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A variable homopolymeric G-repeat defines small RNA-mediated posttranscriptional regulation of a chemotaxis receptor in *Helicobacter pylori*

Sandy R. Pernitzsch[a], Stephan M. Tirier[a], Dagmar Beier[b], and Cynthia M. Sharma[a,1]

[a]Research Center for Infectious Diseases (ZINF), University of Würzburg, 97080 Würzburg, Germany; and [b]Chair of Microbiology, Biocenter, University of Würzburg, 97074 Würzburg, Germany

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Phase variation of hypermutable simple sequence repeats (SSRs) is a widespread and stochastic mechanism to generate phenotypic variation within a population and thereby contributes to host adaptation of bacterial pathogens. Although several examples of SSRs that affect transcription or coding potential have been reported, we now show that a SSR also impacts small RNA-mediated posttranscriptional regulation. Based on in vitro and in vivo analyses, we demonstrate that a variable homopolymeric G-repeat in the leader of the TlpB chemotaxis receptor mRNA of the human pathogen *Helicobacter pylori* is directly targeted by a small RNA (sRNA), RepG (Regulator of polymeric G-repeats). Whereas RepG sRNA is highly conserved, the *tlpB* G-repeat length varies among diverse *H. pylori* strains, resulting in strain-specific RepG-mediated *tlpB* regulation. Based on modification of the G-repeat length within one strain, we demonstrate that the G-repeat length determines posttranscriptional regulation and can mediate both repression and activation of *tlpB* through RepG. In vitro translation assays show that this regulation occurs at the translational level and that RepG influences *tlpB* translation dependent on the G-repeat length. In contrast to the digital ON–OFF switches through frame-shift mutations within coding sequences, such modulation of posttranscriptional regulation allows for a gradual control of gene expression. This connection to sRNA-mediated posttranscriptional regulation might also apply to other genes with SSRs, which could be targeting sites of *cis*- or *trans*-encoded sRNAs, and thereby could facilitate host adaptation through sRNA-mediated fine-tuning of virulence gene expression.

homopolymeric repeat | noncoding RNA

For successful survival in the environment or colonization of a host, bacteria must adapt their phenotypes either through sensing and responding to changing conditions or through selection of beneficial mutations. The establishment of long-term infections and evasion of the immune system, in particular, require mechanisms to modulate gene expression, especially of genes encoding products that influence the interaction with the host. Phase variation represents a frequent and stochastic mechanism of genotype switching and facilitates phenotypic variation within bacterial populations (1). Besides a variety of mechanisms including site-specific recombination or epigenetic changes through DNA methylation, phase variation can occur due to highly mutable DNA sequences. These so called contingency loci are often associated with genes involved in LPS biosynthesis, bacterial surface structures, and DNA restriction or modification (2). In addition to deletions, gene conversions or point mutations, highly variable simple sequence repeats (SSRs) have been shown to be the major source of phase variation within these loci (2, 3).

Phase variation of SSRs is in most cases independent of recombination and occurs during replication through slipped-strand mispairing and polymerase slippage which leads to repeat length variation (3). SSRs have been described to affect virulence and host adaptation of several bacterial pathogens such as *Bordetella pertussis* (4), *Neisseria meningitidis* (5), *Haemophilus influenzae* (6), *Campylobacter jejuni* (7), or *Helicobacter pylori* (8, 9). Depending on their location, SSRs can either affect translation through the introduction of frame-shift mutations within coding regions leading to premature translation termination or altered C termini of proteins (intragenic SSRs) (5, 10–13) or influence transcription by changing the spacing of promoter elements or transcription factor binding sites (intergenic SSRs) (14–16). Whereas the mechanisms and roles of SSRs on gene regulation at the DNA level are established, effects on mRNA stability or posttranscriptional control are less understood (3).

Here, we show that length variation of a homopolymeric repeat can determine small RNA-mediated posttranscriptional regulation. Bacterial small regulatory RNAs (sRNAs) are posttranscriptional regulators of gene expression that have been implicated in stress response or virulence control (17, 18). Although some sRNAs can activate gene expression or directly modulate protein activity, most of the functionally characterized sRNAs act as antisense RNAs and repress translation and/or induce degradation of *cis*- or *trans*-encoded target mRNAs. So far, the majority of sRNAs has been investigated in enterobacteria, but genome-wide studies have been reporting an increasing number of sRNA candidates in various bacteria including several important human pathogens. Using RNA-seq, we recently identified more than 60 sRNAs in *Helicobacter pylori*, the causative agent of gastritis, ulcers, and gastric cancer (19, 20). Like 50% of all bacteria, *Helicobacter* lacks a homolog of the RNA chaperone Hfq, a key player in sRNA-mediated regulation in enterobacteria (21). Thus, sRNAs in these bacteria

Significance

To establish long-term infections, bacterial pathogens must adapt their gene expression through sensing and responding to changing conditions or selection of genotypic variations within the population. Hypervariable simple sequence repeats (SSRs) in coding sequences or promoters are a major source of phase variation and often associated with genes involved in host interaction. While their impact on gene regulation at the DNA level is established, we now demonstrate a connection between SSRs and small RNA (sRNA)-mediated posttranscriptional regulation. We show that a homopolymeric G-repeat within the leader of a chemotaxis receptor mRNA in *Helicobacter pylori* is directly targeted by a small RNA. The length of this G-repeat varies among different *Helicobacter* strains and thereby determines sRNA-mediated translational repression or activation and strain-specific regulation.

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[1]To whom correspondence should be addressed. E-mail: cynthia.sharma@uni-wuerzburg.de.

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must act independently of Hfq and their study will provide new insights into mechanisms of sRNA-mediated regulation and virulence control (22).

Here we study a highly conserved and abundant sRNA, RepG (previously HPnc5490; ref. 19), and show that it targets a homopolymeric G-repeat in the mRNA leader of an acid-sensing chemotaxis receptor, TlpB (23, 24). The *tlpB* G-repeat length varies in diverse *Helicobacter* strains ranging from 6 to 16 guanines and was previously described as an intergenic promoter SSR (25). However, our global transcriptome map revealed that the G-repeat is located in a 5′ UTR (19) and, thus, is unlikely to affect *tlpB* promoter strength. We now demonstrate that variation of the G-repeat length influences posttranscriptional control of *tlpB* by RepG at the translational level and that there is strain-specific *tlpB* regulation by this sRNA. This length-dependent targeting of homopolymeric repeats by a *trans*-acting sRNA provides a twist in sRNA-mediated regulation and mechanisms of gene expression control. Considering the steadily increasing number of sRNAs in diverse bacteria, it is likely that also other phase-variable genes might be subject to posttranscriptional control and that SSRs in their mRNA leaders and also coding sequences might be sRNA target sites.

Results

The Abundant RepG sRNA Is Broadly Conserved in *Helicobacter*. The 87-nt-long RepG sRNA (HPnc5490) was identified as one of the most abundant transcripts in our dRNA-seq study of *H. pylori* strain 26695 (19). It is transcribed from an intergenic region between genes encoding an orphan response regulator, HP1043, and a protein of unknown function, HP1044, and is predicted to fold into two stem-loop structures (Fig. 1*A*). Biocomputational searches for RepG homologs in 31 different *H. pylori* strains, *Helicobacter acinonychis*, and *Helicobacter mustelae* revealed that the RepG gene, its genomic context, and the predicted RepG secondary structure are highly conserved (*SI Appendix*, Fig. S1). Using Northern blot analysis, we confirmed RepG expression in diverse *Helicobacter* strains but observed variations in abundance and band patterns of RepG (Fig. 1*B*). Whereas multiple RepG bands were detected in *H. pylori* strain 26695, only one band was observed in G27, although both strains share an overall highly conserved sRNA sequence. Primer extension revealed that the different bands in strain 26695 correspond to RepG versions that vary slightly at their 5′ end (*SI Appendix*, Fig. S2*A*). In strain G27, the sRNA TSS is shifted to one nucleotide downstream compared with the main RepG species in 26695. Nevertheless, both strains showed a similar sRNA expression profile with an increase of RepG in late exponential growth, a decrease in stationary phase, and accumulation in coccoid forms (Fig. 1*C* and *SI Appendix*, Fig. S2*B*).

