



Transcriptome complexity and riboregulation in the human pathogen *Helicobacter pylori*

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The Gram-negative Epsilonproteobacterium *Helicobacter pylori* is considered as one of the major human pathogens and many studies have focused on its virulence mechanisms as well as genomic diversity. In contrast, only very little is known about post-transcriptional regulation and small regulatory RNAs (sRNAs) in this spiral-shaped microaerophilic bacterium. Considering the absence of the common RNA chaperone Hfq, which is a key-player in post-transcriptional regulation in enterobacteria, *H. pylori* was even regarded as an organism without riboregulation. However, analysis of the *H. pylori* primary transcriptome using RNA-seq revealed a very complex transcriptional output from its small genome. Furthermore, the identification of a wealth of sRNAs as well as massive antisense transcription indicates that *H. pylori* uses riboregulation for its gene expression control. The ongoing functional characterization of sRNAs along with the identification of associated RNA binding proteins will help to understand their potential roles in *Helicobacter* virulence and stress response. Moreover, research on riboregulation in *H. pylori* will provide new insights into its virulence mechanisms and will also help to shed light on post-transcriptional regulation in other Epsilonproteobacteria, including widespread and emerging pathogens such as *Campylobacter*.

Keywords: RNA-seq, sRNA, *Helicobacter pylori*, post-transcriptional regulation, transcriptome analysis

INTRODUCTION

For a successful infection of the host or survival in the environment, bacteria have to rapidly adapt their gene expression in response to changing conditions. Besides regulation at the transcriptional level, post-transcriptional regulation is an important layer of gene expression control in both pro- and eukaryotes. Together with several RNA binding proteins which influence RNA structure and stability, the 50- to 400-nt long bacterial small RNAs (sRNAs) act as post-transcriptional regulators under various stress and growth conditions or during virulence (Waters and Storz, 2009; Papanfort and Vogel, 2010; Storz et al., 2011). For example, RyhB and OxyS sRNAs from *E. coli* are involved in iron metabolism and the oxidative stress response, respectively (Altuvia et al., 1997; Masse and Gottesman, 2002).

Although a small number of sRNAs can directly bind and interfere with protein activity, the majority of functionally characterized sRNAs act as antisense RNAs by base-pairing with target mRNAs (Waters and Storz, 2009). *Cis*-encoded sRNAs originate from the opposite DNA strand relative to the target and share fully complementary regions with their target genes. In contrast, *trans*-encoded sRNAs are encoded elsewhere in the genome and interact with their target mRNAs by short and imperfect base-pairing. Most of the *trans*-encoded sRNAs compete with ribosome binding and, thus, lead to translation repression, which is often coupled to mRNA degradation. Furthermore, an increasing number of sRNAs that activate target-gene expression by various mechanisms have been described (Fröhlich and Vogel, 2009). Similar to transcription factors, sRNAs can modulate the expression of multiple

target genes, thereby functioning as key regulators in metabolic pathways or during stress responses (Papanfort and Vogel, 2009; Beisel and Storz, 2010). Besides sRNAs, the RNA chaperone Hfq is a key-player in sRNA-mediated regulation in many bacteria and is required for the stability and activity of sRNAs (Vogel and Luisi, 2011).

A variety of experimental and biocomputational approaches have been developed to identify sRNAs (Altuvia, 2007; Sharma and Vogel, 2009; Backofen and Hess, 2010). Genome-wide computational approaches in particular have predicted hundreds of sRNA candidates in diverse bacteria, which now await experimental validation and functional characterization. Furthermore, massively parallel cDNA-sequencing (RNA-seq) based on next-generation sequencing methods has been revolutionizing transcriptome studies in prokaryotes, including several important pathogens (Wang et al., 2009; Croucher and Thomson, 2010; van Vliet, 2010).

Helicobacter pylori is a Gram-negative, microaerophilic Epsilonproteobacterium which colonizes the stomach of about half of the world's population, where it can lead to peptic ulcer disease, chronic gastritis, or gastric cancer (Cover and Blaser, 2009). Surprisingly, only a few transcriptional regulators have been discovered in the small 1.67 Mbp *H. pylori* genome, including only three sigma factors, namely RpoD, FliA, and RpoN (Tomb et al., 1997). In contrast to enterobacteria, almost nothing is known about post-transcriptional regulation in *Helicobacter*. The systematic identification and subsequent functional characterization of sRNAs will help us to understand the role of

riboregulation in *H. pylori* and provide new insights into its virulence mechanisms.

