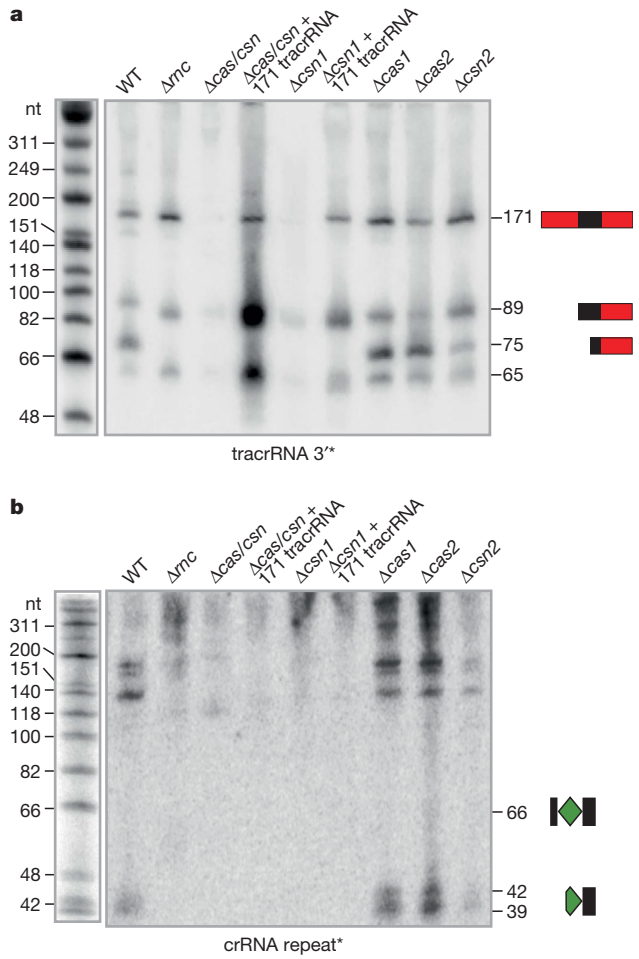


**Figure 2 | Co-processing of tracrRNA and pre-crRNA requires both endogenous RNase III and Csn1 *in vivo*.** **a**, **b**, Northern blot analysis of tracrRNA (**a**) and pre-crRNA (**b**) expression: strains and probes are indicated (Supplementary Figs 6 and 8). Processing of tracrRNA (**a**) into a ~75-nt form and pre-crRNA (**b**) into 39-42 nt mature crRNA forms is abolished in Δ*rnc*, Δ*cas/csn* and Δ*csn1* (refer to Supplementary Figs 6 and 8).

noticed that the repeats of CRISPR/Cas subtype II (Nmeni/CASS4) lack the potential to form stem-loop structures[47]; our findings suggest that tracrRNA overcomes this deficiency by providing an intermolecular RNA structure for pre-crRNA processing. Taken together, RNase III serves as a host factor in tracrRNA-mediated crRNA maturation, and constitutes the first example of a non-Cas protein that is recruited to CRISPR activity.

Next, we entertained the possibility that—in addition to RNase III—Cas proteins facilitate the co-processing of the duplex RNA *in vivo*. Intriguingly, deletion of the *csn1-cas1-cas2-csn2* operon impaired the processing of both tracrRNA and pre-crRNA (Fig. 2 and Supplementary Fig. 8a). In-frame deletions of any of the operon's four genes then revealed Csn1 as the only Cas protein required for the production of mature crRNAs and concomitant tracrRNA cleavage. This was further supported by restored tracrRNA and pre-crRNA processing upon ectopic expression of Csn1 in Δ*cas-csn* or Δ*csn1* mutants (Fig. 2 and Supplementary Fig. 8b–d).

Csn1 (or COG3513) is a large, likely multi-domain protein[32,33] of unknown function except that it is essential for CRISPR-mediated immunity in *Streptococcus thermophilus*[8]. Here, we propose a model wherein Csn1 acts as a molecular anchor facilitating the base-pairing of tracrRNA with pre-crRNA for subsequent recognition and cleavage of pre-crRNA repeats by the host RNase III (Fig. 4). Because Csn1 has predicted motifs of RuvC-like (RNase H fold) and McrA/HNH nucleases[32,33], it might also mediate the second cleavage to occur at a fixed distance within the spacers. Furthermore, Csn1 might help protect tracrRNA and pre-crRNA against other host RNases, as suggested by the strongly reduced accumulation of tracrRNA in the absence of *csn1* (Fig. 2 and Supplementary Fig. 8a, b). Collectively, our results show that in the absence of Cse3 (CasE), Cas6 or Csy4 proteins, CRISPR01 crRNA maturation is achieved by the concerted action of three novel factors, a *trans*-encoded small RNA, a host-encoded RNase and a Cas protein previously not implicated in pre-crRNA cleavage.

