The sibling sRNAs NgncR_162 and NgncR_163 of Neisseria gonorrhoeae participate in the expression control of metabolic, transport and regulatory proteins

Susanne Bauer,† Jonas Helmreich,† Marie Zachary, Marc Kaethner, Elisabeth Heinrichs, Thomas Rudel and Dagmar Beier*

Abstract

Neisseria gonorrhoeae is the causative agent of gonorrhoea, the second most common bacterial sexually transmitted disease. Riboregulation mediated by small regulatory RNAs (sRNAs) is increasingly recognized as an important means of gene expression control in this human-restricted pathogen. sRNAs act at the post-transcriptional level by base-pairing with their target mRNAs which affects translation initiation and/or mRNA stability. In this study we initiated the characterization of a pair of highly conserved sRNAs of N. gonorrhoeae which exhibit redundant functions in the control of a common set of target genes. The identified targets of the sibling sRNAs NgncR_162 and NgncR_163 participate in basic metabolic processes including the methylcitrate and citrate cycle, aa uptake and degradation, and also in transcription regulation. Our data indicate that the sibling sRNAs control their targets via direct base-pairing between the same single-stranded domain(s) of the sRNA and the ribosome binding site in the 5′-untranslated region of the mRNA.

INTRODUCTION

The human-restricted pathogen Neisseria gonorrhoeae is highly adapted to colonization of the urogenital tract thereby causing the sexually transmitted disease gonorrhoea which afflicts more than 100 million individuals per year. Gonorrhoea in men is characterized by acute urethritis and purulent urethral discharge while in women N. gonorrhoeae colonization of the endocervix remains mostly asymptomatic. However, when left untreated, the inflammatory response to ascending infection of the upper genital tract can cause serious sequelae like pelvic inflammatory disease, ectopic pregnancy and infertility. Gonococci may also spread from local infections causing systemic disseminated gonococcal disease with manifestations like endocarditis, arthritis, dermatitis and sepsis [1]. Due to the increasing emergence of antibiotic multi-resistant strains, the role of gonococci in facilitating HIV transmission and hitherto unsuccessful attempts in vaccine development, gonococcal disease has become a major global health concern [2–4].

A prerequisite for successful colonization by a bacterial pathogen is its ability to adapt quickly to fluctuating environmental conditions encountered within the host. A relatively small number of 34 putative DNA-binding transcriptional regulators was found in the N. gonorrhoeae genome (strain FA1090 GenBank AE004969), of which only a few have been studied in some detail. Furthermore, N. gonorrhoeae is endowed with a very restricted repertoire of only three sigma factors and one member of the family of extracytoplasmic function (ecf) sigma factors. Besides the vegetative sigma factor RpoD, N. gonorrhoeae harbours RpoH which controls the expression of at least 12 genes encoding mostly proteins involved in general stress responses [5], and a truncated RpoN, the role of which is still under debate since it lacks the canonical C-terminal DNA-binding helix-turn-helix motif and RpoN-box [6, 7]. The ecf sigma factor of N. gonorrhoeae controls the expression of msrAB, encoding methionine sulfoxide reductase, as well as three other genes encoding proteins of unknown function, presumably in response to oxidative damage [5]. Recent deep sequencing-based transcriptome analyses of N. gonorrhoeae revealed the presence of numerous transcripts which are not derived from coding sequences, but map to intergenic regions or are transcribed antisense to
ORFs [8–10]. Similarly, using both tiling array technology and RNA sequencing, a considerable number of non-coding transcripts was detected in the closely related species Neisseria meningitidis [11–13], indicating that post-transcriptional regulation by small regulatory RNAs (sRNAs) and antisense RNAs (asRNAs) contributes substantially to gene expression control in the pathogenic Neisseria as already described for many other bacterial pathogens [14–16]. Trans-acting sRNAs derived from intergenic regions usually range in size from 50 to 300 nucleotides and affect independently transcribed target mRNAs via short imperfect base-pairing interactions. Most frequently, sRNA binding to the 5′-untranslated region (UTR) impacts the translation initiation by either occluding the RBS of the target mRNA, or by preventing the formation of an inhibitory secondary structure of the mRNA itself which masks the RBS, resulting in repression or activation of translation, respectively. In addition, the mechanisms targeting ribosome binding usually result in mRNA degradation or stabilization [17]. However, mRNA stability can also be affected by sRNAs actively recruiting RNases for transcript degradation, blocking intrinsic RNase cleavage sites or inducing processing by RNases to generate stable transcripts [18]. In Gram-negative bacteria, sRNA function and stability typically depends on the RNA chaperone Hfq [19], which facilitates pairing between the sRNA and mRNA frequently showing only limited complementarity. cis-encoded asRNAs modulate mRNA stability and translation, affect transcription termination or cause transcriptional interference [20].