RNA-seq REVEALS A COMPLEX TRANSCRIPTOME STRUCTURE AND FIRST sRNAs IN *H. pylori*

None of the enterobacterial sRNAs, except for the housekeeping RNAs, tmRNA, SRP RNA, and M1 RNA, are conserved in *H. pylori* (Sharma et al., 2010). Until recently, *Helicobacter* was even regarded as an organism that lacks riboregulation and that carries only basic regulatory circuits, due to its small genome size, a limited number of transcriptional regulators, and the apparent absence of the RNA chaperone Hfq (Mitarai et al., 2007). In addition, based on biocomputational predictions and a small scale cDNA cloning approach only a few natural antisense transcripts and sRNAs were predicted in *Helicobacter* (Livny et al., 2006; Xiao et al., 2009a,b). However, a novel differential RNA sequencing approach (dRNA-seq) selective for the 5' end of primary transcripts allowed for the definition of a global map of transcriptional start sites and operons in *H. pylori* strain 26695, revealing a very complex and compact transcriptome structure (Sharma et al., 2010). Moreover, it led to the discovery of massive antisense transcription as well as an unexpected high number of more than 60 small RNAs including potential regulators of *cis*- and *trans*-encoded mRNA targets, indicating that *H. pylori* uses riboregulation for its gene expression control (Figure 1).

POTENTIAL FUNCTIONS OF sRNAs IN *H. pylori*

Many different *in vitro* and *in vivo* approaches have been established to characterize sRNAs and to validate their targets (Vogel and Wagner, 2007; Sharma and Vogel, 2009). Since sRNAs were discovered only recently in *Helicobacter* our knowledge about their mechanisms, target genes, and their own transcriptional regulators is still limited. The only sRNA that has so far been shown to be essential and required for stress response in *H. pylori* is tmRNA, which is involved in rescue of stalled ribosomes (Thibonnier et al., 2008).

CIS-ENCODED sRNAs

Cis-encoded antisense RNAs can overlap the 5'- or 3'-end, the middle, or entire genes and influence expression of their target genes, e.g., by translation inhibition, transcription interference and attenuation, transcript stabilization, or degradation (Thomason and Storz, 2010). Using dRNA-seq, more than 900 *cis*-encoded antisense RNAs have been identified in *H. pylori*, including at least one antisense transcriptional start site for almost half of all ORFs (Sharma et al., 2010). Similarly, an increasing number of antisense transcripts have been observed in many other prokaryotes (Thomason and Storz, 2010; Georg and Hess, 2011). Whether all of them are functional or rather represent spurious transcription still needs to be clarified. Obviously, these antisense RNAs can in principle harbor a regulatory function, since expression of an artificial