## CRISPR immunity against prophage sequences

To investigate further the role of tracrRNA in CRISPR01-mediated immunity, we developed a plasmid-based read-out system that mimics infection with protospacer-containing lysogenic phages (a protospacer is a DNA target sequence that matches a CRISPR spacer). We assayed transformation rates of a plasmid carrying a protospacer of the *speM* exotoxin gene, expected to be a target because of 100% identity to the second spacer of CRISPR01 (Spyo1h_002; Supplementary Table 2). Consistent with this protospacer being recognized by CRISPR01, wild-type *S. pyogenes* was protected from plasmids containing the *speM* gene, with or without its endogenous promoter region. Protection was specific because the wild-type strain was readily transformed with variants of the parental backbone plasmid as control (Fig. 5 and Supplementary Fig. 9). Importantly, in contrast to the wild-type strain, the Δpre-crRNA, ΔtracrRNA, Δ*rnc* and Δ*csn1* mutants invariably tolerated the *speM* plasmid (Fig. 5 and Supplementary Fig. 9). Together, these results demonstrate that tracrRNA, RNase III and Csn1 are essential in CRISPR01-mediated immunity of *S. pyogenes* against lysogenic phages, and further indicate that the tracrRNA/CRISPR01/Cas system, in concert with the host RNase III, limits horizontal virulence gene transfer among pathogenic streptococcal species[34–38].

## tracrRNA homologues in CRISPR/Cas systems

How widely spread is the tracrRNA-mediated CRISPR activation? Sequence analysis revealed anti-CRISPR repeat sequences, thus candidate tracrRNA homologues, in the vicinity of system II (Nmeni/CASS4) CRISPR/Cas loci of other bacterial genomes (Supplementary Table 7). We probed selected loci of *Listeria innocua*, *Neisseria meningitidis*, *Streptococcus mutans* and *S. thermophilus* (Fig. 6 and Supplementary Figs 12–16) and consistently observed both expression and processing of the homologous tracrRNAs and respective pre-crRNAs (Supplementary Figs 12–16). In addition, our analysis indicates potential co-evolution of the tracrRNA anti-repeat and CRISPR repeat sequences (Supplementary Table 7 and Supplementary Fig. 11). Thus, RNA base-pairing might generally determine crRNA maturation in type II CRISPR/Cas systems, and based on RNA probing results, these systems seem to be constitutively activated to target and affect the maintenance of invader genomes.

No putative tracrRNA homologue was found in the vicinity of other CRISPR/Cas subtypes, and the two additional degenerated repeats identified near the type III-A (Mtube/CASS6) CRISPR/Cas locus in *S. epidermidis* RP62a[25] lacked a corresponding tracrRNA homologue (Supplementary Fig. 17). Thus, the requirement of a *trans*-encoded small RNA for pre-crRNA processing into active crRNAs is a general RNA maturation mechanism shared by the type II (Nmeni /CASS4) CRISPR/Cas systems that lack the *cse3* (*casE*), *cas6* or *csy4* gene but possess *csn1*. Whether all of the type II CRISPR/Cas loci require RNase III as a host factor remains to be seen.

In summary, *trans* RNA-mediated activation of crRNA maturation to confer sequence-specific immunity against parasite genomes represents a novel RNA maturation pathway, and highlights the remarkable diversity and complexity of molecular mechanisms of CRISPR/Cas systems[9–14,26,28,29]. Importantly, CRISPR loci have been generally regarded as autonomous genetic units, encoding all the proteins and RNAs required for their activity. Our identification of RNase III as the