RepG Represses *tlpB* via a G-Repeat. Sequence alignment of RepG homologs showed that the predicted C/U-rich terminator loop of the sRNA is highly conserved, even in more distant species such as the ferret colonizing *H. mustelae* (*SI Appendix*, Fig. S1*B*). Predictions for potential target mRNAs using the TargetRNA program (26) indicated that RepG might base pair with its C/U-rich sequence to a homopolymeric G-repeat in the 5′ UTR of the mRNA encoding the chemotaxis receptor TlpB (HP0103) (Fig. 1*A*), and preliminary analysis of a *repG* deletion mutant indicated that the sRNA represses *tlpB* expression at the mRNA and protein level (19). To study the potential regulation of *tlpB* by RepG, we complemented the Δ*repG* mutant of *H. pylori* 26695 with wild-type (C_{RepG}) or mutant RepG sRNAs expressed from the P_{repG} promoter at the unrelated *rdxA* locus. Mutants we tested were SL 2, which expresses only the second stem-loop; ΔCU, in which the C/U-rich region of the RepG terminator loop was replaced by an extrastable tetraloop; and 3xG and 1xG*, in which three or one C residue(s) in the predicted interaction site were exchanged to G(s) (Fig. 2*A*).

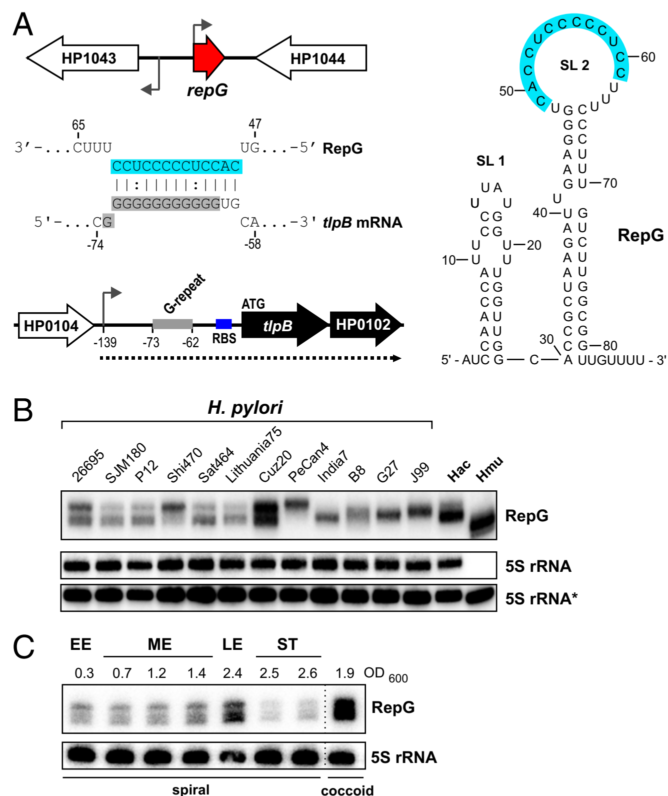

Fig. 1. Conservation and expression of *Helicobacter* RepG sRNA. (*A*) The 87-nt-long RepG sRNA is transcribed from the intergenic region between an orphan response regulator, HP1043, and a hypothetical protein, HP1044. A C/U-rich single-stranded region (marked in blue) in the RepG terminator loop (SL 2) was predicted to base pair with a G-repeat (marked in gray) in the 5′ UTR of the dicistronic *tlpB*-HP0102 mRNA (dotted line) encoding a chemotaxis receptor and a hypothetical protein. Transcriptional start sites (19) are indicated by arrows and the *tlpB* G-repeat and ribosome binding site (RBS) by gray and blue bars, respectively. Numbers indicate the distance to the *tlpB* start codon. (*B*) Northern blot analysis of RepG expression at exponential growth phase in diverse *H. pylori* strains, *H. acinonychis* (Hac), and *H. mustelae* (Hmu) using ^{32}P-labeled oligonucleotide CSO-0003. 5S rRNA served as loading control and was probed with two oligonucleotides: JVO-0485 (5S rRNA) for *H. pylori* and *H. acinonychis* and CSO-0053 (5S rRNA*) for *H. mustelae*, respectively. (*C*) Expression of RepG was analyzed during growth in *H. pylori* 26695 (EE, early exponential; ME, mid exponential; LE, late exponential; ST, stationary phase) at indicated optical densities (OD_{600}). After ~60 h, morphology changed from spiral to coccoid.

Analysis of whole cell protein fractions and RNA samples of these strains by SDS/PAGE and Northern blot, respectively, showed that complementation with the wild-type sRNA and the SL 2 mutant which harbors the predicted C/U-rich *tlpB* interaction site both restore *tlpB* repression (Fig. 2*A*). In contrast, deletion of the C/U-rich binding site and introduction of triple or single point mutations abolished *tlpB* regulation, confirming that the sRNA terminator loop is important for *tlpB* repression. Western blot analysis using an antiserum against all four chemotaxis receptors of *H. pylori* gave results consistent with the SDS/PAGE. RepG specifically represses *tlpB* ~fivefold, whereas the levels of the other chemotaxis receptors TlpA, TlpC, and TlpD remained unaltered upon deletion of the sRNA. Analysis of Δ*tlpB* and Δ*tlpB*/Δ*repG* double deletion mutants confirmed that the up-regulated band is indeed TlpB (Fig. 2*A*). Despite threefold lower levels, the SL 2 mutant represses *tlpB* expression to the same extent as the wild-type sRNA, indicating that RepG levels are not limiting for *tlpB* regulation under this condition. Moreover, this simple stem-loop structure of 58 nt, corresponding