Hitherto, very few sRNAs of the pathogenic Neisseria have been analysed in detail. Cahoon and Seifert [21] proposed a new function mode of a cis-encoded sRNA which is transcribed with opposite orientation from a promoter located upstream of the pilE expression locus and is required for pilin antigenic variation in gonococci. This sRNA is derived from a region harbouring 12 GC base pairs with the potential to form a guanine quadruplex (G4) structure which is required for the homologous recombination reaction by which silent pilIS copies are inserted into the pilE expression locus [22]. The authors hypothesized that synthesis of the sRNA creating a DNA-RNA duplex at the transcription bubble might liberate the non-template DNA single strand to form the G4 structure, initiating pilin antigenic variation. In N. meningitidis, antigenic variation is additionally modulated by an antisense complementary to the complete pilE coding sequence and 5′-UTR [23]. NrrF is a Fur-regulated sRNA of gonococci and meningococci which is transcribed upon iron depletion and controls the expression of succinate dehydrogenase by destabilization of the sdhCDAB mRNA [24–27]. The NrrF-dependent regulon of N. gonorrhoeae was shown to also include genes involved in DNA metabolism and oxidative stress response, aa biosynthesis, antibiotic resistance as well as several genes encoding proteins of unknown function [27]. Recently it was also reported that NrrF is involved in the iron-dependent expression control of other sRNAs [28]. Furthermore, a sRNA which is induced anaerobically via the transcriptional regulator FNR was described in the pathogenic Neisseria [29, 30] and was shown to negatively regulate the expression of oligopeptidase A and a conserved lipoprotein of unknown function in N. meningitidis [29]. In N. gonorrhoeae, cysteine desulphurase and the YhhF RNA methyltransferase were identified as additional targets of FnrR [31]. Transcription of the sRNA Bns1, which is induced upon culture of N. meningitidis in human blood [11], is under control of a GntR family transcriptional regulator and responds to carbon source availability. Deletion of Bns1 negatively affects the transcription of a metabolic operon which encodes enzymes of the methylcitrate cycle, enabling propionic acid utilization. Interestingly, Bns1 knock-out mutants of N. meningitidis show attenuated virulence in the infant rat model [12]. Recently a pair of sibling sRNAs, also targeting genes from the methylcitrate cycle, was described in N. meningitidis [32, 33]. In this study we initiated the characterization of the gonococcal homologues of these sibling sRNAs.

METHODS

Bacterial strains and growth conditions

The Neisseria gonorrhoeae mutants used in this study were derived from wild-type strain MS11 (GenBank accession number NC_022240.1) and are listed in Table S1 (available in the online Supplementary Material). N. gonorrhoeae was grown on GC agar (Oxoid) plates with 1 % vitamin mix (see Supplementary Material) for 14–16 h at 37 °C in a humidified 5 % CO2 atmosphere. Liquid cultures were grown in PPM medium (proteose peptone #3 (15 g), soluble starch (1 g), KH2PO4 (4 g), K2HPO4 (1 g), NaCl (5 g)/l dH2O) containing 1 % vitamin mix and 0.04 % (w/v) NaHCO3. Media were supplemented with kanamycin or erythromycin at final concentrations of 40 and 7 µg ml−1, respectively, when required. Escherichia coli TOP10 (Thermo Fisher Scientific) and E. coli DH5α [34] were cultured in Luria–Bertani (LB) broth. When required, antibiotics were added to the following final concentrations: ampicillin 100 µg ml−1, kanamycin 30 µg ml−1, chloramphenicol 30 µg ml−1, erythromycin 200 µg ml−1.