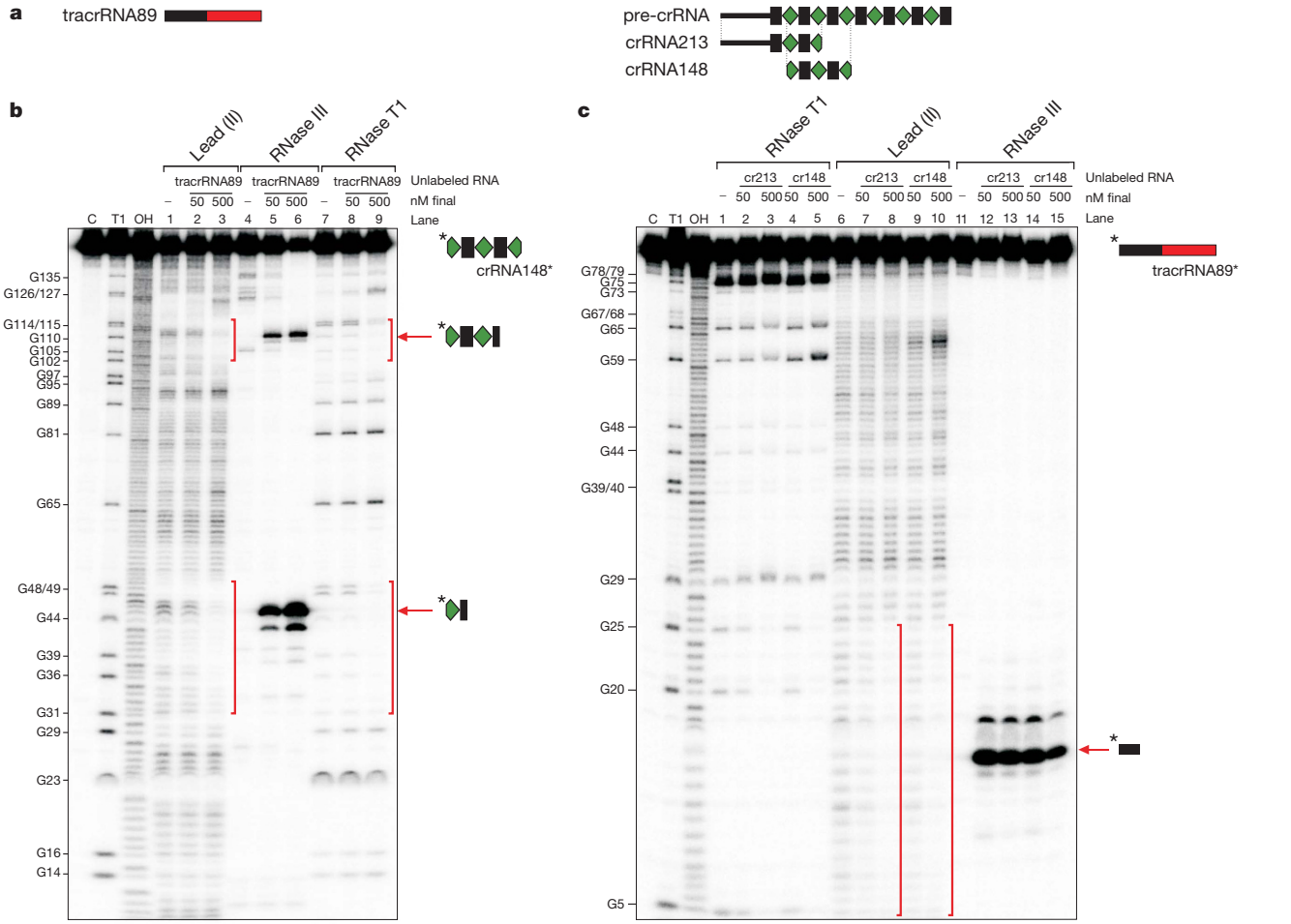


**Figure 3 | tracrRNA directs pre-crRNA cleavage by RNase III *in vitro*.** **a**, Schematic representation of tracrRNA89 corresponding to 89-nt-long tracrRNA, and crRNA213 and crRNA148 corresponding to a 213-nt-long leader-repeat-spacer1-repeat-spacer2 fragment and a 148-nt-long spacer1-repeat-spacer2-repeat-spacer3 fragment, respectively. **b**, Identification of tracrRNA89 binding sites on crRNA148*. 5′ end-labelled crRNA148* (~10 nM) was subjected to lead(II), RNase III and RNase T1 cleavage in the absence (lanes 1, 4, 7) and presence of cold tracrRNA89 (final concentration in lanes 2, 5, and 7: ~50 nM; lanes 3, 6, and 9: ~500 nM). Lane C: untreated crRNA148*; lane T1: RNase T1 digest of crRNA148* under denaturating conditions; lane OH: alkaline ladder; cleaved G residues are labelled. Vertical bars: crRNA148 region protected by tracrRNA89. Arrows denote specific RNase III cleavages in the two repeat regions of crRNA148 in the presence of tracrRNA89. **c**, Identification of crRNA148 and crRNA213 binding sites on tracrRNA89*. 5′ end-labelled tracrRNA89* (~10 nM) was subjected to RNase T1, lead(II) and RNase III cleavage in the absence (lanes 1, 6, 11) and presence of cold crRNA148 or crRNA213 (final concentration in lanes 2, 4, 7, 9, 12 and 14: ~50 nM; lanes 3, 5, 8, 10, 13 and 15: ~500 nM). Lanes C, T1 and OH, positions of cleaved G residues and vertical bars: as above but referring to tracrRNA89* in the presence of cold crRNA148 or crRNA213.

first host factor in CRISPR activity raises the exciting possibility that a recruitment of non-Cas proteins from the host chromosome might contribute to the rapid evolutionary diversification of CRISPR/Cas systems.