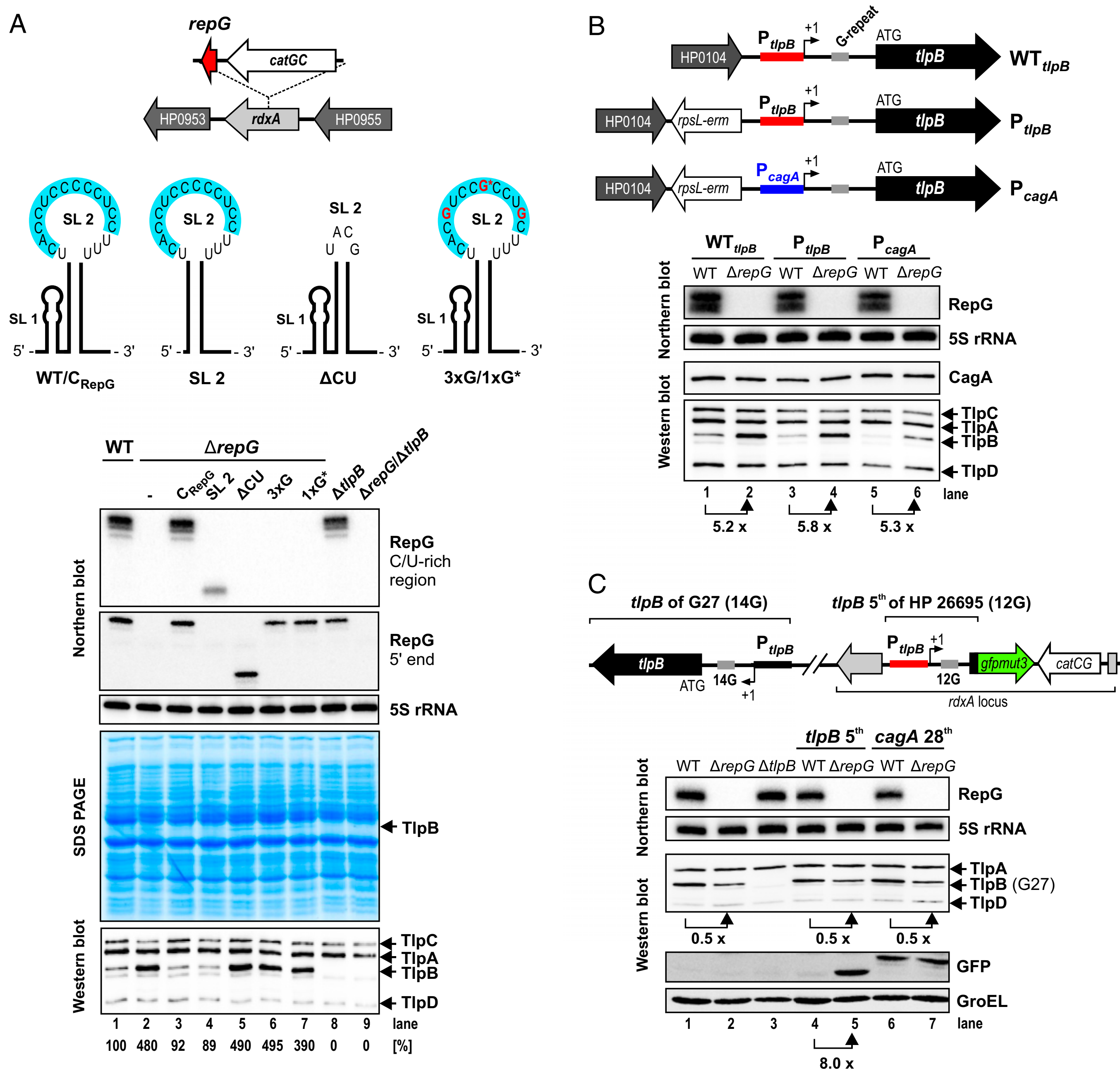

Fig. 2. In vivo validation of *tlpB* regulation by RepG sRNA. (*A*, *Upper*) The Δ*repG* mutant was complemented with wild-type RepG (C_{RepG}) or several mutant sRNAs in the *rdxA* locus. SL 2 consists of only the second stem-loop (nucleotides 30–87) of RepG. In ΔCU the *tlpB* binding site (marked in blue) was replaced by an extrastable tetraloop (UACG). Triple or single* C to G point mutations at position 52, 56, and 60 (3xG) or at position 56 (1xG*) are indicated in red. (*A*, *Lower*) *H. pylori* 26695 wild type (WT), Δ*repG*, and complementation strains (C_{RepG}, SL 2, ΔCU, 3xG, and 1xG*), as well as Δ*tlpB,* and Δ*repG*/Δ*tlpB* double deletion mutants were grown to exponential growth phase and RNA and protein samples were analyzed by Northern blot and SDS-PAGE or Western blot, respectively. RepG was detected with CSO-0003 (binds to the C/U-rich loop) and JVO-2134 (binds to RepG 5′ end). 5S rRNA served as loading control (JVO-0485). Whole cell protein fractions (OD_{600} of 0.1 for Coomassie gel or 0.01 for Western blot) were directly stained with Coomassie or chemotaxis receptors TlpA, B, C, and D were detected by a polyclonal rabbit anti-TlpA22 antiserum. (*B*, *Upper*) Schematic illustration of the *tlpB* locus including its promoter region (WT_{tlpB}). The *cagA* promoter region (blue bar) together with a *rpsL-erm* resistance cassette (P_{cagA}) or *rpsL-erm* alone (P_{tlpB}) were inserted upstream of the *tlpB* promoter (red bar). The *tlpB* G-repeat is marked by a gray box. (*B*, *Lower*) *H. pylori* 26695 strains with either the WT *tlpB* locus (WT_{tlpB}) or mutants that carry the *rpsL-erm* cassette insertion (P_{tlpB}, lanes 3 and 4) or express *tlpB* from the *cagA* promoter (P_{cagA}, lanes 5 and 6) in the wild-type (WT) or *repG* deletion (Δ*repG*) background were grown to exponential growth phase and RNA and protein samples were analyzed on Northern and Western blot, respectively. 5S rRNA and CagA protein served as controls. (*C*, *Upper*) The *H. pylori* 26695 *tlpB* promoter region, its 5′ UTR and the first five amino acids of the *tlpB* coding region were fused to *gfpmut3* and inserted into the *rdxA* locus of *H. pylori* G27. (*C*, *Lower*) *H. pylori* G27 WT, Δ*repG,* and Δ*tlpB* mutant strains, as well as WT and Δ*repG* strains which carry either the *tlpB*-5th::*gfpmut3* (lanes 4 and 5) or *cagA*-28th::*gfpmut3* (lanes 6 and 7) fusions were grown to exponential phase and RNA and protein samples were analyzed by Northern and Western blot, respectively. Note that *H. pylori* strain G27 expresses only three chemotaxis receptors, TlpA, B, and D.

to the RepG terminator, is sufficient to act as a regulatory RNA and, thus, could be used for the optimized design of synthetic RNA regulators, which commonly consist of multiple domains (27).

RepG Acts Posttranscriptionally to Repress *tlpB*. To investigate whether RepG regulates *tlpB* expression at the posttranscriptional level, we exchanged the *tlpB* promoter with the unrelated *cagA* promoter of the major effector protein CagA. To replace the endogenous *tlpB* promoter, we inserted the *cagA* promoter in the chromosome upstream of the *tlpB* transcriptional start site (19, 28) together with an *rpsL-erm* resistance cassette (Fig. 2*B*). Western blot analysis for endogenous CagA confirmed that *cagA* expression itself is not affected by RepG. As a control, the resistance cassette alone was inserted upstream of the *tlpB* promoter (P_{tlpB}), and Western blot analysis confirmed that this did not interfere with *tlpB* expression and its regulation by RepG (Fig. 2*B*, lanes 1–2 and 3–4). When *tlpB* was expressed from the *cagA* promoter (P_{cagA}), we observed a two- to threefold reduced TlpB protein level compared with the wild-type (WT_{tlpB}) and control (P_{tlpB}) strains. Nevertheless, a ∼fivefold increase of the TlpB protein level was observed upon deletion of RepG similar as in WT_{tlpB}, indicating that regulation occurs at the posttranscriptional level (Fig. 2*B*, lanes 5–6).

Translational reporter systems based on *gfp* or *lacZ* have been successfully used to study sRNA-mediated regulation and to define mRNA and sRNA interaction sites (29, 30). Therefore, we adapted the use of a GFP-variant, *gfpmut3*, which was previously used in transcriptional reporter plasmids (31), as a translational reporter in the chromosome of *H. pylori* (Fig. 2*C*). To confirm that the *tlpB* 5′ UTR is sufficient for RepG-mediated repression, we fused the coding region for the first five amino acids, the promoter region, and 5′ UTR of *tlpB* from *H. pylori* strain 26695 to *gfpmut3*. Transformation of *gfpmut3* fusions seemed to be toxic for strain 26695 but not for strain G27. Western blot analysis of the TlpB::GFP fusion protein in *H. pylori* G27 showed a ∼eightfold up-regulation upon *repG* deletion (Fig. 2*C*, lanes 4 and 5). A *cagA*::*gfpmut3* (*cagA*-28th::GFP) control fusion was not affected by *repG* deletion (Fig. 2*C*, lanes 6–7), which is in line with what we observed for endogenous CagA (Fig. 2*B*). Together, our in vivo results indicate that the terminator loop of RepG contains the *tlpB* binding site and that RepG represses *tlpB* at the posttranscriptional level by interacting with its 5′ UTR.

In contrast to the repression of the translational *tlpB*-5th::*gfpmut3* fusion from strain 26695 by RepG, we observed that the level of the native TlpB protein of *H. pylori* G27 was decreased about twofold upon *repG* deletion (Fig. 2*C*, lanes 1–2). Because RepG is highly conserved and the *tlpB* 5′ UTRs are overall very similar in both strains but carry different G-repeat lengths, namely 12 and 14 Gs, we reasoned that the G-repeat length could determine strain-specific RepG-mediated *tlpB* regulation, which we examined in more detail below.