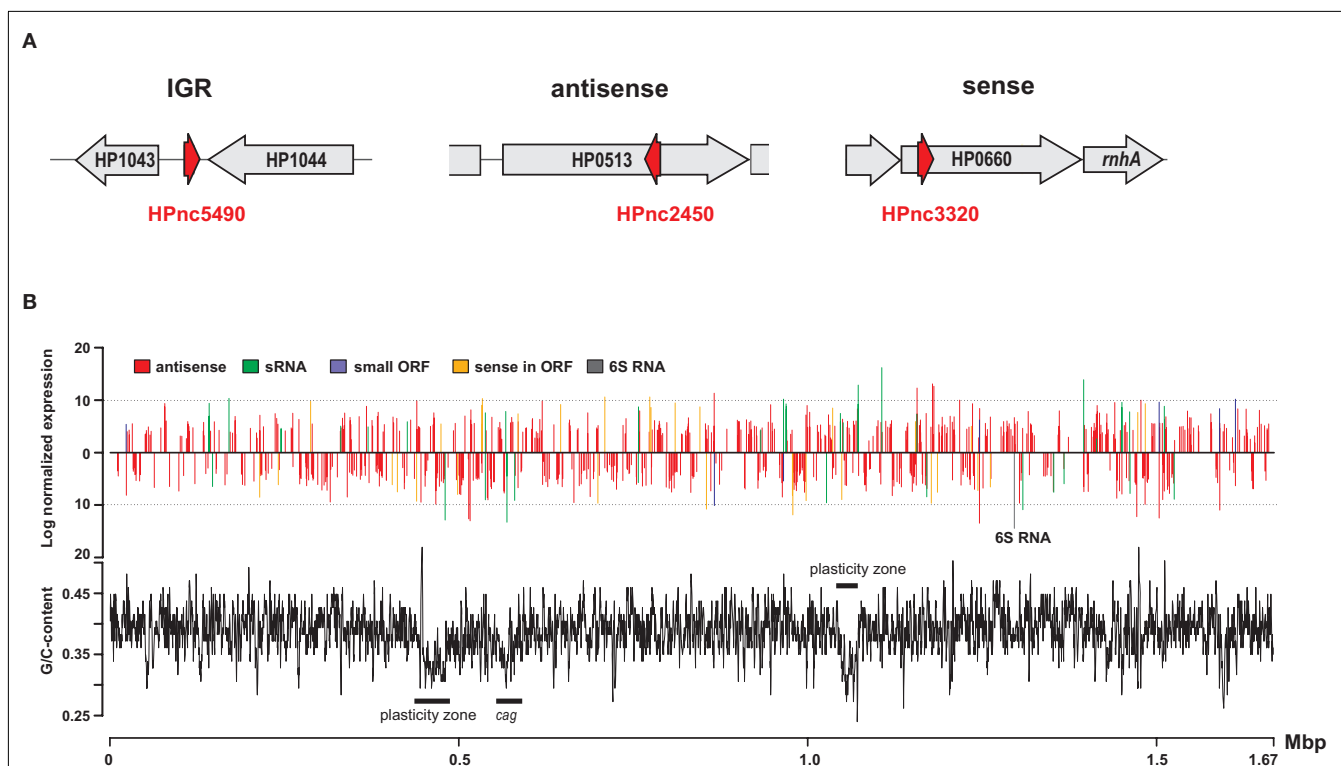


FIGURE 1 | *Helicobacter pylori* sRNA candidates identified by dRNA-seq.

(A) A differential RNA-seq analysis of the primary transcriptome of *H. pylori* strain 26695 revealed hundreds of novel sRNA candidates (denoted as HPnc). These are located either in intergenic regions (left), antisense to ORFs (middle), or sense to ORFs (right). (B) Relative expression of the different

types of sRNA candidates is given as log2 values of read numbers of all sequenced libraries (upper part) and plotted together with local G/C-content (lower part) across the *H. pylori* strain 26695 genome. One of the most abundant sRNAs is the previously missed 6S RNA homolog from the Epsilon subdivision (gray bar). The Figure was adapted from Sharma et al. (2010).

TRANS-ENCODED sRNAs

One of the most abundant transcripts that was identified in the *H. pylori* dRNA-seq study is the previously missed homolog of 6S RNA (**Figure 1B**), a ubiquitous riboregulator, which mimics an open promoter complex and thereby sequesters RNA polymerase (Wassarman and Storz, 2000; Barrick et al., 2005; Sharma et al., 2010). Despite only little sequence conservation to *E. coli* 6S RNA, the 180-nt long RNA from *H. pylori* can fold into the characteristic long hairpin structure of 6S RNA (Trotochaud and Wassarman, 2005). The dRNA-seq data also revealed 12 to 15-nt long product RNAs, which are transcribed using 6S RNA as a template (Wassarman and Saecker, 2006), providing further evidence that this RNA is a functional 6S homolog in *H. pylori*. Deletion of 6S RNA results in no obvious phenotype during exponential growth but altered cell survival during stationary phase and under extreme stress conditions in *E. coli* (Wassarman, 2007). Whether 6S RNA has a role during stress response or stationary phase growth in *H. pylori* or, like in *Legionella* (Faucher et al., 2010), impacts on its virulence still needs to be investigated.

Several of the newly identified sRNAs in *Helicobacter* are potential candidates for *trans*-encoded antisense RNAs. For example, the abundant 87-nt long HPnc5490 sRNA, was predicted to interact by a C/U rich stretch with a G-repeat in the 5' UTR of *tlpB* mRNA encoding for one of the four chemotaxis receptors in *H. pylori* (**Figure 2B**; Sharma et al., 2010). Comparison of *tlpB* expression in the wild-type and a HPnc5490 deletion strain confirmed down-regulation of the *tlpB* mRNA as well as TlpB protein levels by HPnc5490. It has been suggested that TlpB senses protons and diverse studies have demonstrated its potential role in pH-taxis, quorum sensing as well as colonization, and inflammation of the gastric mucosa (McGee et al., 2005; Croxen et al., 2006; Williams et al., 2007; Rader et al., 2011). Therefore, HPnc5490, and probably additional *H. pylori* sRNAs, could play important roles during stress responses or infection, as described for other bacterial pathogens (Papenfert and Vogel, 2010).