We suggest that Csn1 together with RNase III forms a microprocessor complex responsible for tracrRNA-mediated pre-crRNA processing (Fig. 4). As such, the requirement of RNase III in the process seems reminiscent of the key roles of related nucleases (Dicer, Drosha) in eukaryotic RNA–protein complexes that mediate the production of small interfering RNAs and maturation of microRNAs. However, the eukaryotic pathways employing RNase III-like enzymes for pre-RNA processing do not rely on *trans*-encoded RNA factors. More studies are needed to determine whether an RNase III-mediated activation of a small effector RNA by co-processing with a *trans*-acting non-coding RNA is also used in other biological systems.

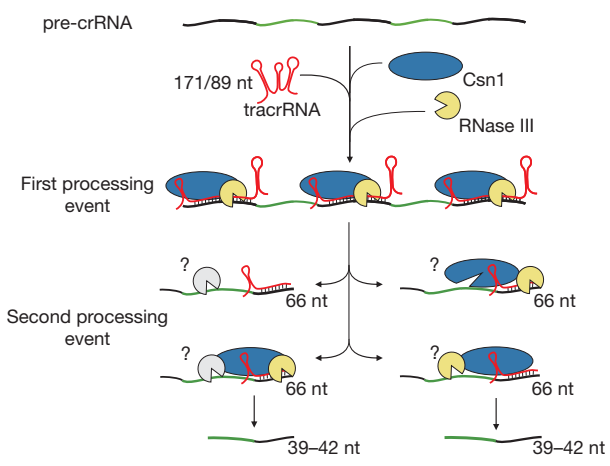


**Figure 4 | Model for tracrRNA-mediated crRNA maturation involving RNase III and Csn1.** Black, repeat; green, spacer. tracrRNA can bind with almost perfect complementarity to each repeat sequence of the pre-crRNA. The resulting RNA duplex is recognized and site-specifically diced by RNase III in the presence of Csn1, releasing the individual repeat-spacer-repeat units (first processing event). The generated units undergo further processing within the spacer sequences resulting in mature crRNA species consisting of unique spacer-repeat sequences (second processing event) by a yet-to-be elucidated mechanism. Csn1 may also be involved in the silencing of invading sequences.

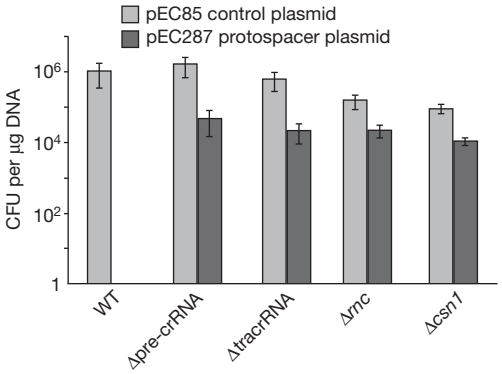


**Figure 5 | Both tracrRNA and pre-crRNA confer immunity against acquisition of a protospacer gene derived from a lysogenic phage.** Transformation efficiencies of *S. pyogenes* with *speM* protospacer containing plasmid (pEC287) and reference 'backbone' plasmid (pEC85) (Supplementary Fig. 9). Strains: *S. pyogenes* wild type (WT; SF370), Δpre-crRNA, ΔtracrRNA, Δ*rnc* and Δ*csn1*. Graph bars, mean values of colony forming units (CFU) per µg of plasmid DNA; error bars, s.d.; $n \geq 3$. pEC287 is tolerated by the Δpre-crRNA, ΔtracrRNA, Δ*rnc* and Δ*csn1* mutants but not by the WT strain. As control, transformants in all strains were obtained with the backbone plasmid (Supplementary Fig. 9).