RepG and the *tlpB* mRNA Directly Base Pair. To further test for a direct interaction between the C/U-rich region of RepG and the *tlpB* G-repeat, we performed binding studies using in vitro

Fig. 3. In vitro gel-shift and structure probing assays show a direct interaction between RepG sRNA and the *tlpB* leader. (*A*, *Left*) Gel-shift experiments with in vitro synthesized RepG and 26695 *tlpB* mRNA leader (-139 to +78 relative to the annotated start codon). About 0.04 pmol ^{32}P-labeled RNA (RepG* or *tlpB* 5′ UTR*) was incubated with increasing concentrations of unlabeled RNA. Arrows indicate RNA-RNA complex formation. (*A*, *Right*) Predicted 11 bp-long duplex between the C/U-rich loop of RepG and the G-repeat in the *tlpB* 5′ UTR based on structure probing assays. Identified RNase III cleavage sites in the RepG-*tlpB* mRNA duplex are indicated by arrows and nucleotides that are protected from cleavage in in-line probing assays are marked in bold. Note that the interacting nucleotides based on the structure probing results are slightly shifted compared with the predicted interaction in Fig. 1*A*. (*B*) In-line probing of ∼0.2 pmol ^{32}P-labeled RepG in the absence (lane 4) or presence of either 20 nM (lane 5) or 200 nM *tlpB* mRNA leader (lane 6), or 200 nM of *tlpB* leader mutants, ΔG (lacks the G-repeat, lane 7) or 3xC (triple G to C substitutions in the G-stretch, lane 8). Spontaneous cleavages of single-stranded regions were analyzed on a 10% PAA gel under denaturing conditions. Untreated RNA (lane C), partially alkali (lane OH) or RNase T1 (lane T1) digested RepG served as ladders. (*C*, *Left*) In-line probing experiment with ∼0.2 pmol ^{32}P-labeled *tlpB* mRNA leader in the absence or presence of either 20 nM (lane 5) or 200 nM RepG (lane 6), or 200 nM of the RepG mutants, ΔCU (lane 7) or 3xG (lane 8). (*C*, *Right*) In vitro structure mapping of the RepG-*tlpB* mRNA duplex using RNase III cleavage. About 0.1 pmol ^{32}P-labeled *tlpB* mRNA leader was treated with RNase III in the absence or presence of either 100 nM (lane 5) or 1,000 nM (lane 6) RepG or 1,000 nM RepG ΔCU or 3xG mutant RNAs (lanes 7 and 8). RepG-mediated RNase III cleavage sites in the G-repeat and structural rearrangements in the *tlpB* 5′ UTR are indicated by red stars and black bars, respectively.

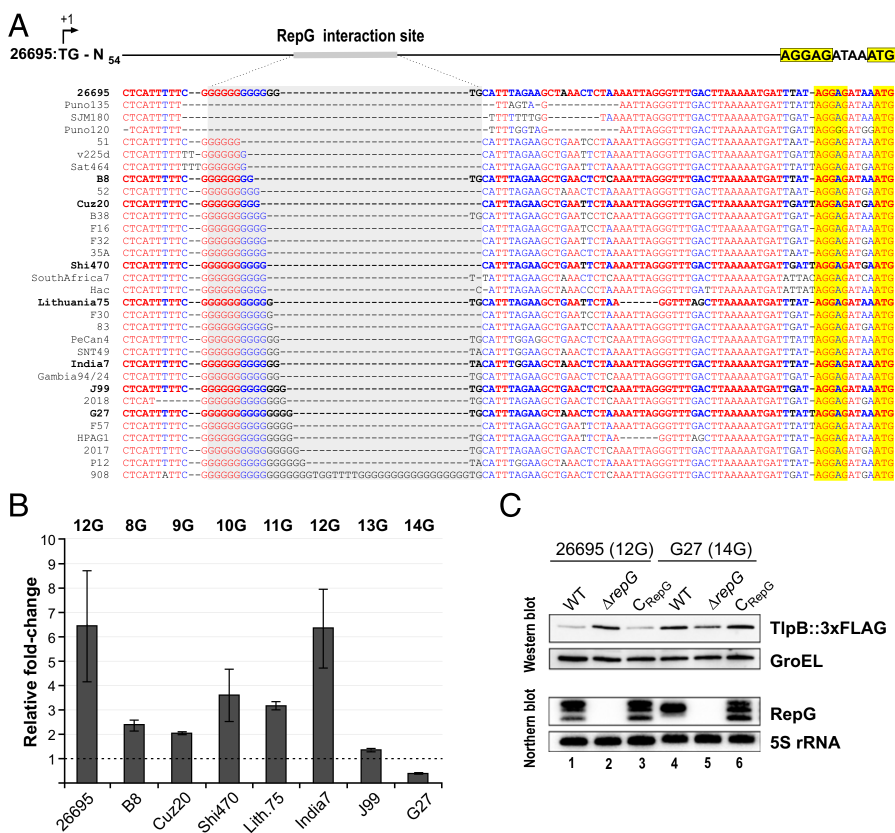

Fig. 4. Variations in the G-repeat length and *tlpB* regulation in diverse *Helicobacter* strains. (*A*) Sequence alignment of the *tlpB* 5′ UTR (+57 to +139 relative to the transcriptional start site) including the RepG binding site (gray), RBS (yellow), and annotated ATG start codon (yellow) from different *H. pylori* strains. Strains that are marked in bold were used for the analysis of *tlpB* regulation shown in *B*. (*B*) Relative fold-changes of *tlpB* expression upon *repG* deletion in diverse *H. pylori* strains were determined by Western blot analysis for TlpB in comparison with the respective wild-type backgrounds (see *SI Appendix*, Fig. S6*B*) and are represented in the bar diagram. Error bars indicate SDs among two or three biological replicates. (*C*) The 26695 and G27 strains comprise *tlpB* mRNA leaders with different G-repeat lengths of 12 and 14Gs, respectively. Protein and RNA samples from strains 26695 and G27 wild-type (WT), *repG* deletion (Δ*repG*), and RepG complementation with RepG from strain 26695 (C_{RepG}), which carried a chromosomally tagged *tlpB*::3xFLAG gene, were harvested at exponential growth phase (OD_{600} of ∼0.9) and analyzed by Western and Northern blot. TlpB::3xFLAG was detected with α-FLAG antibody and GroEL served as loading control. RepG was probed with ^{32}P-labeled CSO-0003 and 5S rRNA with JVO-0485, respectively.

transcribed RNAs. Gel-shift assays with 5′ end-labeled *tlpB* mRNA leader from strain 26695 and increasing amounts of RepG and vice versa revealed a RNA–RNA complex formation at a comparable molecular ratio of 1:15.6 (4 nM:62.5 nM) (Fig. 3*A*). Modifications of either the *tlpB* binding site in RepG (ΔCU, 3xG, 1xG*) or the G-repeat in *tlpB* leader variants, which either lack the G-repeat (ΔG) or contain triple and single nucleotide exchanges in the G-stretch (3xC, 1xC*), abolished RepG–*tlpB* mRNA interaction (*SI Appendix*, Fig. S3). Only gel-shifts with RNA pairs with compensatory base pair exchanges (i.e., RepG 3xG with *tlpB* 3xC or RepG 1xG* with *tlpB* 1xC*) restored the interaction between RepG and *tlpB* mRNA, albeit with lower affinities compared with the respective wild-type versions. Overall, our gel-shifts confirmed a direct interaction between the G-repeat in the *tlpB* 5′ UTR and the C/U-rich terminator loop of RepG in vitro and that mutations in the interaction sites abolish duplex formation.

The C/U-Rich RepG Terminator Loop Region Interacts with the G-Repeat of *tlpB*. To map the sRNA and mRNA interaction in vitro, we performed footprinting assays of in vitro transcribed RepG in the absence or presence of unlabeled *tlpB* leader using in-line probing (32) or enzymatic and chemical cleavages (Fig. 3*B* and *SI Appendix*, Fig. S4*A*). The in-line probing method takes advantage of the fact that single-stranded or unstructured RNA regions undergo spontaneous cleavage of phosphoester linkages faster than structured regions. Thus, besides secondary structure probing, it can be used to monitor ligand binding or RNA–RNA interactions as a reduction in spontaneous RNA degradation events. Cleavage patterns observed in the in-line as well as RNase T1 (cleaves single-stranded G-residues), lead (II)-acetate (cleaves single-stranded nucleotides), and RNase III (cleaves double-stranded RNAs) probing assays agreed with single- and double-stranded regions according to the two biocomputationally predicted stem-loops in RepG (Figs. 1*A* and 3*B*, *SI Appendix*, Figs. S4*A* and S5*A*). The predicted single-stranded 17-nt terminator loop harboring the C/U-rich *tlpB* interaction site showed slight protection against lead (II)-cleavage as well as some RNase III cleavages (*SI Appendix*, Fig. S4*A*), indicating that some of the nucleotides within the C/U-rich loop region might be involved in a tertiary structure. However, upon addition of unlabeled *tlpB* leader, we observed a clear footprint in the RepG terminator loop region in the in-line probing assay, suggesting that the C/U-rich site is indeed involved in the sRNA–mRNA interaction (Fig. 3*B*). In line with the predicted interaction, this protection from spontaneous cleavages within the C/U-rich region was not observed with a *tlpB* mutant RNA that lacks the G-repeat (ΔG) and was only slightly visible upon addition of the 3xC *tlpB* mutant RNA.