PROTEIN FACTORS INVOLVED IN POST-TRANSCRIPTIONAL REGULATION

Besides sRNAs, RNA binding proteins and RNases are involved in post-transcriptional regulation in bacteria (Pichon and Felden, 2007). In *H. pylori*, analysis of the urease operon transcript revealed different mRNA stabilities dependent on the pH value and processing into multiple species, indicating an extensive post-transcriptional regulation of the urease mRNA (Akada et al., 2000). Moreover, it has been shown that a gene of previously unknown function, HP0958, is essential for flagellum biogenesis and encodes for a post-transcriptional regulator of flagellin mRNA by modulating the amount of *flaA* mRNA available for translation (Douillard et al., 2008). Together with the recent identification of sRNAs it is now clear that *H. pylori* uses post-transcriptional regulation to control its gene expression and future identification of the protein factors involved will help to understand the underlying mechanisms in these bacteria.

RNA BINDING PROTEINS

The Sm-like RNA chaperone Hfq is required for the stabilization of sRNAs and facilitates the base-pairing of *trans*-encoded sRNA with

their target mRNAs (Vogel and Luisi, 2011). Deletion of *hfq* leads to pleiotropic phenotypes including reduced fitness and virulence in several bacterial pathogens (Chao and Vogel, 2010). However, as is the case for 50% of all bacterial genomes, *hfq* appears to be absent in Epsilonproteobacteria. Whether a different RNA binding protein replaces the function of Hfq or whether sRNAs act without a chaperone in these bacteria is still an open question.

Besides Hfq, several other RNA binding proteins, such as the carbon storage regulator CsrA/RsmA family or the Crc protein involved in catabolite repression participate in post-transcriptional regulation in bacteria (Sonnleitner et al., 2009; Timmermans and Van Melderen, 2010). The CsrA protein acts as a translation regulator and its own activity is regulated by two sRNAs, CsrB/C, which mimic the RNA substrate of CsrA (Babitzke and Romeo, 2007). The CsrA homolog in *Helicobacter* is required for full motility, survival under oxidative stress, and infection of mice (Barnard et al., 2004). However, neither the target genes of CsrA nor the associated CsrA-regulating sRNAs, CsrB/C, have been identified in *H. pylori*, yet. The recent findings that additional proteins with so far unknown functions or functions unrelated to RNA metabolism, are involved in post-transcriptional control of mRNAs are just one indication that we are far away from knowing all RNA binding proteins and their roles in bacteria (Mitobe et al., 2011; Pandey et al., 2011).

RIBONUCLEASES

Although the exact mechanism of repression is still unclear, both functionally characterized antisense RNAs in *Helicobacter* (**Figure 2**) cause reduced protein levels as well as reduced target-mRNA levels, most likely through transcript destabilization or active recruitment of RNases. In enterobacteria, sRNA-mediated target-mRNA decay mainly depends on the RNA degradosome, a protein complex composed of the endoribonuclease RNase E, polynucleotide phosphorylase PNPase, and the RNA helicase RhlB (Morita et al., 2005; Caron et al., 2010). In *H. pylori*, several RNases are annotated (RNase H, RNase H-II, RNase J, RNase N, RNase P, RNase R, and RNase III) but like all Epsilonproteobacteria it appears to lack a homolog of RNase E. Recent studies on *Bacillus subtilis* and *S. aureus* identified RNase J1 and RNase J2 as the functional orthologs in the RNA degradosome of Gram-positive bacteria (Mathy et al., 2010; Roux et al., 2011). Furthermore, in *S. aureus* the double-strand specific RNase III degrades, mainly in concert with the sRNA RNAIII, several mRNAs encoding virulence factors (Huntzinger et al., 2005; Boisset et al., 2007; Chevalier et al., 2008). Therefore, RNase J and RNase III could be potential RNases participating in sRNA-mediated transcript destabilization in *H. pylori*. Moreover, the 3'-5' exoribonuclease RNase R has been shown to post-transcriptionally down-regulate six virulence related genes (Tsao et al., 2009), but its potential role in sRNA-mediated regulation still needs to be investigated.