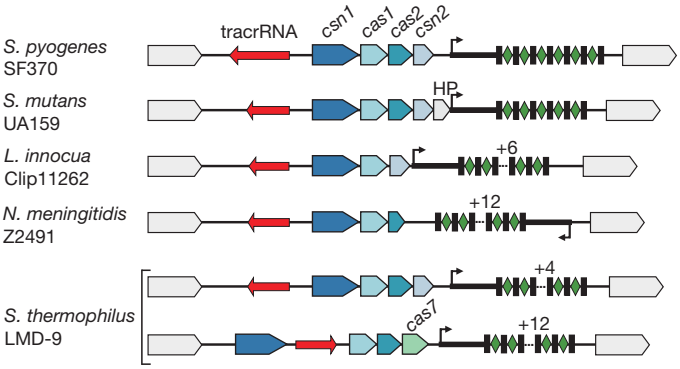


**Figure 6 | tracrRNA-mediated crRNA maturation is conserved among different bacterial species.** tracrRNA-mediated crRNA maturation is inherent to the type II (Nmeni/CASS4) CRISPR/Cas systems. Type II (Nmeni/CASS4) loci from *S. pyogenes* SF370, *S. mutans* UA159, *L. innocua* Clip11262, *N. meningitidis* Z2491 and *S. thermophilus* LMD-9 (Nmeni/CASS4a); red, tracrRNA; rectangles, repeats; diamonds, spacers.

## METHODS SUMMARY

Details of bacteria (culture conditions, transformation), DNA (biocomputational analysis, plasmid construction, in-frame gene deletion mutants) and RNA manipulation (cDNA library construction (vertis Biotechnologie), RNA expression analysis, RNA structure probing and hydrolysis) are provided as Supplementary Information[34,39,48]. In brief, half of DNase I-treated SF370 total RNA was enriched for primary transcripts by treatment with Terminator 5′-phosphate-dependent exonuclease (TEX) (Epicentre), which degrades RNAs with a 5′P (processed RNAs) but not primary transcripts with a 5′PPP RNA[39]. cDNA libraries were constructed from both untreated and TEX-treated RNA[39]. Following 454 pyrosequencing, cDNAs were mapped to the SF370 genome and visualized using the Affymetrix Integrated Genome Browser[39]. Strains, plasmids and primers are listed in Supplementary Tables 8, 9 and 10, respectively.

**Full Methods** and any associated references are available in the online version of the paper at www.nature.com/nature.

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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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## METHODS

**Bacterial strains and culture conditions.** Bacterial strains used in the study are listed in Supplementary Table 8. *Streptococcus pyogenes* and *Streptococcus mutans* were cultured in THY medium (Todd Hewitt Broth (THB, Bacto, Becton Dickinson) supplemented with 0.2% yeast extract (Oxoid)) or on TSA (trypticase soy agar, BBL, Becton Dickinson) supplemented with 3% sheep blood. BHI broth (brain heart infusion) and BHI agar were used for growth of *Listeria innocua* and *Staphylococcus epidermidis*. *Streptococcus thermophilus* was grown in M17 broth supplemented with 0.5% (w/v) lactose (LM17)[49] or on BHI agar. *Escherichia coli* was cultured in Luria–Bertani (LB) medium and agar. *Neisseria meningitidis* cells were grown on GC-agar and in PPM liquid medium including 1% vitamin-mix and 0.5% $NaHCO_3$. Cultures of *S. mutans*, *S. pyogenes* and *S. thermophilus* were incubated at 37 °C (*S. mutans*, *S. pyogenes*) or 42 °C (*S. thermophilus*) in an atmosphere supplemented with 5% $CO_2$ without shaking. Strains of *E. coli*, *L. innocua*, *N. meningitidis* and *S. epidermidis* were grown aerobically at 37 °C with shaking. Whenever required, suitable antibiotics were added to the medium at the following final concentrations: ampicillin, 100 μg ml$^{-1}$ for *E. coli*; kanamycin, 25 μg ml$^{-1}$ for *E. coli* and 300 μg ml$^{-1}$ for *S. pyogenes*. Bacterial cell growth was monitored periodically by measuring the absorbance (*A*) of culture aliquots at 620 nm using a microplate reader (SLT Spectra Reader).

**Bacterial transformation.** Transformation of *E. coli* with plasmid DNA was carried out according to standard protocols[50]. Transformation of *S. pyogenes* was performed as previously described[51] with some modifications. For the transformation read-out experiment, *S. pyogenes* electrocompetent cells were equalized to the same $A_{620\,nm}$ and subsequently used for electroporation with 500 ng of plasmid. Transformations were done in triplicate with the same batch of electrocompetent cells. Each transformation was plated three to four times. Experiments were performed at least three times independently for statistical analysis. Transformation efficiencies were calculated as colony-forming units per μg of DNA. Mock and control transformations were done with sterile $dH_2O$ and backbone 'empty' plasmid pEC85, respectively.