In a reciprocal experiment, we mapped the structure of 5′ end-labeled *tlpB* leader in the absence or presence of unlabeled RepG sRNA (Fig. 3*C* and *SI Appendix*, Fig. S4*B*). In combination with secondary structure predictions using RNAstructure (33), our structure probing results indicated a stem-loop structure upstream of the ribosome binding site (RBS) of the 139-nt-long *tlpB* mRNA leader (*SI Appendix*, Fig. S5*B*). Moreover, we observed only minor cleavage events within the G-repeat in the in-line probing assay and a protection against RNase T1 and lead (II)-cleavages as well as two RNase III cleavage sites, indicating a potential intra- or intermolecular structure, which could not be resolved with the applied methods (Fig. 3*C* and *SI Appendix*, Fig. S4*B*). Apart from this potentially structured region, the cleavage patterns in the in-line and lead (II) probing assays indicate a rather flexible structure or multiple conformations of the *tlpB* leader. Upon addition of increasing amounts of RepG, a footprint in the in-line reactions (Fig. 3*C*, *Left*) and RNase III cleavage sites (Fig. 3*C*, *Right*, red asterisks) were observed in the *tlpB* G-repeat, indicating the formation of a RepG–*tlpB* leader

duplex. The footprint as well as RNase III cleavages and several other structural rearrangements in the *tlpB* leader, especially in the stem-loop upstream of the ribosome binding site, were not observed upon addition of ΔCU or 3xG RepG mutant RNAs. In summary, the structure probing results support an interaction between the C/U-rich terminator loop of RepG and the G-repeat in the *tlpB* 5′ UTR (Fig. 3*A*).

Posttranscriptional Regulation of *tlpB* Varies in Different *H. pylori* Strains. Our conservation analysis revealed that RepG and especially its C/U-rich loop are highly conserved among different *H. pylori* strains (Fig. 1 and *SI Appendix*, Fig. S1). In contrast, despite high conservation of the *tlpB* promoter, RBS, and start codon (*SI Appendix*, Fig. S6*A*), the G-repeat in the *tlpB* leader varies among diverse *H. pylori* strains ranging from 6 to 16 guanines (Fig. 4*A*). In strain 26695, in which *tlpB* is repressed by RepG, the G-repeat comprises 12Gs. In contrast, the G-repeat is completely absent in strains SJM180, Puno120, and Puno135, or contains a duplication of two sets of 17Gs separated by TGGTTTT in strain 908. Because the G-repeat is located in a 5′ UTR, its variation could neither lead to frame-shift mutations nor affect transcription of *tlpB*.

Based on the observed RepG-mediated regulation of *tlpB* expression, we predicted that variations in the G-repeat length could influence posttranscriptional regulation of *tlpB* by RepG. Therefore, we deleted *repG* in strains B8, Cuz20, Shi470, Lithuania75, India7, J99, and G27, which comprise G-repeat lengths from 8 to 14Gs. Western blot analysis showed that, although the basal protein levels of the four chemotaxis receptors vary slightly among the different *H. pylori* strains, only the TlpB level was affected by *repG* deletion (Fig. 4*B* and *SI Appendix*, Fig. S6*B*). We detected a ∼two- to tenfold increase in TlpB protein level in the Δ*repG* mutants of B8, Cuz20, Shi470, Lithuania75, and India7, which harbor homopolymeric repeats of 8–12Gs. However, the TlpB protein level was not significantly affected in a Δ*repG* mutant of strain J99 with a 13G-long repeat.

In contrast to *tlpB* repression for strains with 8–12Gs, we observed that longer G-repeat lengths lead to activation of *tlpB* expression by RepG. For example, in strain G27 with a 14G repeat, *tlpB* was about twofold down-regulated upon deletion of *repG*, which we had also noticed for the endogenous TlpB levels in G27 during our GFP–reporter fusion experiments (Fig. 2*C*). Despite the small differences at their 5′ ends, RepG sRNAs from strains 26695 and G27 are very similar (*SI Appendix*, Figs. S1*B* and S2). Complementation of the sRNA deletion mutant in G27 with RepG from strain 26695 restored the TlpB protein level to the wild-type level of G27, indicating that the difference in regulation is not due to sRNA variations but rather due to differences within the G-repeat length (Fig. 4*C*). In line with this finding, RepG from strain G27 represses the *tlpB::gfpmut3* fusion with the 12G *tlpB* 5′ UTR from strain 26695 (Fig. 2*C*). Overall, the differences in RepG regulation in diverse *H. pylori* strains indicate that variations in the G-repeat length could influence sRNA-mediated regulation of *tlpB*.

The Length of the Homopolymeric G-Repeat Determines Posttranscriptional Regulation of *tlpB*. To test whether different G-repeat lengths can lead to the observed strain-specific *tlpB* regulation, we varied the length of the G-repeat in the *tlpB* 5′ UTR of strain 26695, which normally contains 12Gs. We either completely deleted the G-repeat (ΔG) or modulated the G-repeat length of the *tlpB* leader from 6 to 16 guanines (6G to 16G) (Fig. 5*A*). Western blot analysis of TlpB::3xFLAG of these G-repeat variants in the wild-type and the Δ*repG* deletion background revealed that RepG-mediated *tlpB* regulation is dependent on the length of the G-repeat (Fig. 5 *B* and *C*). Whereas a lack of the G-repeat had only a minor influence on TlpB protein levels compared with 26695 wild type (12G), the TlpB protein level was increased in the variant carrying 6Gs. A gradual RepG-dependent decrease in TlpB protein level was observed with an increased number of guanines in the *tlpB* leader in the wild-type background that reaches its minimum in mutants with a 9- to 11-nt-long G-repeat. Further extension of the G-repeat from 12 to 16Gs resulted in an increase in TlpB protein levels. Although deletion of *repG* did not affect *tlpB* expression in the ΔG, 6G, and 13G mutants, an increased TlpB protein level was observed for G-repeat lengths of 7–12Gs, indicating these lengths as an optimal window for RepG-mediated repression. In line with our observations for strain G27, a homopolymeric repeat of 14–16Gs resulted in a slight down-regulation of TlpB levels upon *repG* deletion. Because RepG is expressed at similar levels in all G-repeat mutants, the differences in TlpB expression are likely a result of the variation in the G-repeat length rather than sRNA levels.

To investigate whether the different G-repeat lengths influence the interaction between RepG and the *tlpB* leader, we performed gel-shift assays and in-line probing experiments with RepG and different *tlpB* mRNA leader variants (Fig. 5 *D* and *E*). Gel-shift assays showed that RepG efficiently base pairs with *tlpB* leaders with a repeat of 9–14Gs, with the strongest affinity for variants with 10–13Gs. In contrast, shorter (ΔG, 6–8Gs) or longer G-repeats (>14Gs) abolished or reduced the interaction between RepG and the *tlpB* leader. Reciprocal experiments with selected labeled *tlpB* G-repeats variants (10, 12, 13, and 14G) and increasing concentrations of RepG confirmed that the different *tlpB* leaders bind RepG with different affinities (*SI Appendix*, Figs. S3 and S7). Differences in the footprint strength observed in the terminator loop of RepG upon addition of different G-repeat variants in in-line probing experiments confirmed that the strength of the interaction between both RNAs is influenced by the *tlpB* G-repeat length (Fig. 5*E*). Overall, the pattern of different binding affinities for different G-repeat variants closely correlates with the observed pattern of RepG-mediated *tlpB* regulation in vivo, indicating that the G-repeat length influences the interaction with RepG and in turn posttranscriptional *tlpB* regulation (Fig. 5 *B* and *C*).