Construction of N. gonorrhoeae mutants

Primers used for the amplification of all DNA segments relevant for the construction of N. gonorrhoeae mutants are listed in Table S2. For the synthesis of N. gonorrhoeae-specific fragments, chromosomal DNA of strain MS11 was used as the template and all PCR fragments were checked for proper amplification by DNA sequencing. Clonings were performed in E. coli DH5α. In mutants MS11 Δ162, MS11 Δ163 and MS11 ΔΔ162/163, the sRNA genes Ngncr_162, Ngncr_163 or both sRNA genes were substituted by a kanamycin resistance cassette which was PCR-amplified from transposon EZ-Tn5 <KAN-2> (Epicentre Biotechnologies). PCR fragments for transformation of N. gonorrhoeae MS11 were obtained by recombinant PCR or Gibson assembly (New England Biolabs) and were composed of the kanamycin cassette flanked at the 5′- and 3′-
end by the DNA sequences which on the MS11 chromosome flank the region to be deleted. Flanking regions for the deletion of NgncR_162 comprised 500 and 424 bp, respectively, and were amplified with primer pairs Δ162-1/Δ162-2 and Δ162-3/Δ162-4. The DNA fragment designated for the deletion of NgncR_163 was composed of 475 and 461 bp flanking regions which were amplified with primer pairs Δ163-1/Δ163-2 and Δ163-3/Δ163-4. To generate the double deletion mutant MS11 was transformed with a DNA fragment, comprising the amplification products of primer pairs Δ162-1/Δ162-2 and Δ163-3/Δ163-4. For complementation of MS11 ΔΔ162/163 Sall/Xbal DNA fragments, comprising NgncR_162 or both sRNA genes, were cloned into vector pMR68 [35], thereby replacing the F$_{\text{set}}$ promoter and the tet repressor gene. The resulting plasmids, pMR-162 and pMR-162/163, were individually transformed into MS11 ΔΔ162/163 and erythromycin-resistant transformants resulting from the integration of the sRNA genes in the iga-trpB locus of N. gonorrhoeae were selected. It should be noted that NgncR_163 in pMR-162/163 exhibited a point mutation at position 34 (T to C) which according to RNAfold does not affect the secondary structure of the sRNA. Attempts to clone NgncR_163 separately into pMR68 resulted in the accumulation of nucleotide exchanges in the sRNA sequence or promoter region. To circumvent this problem, a DNA fragment suitable for transformation of N. gonorrhoeae was generated by overlapping PCR performed with DNA subfragments exhibiting wild-type sequence which were PCR-amplified by the use of appropriate primer combinations from pMR-163 template DNAs harbouring mutated inserts.

To analyse the effect of sRNAs NgncR_162 and NgncR_163 on the expression of putative targets the gene for a FACS-optimized variant of green fluorescent protein (gfp-mut2) [36] was fused via a 27 bp linker to the sequence encoding the N-terminus of PrpB (NGFG_0243; 13 aa), PrpC (30 aa) and Ack (25 aa), respectively, and the resulting gfp-fusions were integrated into the chromosome of both N. gonorrhoeae MS11 and MS11 ΔΔ162/163. For this purpose, DNA fragments approximately 500 bp in size comprising the upstream region and 5′-end of the respective gene (amplified with primers prpB-up5/prpB-up3, prpC-up5/prpC-up3 and ack-up5/ack-up3) were combined via recombinant PCR with gfp-mut2 (amplified with primers Lfgp-5/gfp-3(KpnI)) and the resulting DNA fragments were cloned into pSL1180 vector DNA [37] via BamHI/KpnI restriction. The resulting plasmids were transformed into the subsequent ligation of the ermC gene (amplified with primers ermC-5 (KpnI)/ermC-3(PstI) from pMR68) and an approximately 500 bp DNA fragment derived from the downstream region of the putative target genes (amplified with primers prpB-down5/prpB-down3, prpC-down5/prpC-down3 and ack-down5/ack-down3) and then were transformed into N. gonorrhoeae MS11 and MS11 ΔΔ162/163. Erythromycin-resistant transformants were selected yielding mutants MS11 prpB-gfp, MS11 prpB-gfp/ΔΔ, MS11 prpC-gfp, MS11 prpC-gfp/ΔΔ and MS11 Δgfp/ΔΔ in which the respective translational target-gfp fusion replaces the wild-type target gene. To create MS11 gdhR-gfp and MS11 gdhR-gfp/ΔΔ, a DNA fragment comprising the upstream region of gdhR as well as the sequence encoding the first 18 aa was combined with gfp-mut2 via recombinant PCR and the resulting fragment was ligated into pMR68 via SalI/XbaI restriction. The plasmid was then used for transformation of the parental strains MS11 and MS11 ΔΔ162/163 and erythromycin-resistant clones were selected. Similarly, mutants MS11 1721-gfp and MS11 1721-gfp/ΔΔ were constructed by transformation of the respective parental strains with a pMR68-derived plasmid carrying a fusion of the upstream region and 5′-end (encoding aa 1–20) of NGFG_01721 to gfp-mut2. For complementation of MS11 gdhR-gfp/ΔΔ and MS11 1721-gfp/ΔΔ, the respective plasmids were modified by the insertion of an XbaI-fragment comprising sRNA gene NgncR_162.