**DNA manipulations.** DNA manipulations including DNA preparation, amplification, digestion, ligation, purification, agarose gel electrophoresis and Southern blot analysis were performed according to standard techniques[50] with some modifications. Site-directed mutagenesis in plasmid inserts was done using QuickChange II XL kit (Stratagene). Kits (Peqlab Biotechnology and Qiagen) were used for plasmid preparation and DNA purification with minor modifications. Synthetic oligonucleotides used in this study were supplied by VBC-Biotech Services and Sigma-Aldrich and are listed in Supplementary Table 10. When required (for example, further use in cloning experiments, verification of in-frame gene deletion mutants), PCR-generated DNA fragments and plasmid inserts were sequenced at VBC-Biotech Services and LGC Genomics. All plasmids generated in this study are listed in Supplementary Table 9.

**In-frame gene deletion in *S. pyogenes*.** The following procedure was used for the generation of ΔtracrRNA, Δpre-crRNA, Δ*csn1-cas1-cas2-csn2*, Δ*csn1*, Δ*cas1*, Δ*cas2*, Δ*csn2* and Δ*rnc* mutants in *S. pyogenes* wild-type (WT) SF370 (M1 serotype). Briefly, we constructed the shuttle vector pEC214 that includes a thermosensitive origin of replication (pWV01-*repAts*) specific for Gram-positive bacteria[48], the ColE1 replicon specific for *E. coli*[52], a kanamycin resistance cassette (*aphIII*) for plasmid selection in both *E. coli* and Gram-positive bacteria[52], a thermostable β-galactosidase cassette (*bgaB*) under the control of the *Staphylococcus aureus clpB* promoter for plasmid loss selection[53] and a multiple-cloning site (MCS)[52] (Supplementary Table 9). For each in-frame gene deletion, DNA fragments located upstream and downstream of the gene of interest were amplified by PCR using genomic DNA of *S. pyogenes* SF370 and primers containing flanking restriction sites (Supplementary Table 10). The amplified fragments were then cloned in the MCS of pEC214, thus generating thermosensitive plasmids containing a DNA region with in-frame deletion of the gene of interest (Supplementary Table 9). Each recombinant plasmid was introduced in *S. pyogenes* SF370 by electroporation at the permissive temperature (28 °C). A series of incubation at the non-permissive temperature (37 °C) and the permissive temperature (28 °C)[48] led to the selection of in-frame gene deletion mutants sensitive to kanamycin (Supplementary Table 8). Kanamycin-sensitive wild-type (WTts) clones issued from the same temperature shift procedure were also kept for comparative analysis. In this study, the phenotypes of all control WTts strains were identical to that of the wild-type (WT) strain. This indicated that the phenotypes observed in the in-frame gene deletion mutants were not caused by mutations, which would have occurred somewhere else on the chromosome during the recombination procedure (Supplementary Table 8). The correct deletion and non-deletion events in selected clones were verified by PCR, sequencing and Southern blot analyses (Supplementary Table 10). Reverse transcription–PCR (RT–PCR ) analysis of the in-frame Δ*rnc*, Δ*csn1*, Δ*cas1*, Δ*cas2* and Δ*csn2* mutants demonstrated that each deletion of the single genes did not affect the expression of the other genes in the operons (operon *rnc-smc*, Supplementary Fig. 6, and operon *csn1-cas1-cas2-csn2*, Supplementary Fig. 8) (data not shown). RT–PCR analysis also showed *rnc* expression in Δ*csn1* and *csn1* expression in Δ*rnc* (data not shown).

**Construction of plasmids for complementation studies in *S. pyogenes*.** Plasmid pEC85 containing an origin of replication specific for Gram-positive bacteria (*repDEG*-pAMβ1)[54], the ColE1 replicon specific for *E. coli*[52], a kanamycin resistance cassette (*aphIII*) for selection in both *E. coli* and Gram-positive bacteria[52], and an expanded MCS[54] was used as vector for complementation studies. For tracrRNA, CRISPR01 pre-crRNA and *rnc* complementation plasmids, a RNA-(tracrRNA and pre-crRNA) or protein-(*rnc*) encoding DNA fragment of interest under the control of the native promoter was cloned in pEC85 (Supplementary Tables 9, 10). For *csn1*, *cas1*, *cas2* and *csn2* complementation plasmids, a DNA fragment containing the coding sequence of the gene of interest under the control of the promoter of the *csn1-cas1-cas2-csn2* operon was cloned in pEC85 (Supplementary Tables 9, 10). The generated recombinant plasmids were introduced in *S. pyogenes* mutant strains selecting for kanamycin resistant clones (Supplementary Table 8). The integrity of plasmids was verified by plasmid digestion and sequencing analysis. In addition, RT–PCR analysis was conducted to verify that the genes (encoding RNAs or proteins) in the inserts of the recombinant plasmids were expressed in the recombinant *S. pyogenes* strains (data not shown).