RepG Regulates *tlpB* mRNA Translation Depending on the G-Repeat Length. To further study the influence of the G-repeat length on RepG-mediated posttranscriptional control of *tlpB*, we investigated the underlying molecular mechanism. Our preliminary quantitative RT-PCR data indicated that the *tlpB* mRNA is fivefold up-regulated in a Δ*repG* mutant (19). To examine a potential effect of RepG on *tlpB* mRNA stability, we determined the half-life ($t_{1/2}$) of *tlpB* mRNA in *H. pylori* 26695 wild-type, Δ*repG* deletion, and complementation (C_{RepG}) strains (Fig. 6*A*). Rifampicin stability assays showed that *tlpB* mRNA was less stable in the wild-type ($t_{1/2}$ WT ∼2 min) and complementation strains ($t_{1/2}$ C_{RepG} ∼1.5 min) than in the *repG* deletion strain ($t_{1/2}$ Δ*repG* ∼8 min), indicating that RepG reduces *tlpB* mRNA stability.

Because changes in mRNA stability could be due to inhibition of translation by sRNAs, which is often coupled to mRNA degradation in vivo, we examined the influence of RepG on *tlpB* translation in an in vitro translation system. In vitro transcribed mRNAs of *tlpB*::3xFLAG and the translational reporter fusion *tlpB*-5th::*gfpmut3* were translated using reconstituted ribosomes, and protein synthesis was monitored on Western blots (Fig. 6*B*). We observed reduced TlpB::3xFLAG or TlpB::GFP protein levels upon addition of increasing concentrations of wild-type RepG, whereas mutant RNAs had no effect on protein synthesis. Translation of the control mRNA of the *cagA*-28th::*gfpmut3* fusion was not affected by RepG, confirming a specific effect on *tlpB* translation by RepG. Overall, the in vitro translation assays fully recapitulated the observed regulation in vivo (Fig. 2 *A* and *C*) and indicate that RepG regulates *tlpB* expression at the translational level.

Next, we performed in vitro translation reactions with different *tlpB* leader variants (ΔG, 10–14G) in the absence or presence of RepG (Fig. 6*C*). In line with our in vivo results (Fig. 5 *B* and *C*), RepG reduced translation for *tlpB* variants with a G-repeat of

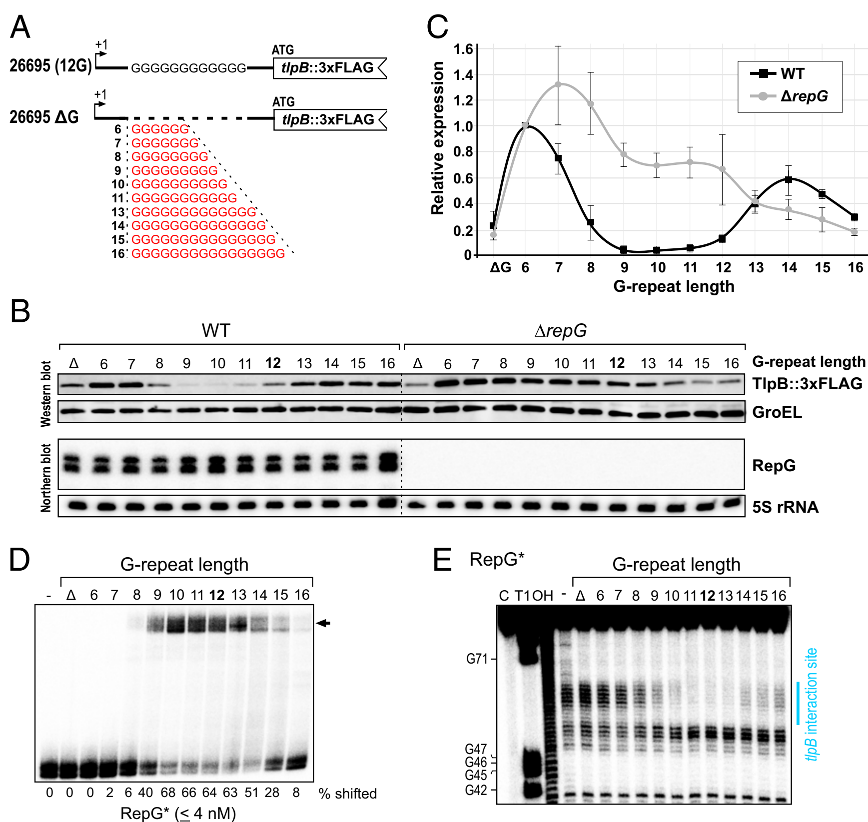

Fig. 5. Variation of the G-repeat length in *H. pylori* 26695 determines *tlpB* regulation by RepG. (*A*) Scheme of *tlpB* mRNA leader mutants, which either lack the homopolymeric G-repeat (ΔG) or comprise diverse G-repeat lengths ranging from 6 to 16 guanine residues (6G to 16G). All mutants were constructed in a *H. pylori* 26695 *tlpB*::3xFLAG strain. (*B*) Western and Northern blot analyses of *tlpB* leader mutants (ΔG, 6G to 16G) in wild-type (WT) or Δ*repG* backgrounds at exponential growth phase. TlpB protein was detected using α-FLAG antibody and GroEL served as loading control. RepG was detected using ^{32}P-labeled CSO-0003 and the loading control 5S rRNA with JVO-0485, respectively. (*C*) Quantification of the relative TlpB protein levels in the different *tlpB* mRNA leader mutants determined by Western blot (*B*) in the WT (black) and Δ*repG* (gray) background. The TlpB protein level in the *tlpB* 6G leader was used as reference and set to 1. (*D*) Gel-shift assay with ~0.04 pmol ^{32}P-labeled RepG* in the absence or presence of 1,000 nM unlabeled *tlpB* leader variants that either lack the homopolymeric G-repeat (ΔG) or comprise different G-repeat lengths (6 to 16G). The arrow indicates RNA-RNA duplex formation and the amount of shifted RepG* for each variant is given in percent. The result of a representative experiment (out of three) is shown. (*E*) In-line probing of ~0.2 pmol ^{32}P- labeled RepG in the absence or presence of 20 nM *tlpB* mRNA leader variants with indicated G-repeat lengths. The footprint in the RepG terminator loop which is observed upon addition of several *tlpB* variants is marked by a blue bar and corresponds to the *tlpB* interaction site.

10–12Gs, had no effect on *tlpB* mRNAs that lack the G-stretch or contain 13Gs, and slightly increased translation of the 14G-long *tlpB* mRNA. Because the RepG binding site is still present in the *tlpB* leader and binding still occurs (although to a lesser extent, Fig. 5 *D* and *E*), longer G-repeats (13–14G) might fold into a structure that affects translation of *tlpB* and thereby lead to the reversal of RepG regulation. Overall, our data indicate that the G-repeat length determines the outcome of RepG-mediated posttranscriptional regulation of the chemotaxis receptor gene *tlpB* and that its activation or repression occurs at the translational level.

Discussion

In our study, we showed that a variable SSR contributes to sRNA-mediated posttranscriptional regulation. We demonstrated that the length of a G-repeat in the leader of *tlpB* mRNA encoding a chemotaxis receptor in *H. pylori* determines *tlpB* repression or activation through the abundant sRNA RepG. This modulation of *tlpB* expression through length variation of a SSR represents an unexpected twist in sRNA-mediated regulation and connects it with gene expression control and phenotypic variation through variable repeats. Such gradual posttranscriptional regulation through a SSR in a 5′ UTR allows for a fine-tuning of gene expression, whereas intragenic SSRs mediate rather digital ON–OFF switches through frame-shift mutations. Promoter associated SSRs (intergenic SSRs) mainly result in strong, moderate or low expression due to changing the spacing of promoter elements or transcription factor binding sites (3). An increase in the number of 7-bp tandem repeats between two promoters has been shown to result in a step-wise decrease in mRNA and protein production of adhesins in *Haemophilus influenzae* and occurs during natural infection in humans (34). Because phase variation by SSRs facilitates host adaptation for a variety of bacterial pathogens (2), the gradual control through SSRs at the transcriptional or posttranscriptional level could be important for the fine-tuning of virulence gene expression. Our work demonstrates the functional characterization of a *trans*-acting sRNA in *Helicobacter* and shows that studying sRNAs in bacteria that lack the common RNA chaperone Hfq can reveal unexpected mechanisms of sRNA-mediated gene regulation.