N. gonorrhoeae strains carrying fusions of the sRNA gene promoters to gfp in the iga-trpB locus were generated via transformation with pMR68-derived plasmids. The respective Sall-XbaI inserts were created via overlapping PCR using DNA fragments derived from the upstream region of NgncR_162 (generated with primer pair C162-5/162gfp-1) or NgncR_163 [generated with primer pair C163-5/163gfp-1 (P$_{\text{gfp}}$-1) or C162-5/163gfp-1 (P$_{\text{gfp}}$-2)] and gfp-mut2 [generated with primer pair 162gfp-2/gfp-3(KpnI) or 163gfp-2/gfp-3(KpnI)]. In these constructs an artificial gfp 5′-UTR comprising an RBS was fused five base pairs downstream of the −10 box of the respective sRNA promoter. To create a knock-out of NGFG_02170, part of the corresponding ORF was replaced by a kanamycin resistance cassette. For this purpose, DNA fragments comprising the upstream region and 3′-part of NGFG_02170 were amplified with primer pairs AS2170c/AS2170d and AS2170g/AS2170h, respectively, and cloned into plasmid pSL1180. Finally, the ermC cassette was inserted between the two fragments via KpnI/PstI restriction.

Transformation of naturally competent N. gonorrhoeae with linear DNA fragments (10 ng) or plasmid DNA (200 ng) harbouring a specific DNA uptake sequence [38] was performed as follows: piliated gonococci were suspended in PPM medium supplemented with 1% vitamin mix, 0.04% NaHCO$_3$ and 10 mM MgCl$_2$. Approximately 10$^8$ bacteria in a volume of 50 µl were carefully mixed with the DNA. The mixture was spotted on a GC agar plate and incubated for 8–18 h at 37 °C and 5% CO$_2$. The bacteria were resuspended in supplemented PPM medium and plated onto GC agar plates containing the appropriate antibiotic for selection of mutants. The kanamycin- or erythromycin-resistant transformants were checked for the desired allelic exchange reaction by PCR with appropriate primers.

**Construction of plasmids for sRNA target validation in E.coli**

For the validation of in silico predicted targets of NgncR_162 and NgncR_163, a GFP-based reporter system
developed by Urban and Vogel [39] was used. For expression of the sRNAs in *E. coli*, plasmid pJV300 [40] was amplified by inverse PCR using primers pJF FW and pJF RV (EcoRI), thereby introducing a EcoRI-site immediately downstream of the P$_{lacO}$ promoter in pJV300. The resulting PCR fragment was cleaved with EcoRI and XbaI and ligated to the sRNA genes NgncR$_{162}$ or NgncR$_{163}$ which were amplified with primer pair 162-1 (EcoRI)/162-2 or 163-1/163-2 yielding plasmid pJV-162 and pJV-163, respectively. Plasmids pJV-162m1 and pJV-162m2, expressing NgncR$_{162}$ derivatives with altered loop 2 (L2) sequences, were constructed similarly. However, in these cases the sRNA-encoding EcoRI/XbaI fragments were synthesized by annealing overlapping PCR fragments amplified from pJV-162 with primers introducing the desired mutations, filling in and amplification of the combined fragment with primer pair 162-1 (EcoRI)/162-2. Overlapping fragments were amplified with primer pairs pJF FWseq/162mut1 and 162mut2/pJF RVseq in the case of pJV-162m1, and primer pairs pJF FWseq/162mut3 and 162mut4/pJF RVseq in the case of pJV-162m2. Primers 162mut1 and 162mut2 thereby introduce an alteration of the 9 nucleotide L2 sequence from TTCTCCTTT to TTCCAAGCT, while primers 162mut3 and 162mut4 create the L2 sequence TACTCCTCC. pJV-162m3 expresses a NgncR$_{162}$ derivative where the single-stranded region between stem-loop 1 and stem-loop 2 (SSR1) was changed from TTCTCCTTT to TTCTCCTTT. Primers 162mut1 and 162mut4 creates the 9 nucleotide L2 sequence from TTCTCCTTT to TTCCAAGCT. 