**Construction of plasmids for transformation studies in *S. pyogenes*.** Plasmid pEC85 described above was used as vector for transformation studies. A control DNA fragment and DNA fragments containing wild-type and mutated protospacers were cloned in pEC85 (Supplementary Tables 9, 10). The integrity of the cloned plasmids was verified by enzymatic digestion and sequencing analysis. The generated recombinant plasmids were introduced in *S. pyogenes* wild-type and mutant strains, selecting for kanamycin resistant clones (Supplementary Table 8). The integrity of plasmids was verified in selected clones by plasmid digestion and northern blot analysis of protospacer expression (Supplementary Table 10 and Supplementary Fig. 9).

**RNA preparation.** Total RNA from streptococcal, staphylococcal, listerial and neisserial cells was prepared from culture samples collected at different time points during growth[48,54,55], using the TRIzol reagent (Sigma-Aldrich) with some modifications. The total RNA samples were treated with DNase I (Fermentas) according to the manufacturer's instructions. The concentration of RNA in each sample was further normalized following spectrophotometry measurements using NanoDrop.

**cDNA library for differential RNA sequencing (dRNA-seq) and 454 pyrosequencing.** In brief, total RNA from *S. pyogenes* SF370 (M1 serotype) cells grown until mid-logarithmic phase was treated with DNase I to remove any residual genomic DNA[39]. The RNA was submitted to a new treatment protocol to deplete processed transcripts and thereby enrich for primary transcripts by using Terminator 5′-phosphate-dependent exonuclease (TEX) (Epicentre)[39]. A cDNA library from the TEX-treated and untreated RNA was constructed by Vertis Biotechnologie and a total of 38,468 DNAs was sequenced. After 5′end-linker and poly(A)-tail clipping, cDNAs longer than 17 nucleotides (87%) were mapped to the *S. pyogenes* M1 GAS genome (NC_02737) using WU-BLAST 2.0 (http://blast.wustl.edu/) as previously described[56]. Afterwards, mapping data were visualized using the 'Integrated Genome Browser" (Affymetrix). Small RNA candidates were selected on the basis of the following criteria: encoded in intergenic regions, size ranging from 50 to 500 nucleotides, presence of a putative promoter (BPROM software) and/or Rho-independent transcription terminator (TransTermHP (v2.04) program) (see below for references).

**Northern blot analysis.** Northern blot analysis was carried out essentially as described elsewhere[55,57,58]. RNAs separated on 10 or 12% polyacrylamide gels containing 8 M urea were blotted onto Hybond-N+ or Hybond-XL membranes (General Electric Healthcare) and then hybridized with specific probes. In general, $^{32}$P-labelled primers specific to the RNAs of interest were used as probes (Supplementary Table 10). For primer labelling, 40 pmol of oligonucleotide were denaturated for 5 min at 95 °C and then mixed with 20 μCi of $^{32}$P-γ-ATP and 10 U of T4 polynucleotide kinase (Fermentas). Labelled probes were purified before hybridization using G25 columns (GE Healthcare) in order to remove unincorporated nucleotides. For all northern blots, 5S rRNA served as loading control.

**RNA metabolic stability analysis.** The metabolic stability of RNAs was determined as previously described[59] with minor modifications. Cells were grown until mid-logarithmic phase, at which time point rifampicin (250 μg ml$^{-1}$, Sigma-Aldrich) was added. Aliquots of cells were collected before treatment (control), immediately after (0 min) and at defined time points after addition of rifampicin. A mixture of ice-cold acetone-ethanol (vol:vol, 1:1) was added and cells were frozen immediately at -80 °C. Total RNA was prepared and analysed by northern blot analysis as described above. RNA half-life ($t_{1/2}$) was calculated by automated pixel counting using the ImageQuant v5.1 software, referring to 5S rRNA as loading control. To ensure absence of rifampicin-resistant bacteria, serial

dilutions of the rifampicin-treated cells were plated on TSA blood plates with or without rifampicin (250 μg ml$^{-1}$), incubated overnight at 37 °C and then visualized for the possible appearance of colonies.

**RT–PCR analysis.** RT–PCR analysis of RNA samples was done using the OneStep RT–PCR Kit (Qiagen) according to the manufacturer's instructions and primers listed in Supplementary Table 10. For the semiquantitative analysis, RNA samples were serial-diluted before reverse transcription and PCR. DNase I-treated RNA samples (8 ng to 1 μg; TURBO DNA-*free*, Ambion) were used. Quantification of RT–PCR fragments was done using 5S rRNA or transfer-messenger RNA (tmRNA) as loading control.