RepG Binds to a G-Repeat in the *tlpB* 5′ UTR and Affects Translation of *tlpB* mRNA. The majority of sRNAs regulate gene expression by base-pairing close to the RBS or the start codon of their target mRNAs (17). Our in vitro translation assays indicate that RepG influences *tlpB* expression at the translational level (Fig. 6). This translational control could either occur at the level of translation initiation or translation elongation. Because the *tlpB* G-repeat, which is the RepG interaction site, is far upstream of the RBS (Fig. 3), we assume that repression of *tlpB* expression is based on structural changes or binding to a ribosome stand-by site rather than on a direct masking of the RBS. Several sRNAs bind far upstream of the RBS and affect gene expression through the sequestration of translational enhancers or ribosome stand-by sites (35, 36). Moreover, structural rearrangements can lead to inhibition of translation, transcript destabilization, transcription attenuation, or termination (17, 37, 38). Following translational inhibition, interacting RNAs often become substrates for endoribonucleases such as RNase E and RNase III (38). Also the observed repression of *tlpB* translation might be coupled to an increased transcript degradation and thereby lead to the RepG-mediated reduction in *tlpB* mRNA stability observed in vivo.

The Homopolymeric G-Repeat Forms an Inter- or Intramolecular Structure. Our in vitro structure probing indicated that the *tlpB* G-repeat might form an inter- or intramolecular structure. Repetitive guanine-rich DNA and RNA sequences have the ability to form so called G-quadruplex structures (39, 40). In eukaryotes, such G-quadruplexes have been implicated in, for example, DNA maintenance, telomere homeostasis, recombination, or gene expression regulation. Introduction of G-quadruplex-

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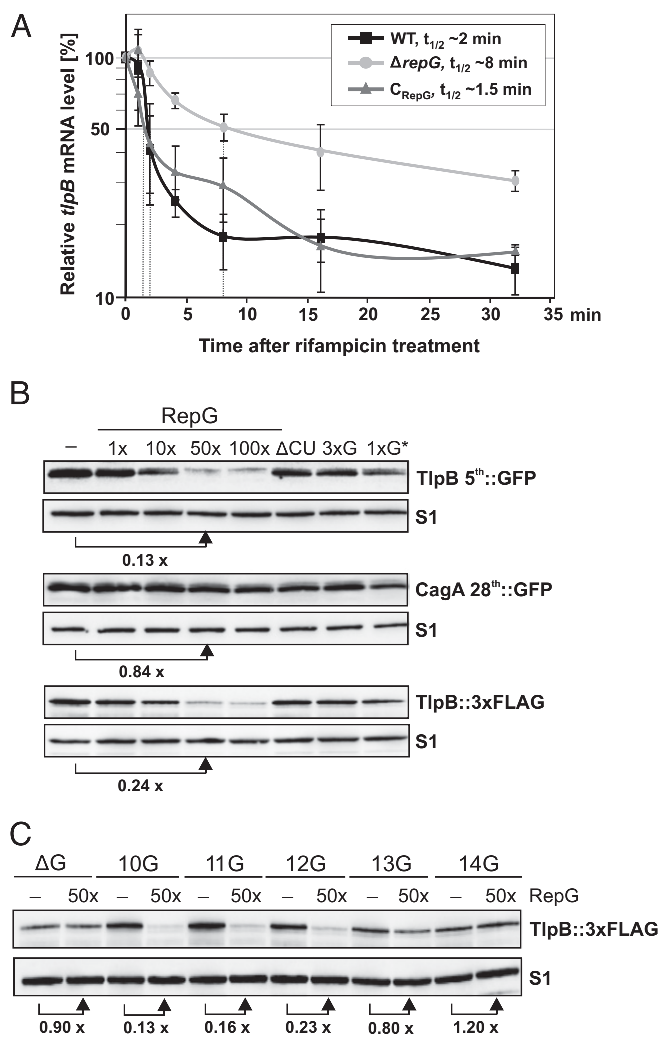

Fig. 6. RepG reduces *tlpB* mRNA stability and regulates translation of *tlpB* mRNA. (*A*) The *tlpB* mRNA half-life at exponential growth phase was determined in *H. pylori* 26695 WT, Δ*repG*, and RepG complementation (C_{RepG}) strains using rifampicin assays and quantitative RT-PCR. The *tlpB* mRNA abundance at 0 min was set to 100% and percentage of *tlpB* mRNA remaining at indicated time points after rifampicin treatment was plotted. The time points at which 50% of *tlpB* mRNA remained (dotted lines) were used to determine the half-lives ($t_{1/2}$) of *tlpB* mRNA in the three strains based on three biological replicates. (*B*) Western blot of TlpB::GFP, TlpB::3xFLAG or CagA::GFP proteins synthesized during in vitro translation assays with 0.1 μM in vitro transcribed *tlpB*-5th::*gfpmut3*, *tlpB*::3xFLAG or *cagA*-28th::*gfpmut3* mRNA in the absence or presence of 0.1, 1, 5, and 10 μM RepG (1- to 100-fold excess). As control, the effect of 10 μM of RepG mutants ΔCU, 3xG or 1xG* on *tlpB* translation was examined in parallel. TlpB::GFP and CagA::GFP were detected using α-GFP antibody and TlpB::3xFLAG with monoclonal α-FLAG antibody, respectively. The ribosomal protein S1 served as loading control. (*C*) In vitro translation assay with 0.1 μM in vitro synthesized mRNAs of FLAG-tagged *tlpB* mRNAs with different leader variants that either lack the homopolymeric G-repeat (ΔG) or comprise a G-repeat length of 10–14Gs in the absence (–) or presence of 50-fold excess (50x) of RepG. For *B* and *C*, a representative Western blot (out of two or three experiments) is shown.

forming sequences close to the RBS in bacterial mRNAs has been shown to affect gene expression in *E. coli* (41). A DNA G-quadruplex in the promoter of the pilin locus in *Neisseria gonorrheae* also has been shown to be required for antigenic variation (42). Interestingly, transcription of a *cis*-encoded antisense RNA that originated within the guanine-rich sequence has been suggested to be crucial for formation of this DNA G-quadruplex and antigenic variation (43). Future studies will be required to determine the exact structure of the G-repeat in the *tlpB* 5′ UTR, its interaction with RepG, and to resolve RepG-mediated structural rearrangements that could be the underlying mechanism of RepG-mediated *tlpB* translational regulation.

RepG and Its Genomic Context Are Highly Conserved in Diverse *H. pylori* Strains. RepG is one of the most conserved small RNAs in *Helicobacter*, particularly its C/U-rich terminator loop, indicating that RepG uses this loop to interact with other mRNAs. Our own unpublished whole transcriptome analyses indicate that multiple genes are affected upon *repG* deletion and potentially targeted at G-rich sequences. Consequently, a mutation in the highly conserved C/U-rich region would abolish the global regulatory function of RepG. In contrast, variation of the *tlpB* G-repeat length and its influence on RepG regulation facilitates uncoupling of a single target from a sRNA regulon through modification of its targeting site.

During our search for RepG homologs, we observed that *repG* in *H. pylori* is always encoded upstream of homologs of the orphan response regulator HP1043 (*SI Appendix*, Fig. S1). Because sRNAs are often encoded next to their transcriptional regulators (44, 45), HP1043 might control *repG* expression. In addition, a positive control of *tlpB* by this regulator was suggested based on in vitro promoter binding studies (28), indicating a potential feed-forward loop of *tlpB* regulation involving HP1043 and RepG. Examination of *repG* expression under various stress conditions or in transcriptional regulator mutants will provide further insights into its own regulation.