**Immunoblot analysis**

For the analysis of GFP expression in *E. coli* bacteria were grown to an OD$_{600}$ of 1.0 in LB broth. Bacteria from a culture volume of 2 ml were pelleted, resuspended in 200 µl of Laemmli buffer and incubated for 5 min at 95°C. *N. gonorrhoeae* were harvested from GC agar plates and resuspended in PBS to an OD$_{550}$ of 1.0. Bacteria from 1 ml cell suspension were pelleted, resuspended in 50 µl of Laemmli buffer and incubated for 7 min at 95°C. For Western blot analysis, equal volumes of the samples were loaded on 12% SDS polyacrylamide gels. Proteins separated by SDS-PAGE were transferred onto nitrocellulose membrane (Amersham) using a semidyblotting chamber. After blocking for 1 h in 5% skim milk solution (137 mM NaCl, 20 mM Tris-HCl, pH 7.6, 0.05% Tween-20), the membranes were cut into two pieces: the upper part of the membrane covering proteins with a molecular weight higher than 50 kDa was hybridized with an anti-Hsp60 antibody (Santa Cruz Biotechnology) serving as the loading control, while the lower part was hybridized with an anti-GFP antibody (Santa Cruz Biotechnology). Primary antibody staining in 1:1000 dilutions was performed overnight. The membranes were washed and incubated with the secondary antibody (1:3000 dilution) conjugated to horseradish peroxidase for 1 h. Signal detection was performed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). Quantification of immunoblot images was performed using ImageJ [42].

**RNA preparation, Northern blot analysis and quantitative real-time PCR**

RNA of *N. gonorrhoeae* grown on GC plates or in liquid culture was prepared using the miRNeasy Micro Kit (Qiagen) according to the manufacturer’s instructions. Northern blot analysis was performed as described previously [9]. For quantitative real-time (qRT)-PCR experiments 1 µg of RNAse-free DNase-treated RNA was reverse transcribed with random hexamer primers using RevertAid first strand cDNA Synthesis Kit (Thermo Scientific). All qRT-PCR reactions were performed in triplicate in a 20 µl mixture containing cDNA (5 µl of 1:20 dilution), PerfeCTa SYBR Green FastMix containing ROX (Quanta Biosciences) and...
RESULTS

NgncR_162/163 are abundantly expressed during growth of \textit{N. gonorrhoeae} on a rich medium