**RNA mapping.** Head-to-tail RNA circularization and/or primer extension were used to map the RNA termini. (1) Head-to-tail RNA circularization: this method and its suitability to simultaneously map the 5′ and 3′ ends of RNAs was described elsewhere[60]. Briefly, total RNA was treated with tobacco acid pyrophosphatase (TAP, Epicentre Biotechnologies) according to the supplier's instructions and the treated RNA was purified by acid:phenol:chloroform (Ambion) extraction and precipitation. RNAs were circularized using T4 RNA Ligase I (New England Biolabs) followed by purification using acid phenol:chloroform extraction and precipitation. The purified circularized RNAs were reverse transcribed and amplified by PCR using inward RNA-specific primers (Supplementary Table 10) and the OneStep RT–PCR Kit (Qiagen). The generated PCR fragments were cloned into pCR2.1-TOPO vector using TOPO TA cloning kit (Invitrogen) and the inserts of three to five clones were sequenced. (2) Primer extension: 5 to 10 μg of total RNA were denaturated in presence of 5′ radiolabelled reverse primer. Reverse transcription was done using ThermoScript Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. The reverse transcription reaction was subsequently separated on 8% polyacrylamide/8 M urea gel and analysed in reference to the PhiX174/HinfI ladder (Fermentas).

***In vitro* transcription, purification and 5′ end labelling of RNA.** DNA templates carrying a T7 promoter sequence for *in vitro* transcription were generated by PCR using genomic DNA of *S. pyogenes* SF370 and primers listed in Supplementary Table 10. RNA was *in vitro* transcribed, gel-purified and quality-checked as described earlier[61]. 5′ labelling of RNA was done according to a previously published protocol[62].

***In vitro* RNA structure mapping and footprinting.** Secondary structure probing and mapping of RNA complexes was conducted on 5′-end-labelled RNA (~0.1 pmol) in 10 μl reactions. RNA was denatured 1 min at 95 °C and chilled on ice for 5 min, upon which 1 μg yeast RNA and 10× structure buffer (0.1 M Tris pH 7, 1 M KCl, 0.1 M $MgCl_2$, Ambion) were added. Concentrations of unlabelled tracrRNA/crRNA fragments added to the reactions are given in the figure legends. Following incubation for 10 min at 37 °C, 2 μl of a fresh solution of lead(II) acetate (25 mM; Fluka), or 2 μl of RNase T1 (0.05 U μl$^{-1}$; Ambion) were added and incubated for 1 and 3 min at 37 °C, respectively. RNase III cleavage reactions contained 1 mM DTT and 0.0026 units (1 μl of a 1:500 dilution of the 1.3 U μl$^{-1}$ enzyme stock) enzyme (RNase III from *E. coli*, New England Biolabs), and were incubated for 3 min at 37 °C. Reactions were stopped by direct addition of 12 μl loading buffer on ice. RNase T1 ladders were obtained by incubating labelled RNA (~0.2 pmol) in 1× sequencing buffer (Ambion) for 1 min at 95 °C. Subsequently, 1 μl RNase T1 (0.1 U μl$^{-1}$) was added and incubation was continued at 37 °C for 5 min. OH ladders were generated by 5 min incubation of 0.2 pmol labelled RNA in alkaline hydrolysis buffer (Ambion) at 95 °C. Reactions were stopped with 12 μl loading buffer II (Ambion). Samples were denatured for 3 min at 95 °C before separation on 6% or 8% polyacrylamide/7 M urea sequencing gels in 1× TBE. Gels were dried and analysed using a PhosphorImager (FLA-3000 Series, Fuji) and AIDA software (Raytest).

**Computational analysis of DNA and protein sequences.** Gene annotations are according to NCBI genome browser and KEGG (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/). DNA sequence analysis for genetic locus organization and plasmid generation was done using the Vector NTI software (Invitrogen). Comparative sequence analysis of DNA and putative proteins was done using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/ index.html) and AlignX (Invitrogen) were used for sequence alignments. Putative rho-independent transcription terminators (RITs) were annotated using the TransTermHP (v2.04) program (http://transterm.cbcb.umd.edu/)[63]. Putative promoters were predicted using the BPROM software (http://www.softberry.com/) and BDGP Neural Network Promoter Prediction NNPP version 2.2 (http://www.fruitfly.org/seq_tools/promoter.html).

**Computational predictions of RNA structure and co-folding.** Computational predictions were done using algorithms of the Vienna RNA package[64,65]. RNA secondary structures were predicted with RNAfold, co-folding was done using RNAcofold and co-folded secondary structures were drawn using VARNA[66].


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