Possible Role for Phase Variation of the *Helicobacter* Chemotaxis Receptor TlpB in Virulence. Motility and chemotaxis are important for virulence and efficient colonization by *H. pylori* (20, 46–48). *H. pylori* strain 26695 carries four methyl-accepting chemotaxis receptors, TlpA, TlpB, TlpC, and TlpD. Our study shows that RepG sRNA specifically regulates *tlpB* expression whereas the other chemotaxis receptors are not affected (Fig. 2). TlpB has been shown to sense quorum sensing molecules and pH, whereby acid acts as a repellant (49). Moreover, TlpB has been implicated in colonization and inflammation during mice and gerbil infections (23, 47, 48). We observed that the length of the G-repeat in the *tlpB* 5′ UTR determines the outcome of sRNA-mediated *tlpB* regulation in different *H. pylori* strains (Fig. 4). Analysis of *tlpB* sequences of sequential *H. pylori* isolates from human patients (50–52) and from strains reisolated from animal colonization experiments (46, 53) indicates that the G-repeat not only varies between strains from different patients but also between isolates from the same host, suggesting that *tlpB* can undergo phase variation during infection (*SI Appendix*, Table S1). How differential *tlpB* expression is connected to host adaptation remains to be shown. Because pH-taxis is crucial for the spatial orientation of *H. pylori* along the mucus pH gradient (54), sRNA-mediated regulation and fine-tuning of *tlpB* expression could be important for colonization of different niches within the stomach. It is also possible that the gene downstream of *tlpB*, HP0102, which encodes for a putative glycosyltransferase and is coregulated with *tlpB* in the dicistronic *tlpB*-HP0102 mRNA by RepG (19), is important for host adaptation. In a global transposon screen both genes were identified as candidate loci that contribute to stomach colonization of mice (55). Thus, RepG could have an impact on virulence of *H. pylori*, which needs to be addressed in future studies.

***H. pylori* Exploits Phase Variation for Host Adaptation and Persistent Colonization.** It has been suggested that persistent colonization of the human host by *H. pylori* is facilitated through its extensive genetic diversity due to an elevated mutation rate, impaired DNA repair system, horizontal gene transfer, frequent recombination

events, and phase variation (56, 57). Based on the presence of simple sequence or tandem repeats, around 50 candidate phase-variable genes have been identified in *H. pylori* (25, 53, 58). The products of these phase-variable genes are often involved in surface structures and, in turn, host recognition or adhesion (8, 9, 59–61), motility (13), or in DNA restriction and modification (62, 63). Although the influence of phase variation on transcription and translation has mainly been attributed to length variation of SSRs within promoters or coding regions, we now showed that the length of a G-repeat in an mRNA leader affects expression of a chemotaxis receptor through posttranscriptional regulation by a sRNA. The only other example of a 5′ UTR-associated G-repeat so far has been described for the UspA1 adhesin in *Moraxella catarrhali* and has been shown to influence *uspA1* mRNA levels (64). Moreover, the length of a heteropolymeric tetranucleotide repeat in the leader of *uspA2* mRNA was shown to affect mRNA stability and protein level of this adhesin and thereby contributes to serum resistance in *M. catarrhali* (65). However, in both cases the underlying mechanism remained unclear and it is possible that also these SSRs might be targeted by sRNAs. Apart from posttranscriptional control through SSRs in 5′ UTRs, a phase-variable invertible element in the *cwpV* leader of *Clostridium difficile* has been shown to determine transcription elongation through formation of an intrinsic transcription terminator depending on the orientation of the DNA element (66).

Besides in *Helicobacter*, length variations of poly-G tracts have been observed under selective environmental conditions and passage through animals also in other Epsilonproteobacteria such as the food-borne pathogen *Campylobacter jejuni* (7, 67). These G-repeats could also be potential target sites of sRNAs which have recently been identified in this pathogen (68). Comparisons of homopolymeric SSR locations with our global transcriptional start site maps of *H. pylori* and *C. jejuni* (19, 68) showed that the majority of SSRs are found in promoter or coding regions, but revealed about 10 genes that carry a SSR in their 5′ UTR which might act by influencing posttranscriptional regulation (*SI Appendix*, Table S2). Besides base-pairing with translation initiation regions, bacterial sRNAs can also regulate gene expression by targeting coding sequences (69). Therefore, several of the intragenic SSRs could also be targeted by *trans*-encoded sRNAs and our previous transcriptome study also identified several *cis*-encoded antisense RNAs to SSRs in *H. pylori* (19). Overall, this posttranscriptional mode of gene regulation through homopolymeric repeats is likely to be more widespread and SSRs not only in 5′ UTRs but also within the coding sequence could be targeting sites of sRNAs.

Materials and Methods

Bacterial Strains, Oligonucleotides, and Plasmids. *Helicobacter* and *Escherichia coli* strains used in this study are listed in *SI Appendix*, Table S3. DNA oligodesoxynucleotides used for cloning, T7 transcription template generation, and Northern blot probes are listed in *SI Appendix*, Table S4. Plasmids are summarized in *SI Appendix*, Table S5, and sequences of all RepG and *tlpB* variants in *SI Appendix*, Tables S6 and S8.

Bacterial Growth and Construction of *Helicobacter* Mutants. *E. coli* strains were grown in Luria Bertani (LB) medium supplemented with 100 μg/mL ampicillin and/or 20 μg/mL chloramphenicol if applicable. *H. pylori* media used for growth on plates or in liquid cultures as well as culture conditions are described in *SI Appendix*. Details about the generation of *H. pylori* mutant strains are also listed in *SI Appendix*.

RNA Preparation, Northern Blot Analysis, and Stability Assays. If not mentioned otherwise, *H. pylori* was grown in liquid culture to midexponential growth phase ($OD_{600\ nm}$ 0.5–0.9) and cells corresponding to an OD_{600} of 4 were harvested, mixed with 0.2 volumes stop mix [95% (vol/vol) EtOH/5% (vol/vol) phenol], and immediately shock-frozen in liquid nitrogen. Frozen cell pellets were thawed on ice, centrifuged for 10 min at 3,250 × *g* at 4 °C, and resuspended in TE buffer (pH 8.0) containing 0.5 mg/mL lysozyme and 1% SDS. Cell lysis was completed by incubation at 65 °C for 2 min. RNA was extracted using the hot phenol method as described (19). For Northern blot analysis, 5–10 μg of total RNA was separated on 6% (vol/vol) polyacrylamide (PAA) gels containing 7 M urea and subsequently blotted to Hybond-XL membranes (GE-Healthcare). After blotting, total RNA was UV cross-linked to the membrane and hybridized with 5′ end-labeled (γ^{32}P) DNA oligonucelotides as described (29). Details about rifampicin stability assays and quantitative RT-PCR are listed in *SI Appendix*.

SDS/PAGE and Immunoblotting. For protein analysis, cells corresponding to an OD_{600} of 1 from *H. pylori* cells grown to midexponential growth phase were collected by centrifugation at 16,100 × *g* at 4 °C for 2 min. Cell pellets were dissolved in 100 μL of 1× protein loading buffer [62.5 mM Tris·HCl pH 6.8, 100 mM DTT, 10% (vol/vol) glycerol, 2% (wt/vol) SDS, 0.01% bromophenol blue]. After boiling at 95 °C for 8 min, protein samples corresponding to 0.1 OD_{600} were separated by 12% (vol/vol) one-dimensional SDS-PAA gels and stained by Coomassie (Fermentas, #R0571). For Western blot analysis, protein samples corresponding to an OD_{600} of 0.01 or 0.005 were separated by 12% or 10% (vol/vol) SDS/PAGE and transferred to a PVDF membrane by semidry blotting. Membranes were blocked for 1 h with 10% (wt/vol) milk powder/TBS-T and incubated over night with primary antibody at 4 °C. Afterward, membranes were washed with TBS-T, followed by 1 h incubation with secondary antibody linked to horseradish peroxidase. After additional washing steps, chemiluminescence was detected using ECL-reagent. Details about the used antisera and antibodies are listed in *SI Appendix*.

In Vitro Structure Probing and Gel Mobility Shift Assays. DNA templates that contain the T7 promoter sequence for in vitro transcription using the MEGAscript T7 Kit (Ambion) were generated by PCR. Oligos and DNA templates used to generate the individual T7 templates are listed in *SI Appendix*, Tables S7 and S8. Details about in vitro T7 transcription, structure probing, and footprinting assays, as well as gel-shift experiments and in vitro translation reactions, are listed in *SI Appendix*.

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