TRANSCRIPTIONAL REGULATION OF SMALL RNAs IN *H. pylori*

In enterobacteria, several of the functionally characterized sRNAs have been shown to be key regulators of larger regulatory networks (Papenfert and Vogel, 2009; Beisel and Storz, 2010). Moreover, some sRNAs are controlled by key transcriptional regulators of

certain metabolic pathways and several transcriptional regulators have been shown to be themselves regulated at the post-transcriptional level (Storz et al., 2011). Thus, besides the identification of their target genes, the investigation of transcriptional regulators of sRNAs and antisense RNAs will help provide insights in the conditions in which their expression is required and what are their physiological roles. As the *H. pylori* genome contains only a few transcriptional regulators, it is likely that some of the sRNAs are regulated by one or more of these transcription factors and *vice versa*.

PERSPECTIVE

The discovery of an unexpected wealth of sRNAs in *Helicobacter* opens an additional area of gene expression control in this pathogenic Epsilonproteobacterium. The characterization of selected *cis*- and *trans*-encoded sRNAs will uncover their potential regulatory functions in virulence and stress response of *H. pylori*. Moreover, these studies could reveal novel mechanisms of post-transcriptional regulation independent of the RNA chaperone Hfq. The identification of novel ribonucleoprotein complexes will expand our knowledge about cellular regulators and could provide new targets for antimicrobial therapies against emerging pathogens.

Besides mechanistic aspects of riboregulation, research on sRNAs in *H. pylori* could also help to understand phenotypes observed in previous genetic screens. Global transposon screens failed to hit around 340 genes indicating their potential essentiality and revealed about 220 candidate mutants with a colonization defect in mice (Salama et al., 2004; Baldwin et al., 2007). Since at least 60 sRNAs have been validated in *H. pylori* and considering the high fraction of *cis*-encoded antisense RNAs, several of the virulence associated genes could be subjected to post-transcriptional regulation. For example, the above mentioned first examples of functional sRNAs in *H. pylori* prove post-transcriptional regulation of the virulence factors, urease, and TlpB in *H. pylori* (Sharma et al., 2010; Wen et al., 2010). In addition, a transposon screen for virulence and colonization factors in *H. pylori* revealed several mutants with a transposon hit at the end of genes or in intergenic regions, indicating that there might be additional sRNAs with a role in virulence (Baldwin et al., 2007).

Next-generation sequencing methods have turned out to be an effective tool to uncover the transcriptome structure and identify novel transcripts in prokaryotes. RNA-seq based expression profiling of bacteria grown under different conditions or in comparison to mutant strains will help to identify sRNAs, which are induced under certain stress or virulence conditions. Moreover,

the full understanding of infection processes requires the parallel investigation of gene expression in both, pathogen and host. Microarray-based techniques are limited by the requirement of relatively high amounts of starting material as well as cross-hybridization problems of host and pathogen RNA. RNA-seq methods could overcome those limitations by parallel sequencing of host and pathogen RNA without any prior separation steps followed by mapping of the cDNA reads to the host and pathogen genomes. Furthermore, the investigation of single cell transcriptomes (Tang et al., 2009; Kang et al., 2011) as well as the study of expression profiles of several strains by comparative RNA-seq approaches could lead to new insights into gene expression in bacterial sub-populations or differences between pathogenic and non-pathogenic species.

The study of riboregulation in *H. pylori* will also help to understand post-transcriptional regulation in other Epsilonproteobacteria, such as the emerging pathogen *Campylobacter jejuni*, which is considered as the leading cause of bacterial gastroenteritis (Young et al., 2007). Recently, first sRNA candidates have also been identified in *C. jejuni* NCTC11168 based on conventional RNA-seq combined with predictions of conserved RNA structures (Chaudhuri et al., 2011). A dRNA-seq analysis of the same strain revealed more than 20 candidate sRNAs as well as a complex and compact transcriptional output (Porcelli and van Vliet, personal communication). A comparative dRNA-seq analysis of multiple *C. jejuni* strains indicates that many of the sRNAs are conserved among *C. jejuni* strains and show similar expression profiles (Dugar and Sharma, unpublished). However, some conserved sRNAs show distinct expression patterns in certain strains indicating that they could have varying function. Overall, *Helicobacter* and *Campylobacter* seem to have evolved their own specific sRNA repertoires, but with respect to their smaller genome sizes compared to *E. coli* express a comparable number of sRNAs. Research on sRNAs in *Helicobacter* and *Campylobacter* as well as parallel transcriptome analysis of host and pathogen during the time-course of infection will help to shed light on post-transcriptional regulation and virulence mechanisms not only in Epsilonproteobacteria, but also other bacterial pathogens, including species that lack Hfq.

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