Recently two paralogous sRNAs termed RcoF2/F1 or NmsR/R which are transcribed from the intergenic region between genes, encoding disulfide bond formation protein B and a Lrp/AsnC family transcriptional regulator, were identified in \textit{N. meningitidis} \cite{32,33}. These sRNAs are encoded at the same genomic location in gonococci (Fig. S1) and could be detected in a deep sequencing-based transcriptome analysis of \textit{N. gonorrhoeae} MS11 \cite{9}. BLAST analysis revealed that the sibling sRNAs are also conserved in commensal \textit{Neisseria} species like \textit{N. lactamica}, \textit{N. polysaccharaee}, \textit{N. cinerea}, \textit{N. flavescens}, \textit{N. subflava}, \textit{N. mucosa}, \textit{N. sicca} and \textit{N. elongata}. According to RNA-seq data the respective gonococcal sRNAs NgncR_162 and NgncR_163 have a length of 88 and 91 nucleotides \cite{9}. They exhibit 78\% sequence identity and, like their meningococcal counterparts, are predicted to fold into a secondary structure consisting of three stem-loops (SL1, SL2, SL3) which are separated by short single-stranded regions (Fig. 1a, b). The corresponding genes which are separated by 111 bp have the same transcriptional orientation and are preceded by promoter Pribnow boxes, exhibiting one mismatch compared to the consensus sequence (NgncR_162: GATAAT; NgncR_163: CATAAT). As observed for most gonococcal promoters \cite{9} no \textit{E. coli} σ70 –35 promoter element is present in the upstream region of NgncR_163, while a consensus –35 element with proper spacing is located upstream of the NgncR_162 -10 box (Fig. S1). In order to analyse the role of the sibling sRNAs in gonococci, either the individual or both sRNA genes were replaced with a kanamycin resistance cassette in \textit{N. gonorrhoeae} MS11. Deletion of the sRNA genes did not affect the proliferation of \textit{N. gonorrhoeae} MS11 under standard culture conditions since wild-type and mutants exhibited similar growth curves in PPM (data not shown). In Northern blot experiments with a hybridization probe with complementarity to both NgncR_162 and NgncR_163 a signal corresponding to RNAs of the expected size could be detected in RNA extracted from wild-type MS11 which was missing in RNA samples from the double deletion mutant (Fig. 2). In addition, RNA molecules with a length of approximately 70 nucleotides, which might represent processing or degradation products of NgncR_162/163, were detected. The hybridization signals obtained with RNA preparations from MS11 Δ162 and MS11 Δ163 indicated that NgncR_163 is approximately threefold more abundant than NgncR_162. Higher abundance of NgncR_163 under standard growth conditions was also detected by deep sequencing \cite{9}. Likewise, it was reported that the NgncR_163 homologue (RcoF1/NmsR\textsubscript{a}) is the predominant sibling sRNA in \textit{N. meningitidis} \cite{32,33}. Heidrich \textit{et al.} \cite{32} detected identical half-lifes of RcoF2/F1 indicating similar stability of the sibling sRNAs. Therefore, we investigated whether differences in sRNA abundance result from differences in promoter strength using reporter gene fusions where the upstream regions of the sibling sRNAs were combined with the gfp-mut2 gene \cite{36} comprising an artificial 5′-UTR. For the analysis of the NgncR_163 promoter, the intergenic region between the sibling sRNA genes (P\textsubscript{163}1) as well as a larger fragment comprising also NgncR_162 and its upstream region (P\textsubscript{163}2) were fused to gfp, in order not to miss the influence of putative regulatory elements present in the NgncR_162 sequence. Surprisingly, we did not observe differences in promoter strength when gfp expression was monitored on mRNA and protein level via qRT-PCR and immunoblot analysis (Fig. S2). Since sRNA genes are frequently encoded adjacent to a transcriptional regulator controlling their expression, we created a knock-out mutant of the Lrp/AsnC family transcriptional regulator NGFG_02170. However, inactivation of NGFG_02170 did not affect the amount of NgncR_162/163 when \textit{N. gonorrhoeae} was cultivated under standard conditions (Fig. 2).

\textbf{In silico prediction of NgncR_162/163 target genes}

The bioinformatics tool TargetRNA (version 2.01) \cite{44} was applied for the prediction of putative targets of the gonococcal sibling sRNAs. The full length sequence of NgncR_162 was matched with the genome of \textit{N. gonorrhoeae} FA 1090 using default settings. Strikingly, from a total of 43 predicted targets with a P-value <0.05 a subset of eight genes exhibited complementarity of the 5′-UTR including the RBS with the loop sequence of SL2 of NgncR_162 (Figs 3 and S3). Since the SL2 sequences of NgncR_162 and NgncR_163 are identical, both sRNAs might inhibit translational initiation at the respective target mRNAs. Thus the genes, \textit{prpB} encoding 2-methylisocitrate lyase, \textit{prpC} encoding methylcitrate synthase, \textit{ack} encoding acetate kinase, NGO1243 (NGFG_02049) encoding 3-hydroxy isobutyrat dehydrogenase, NGO1807 (NGFG_01721) encoding a sodium alanine symporter, NGO1360 (NGFG_01559) encoding the GntR family transcriptional regulator GdhR \cite{45}, NGO1220 (NGFG_02025) encoding a membrane protein of unknown function and NGO0949 (NGFG_00785) encoding proline iminopeptidase, were considered for further analysis. In \textit{N. meningitidis} and \textit{N. gonorrhoeae} \textit{prpB}, \textit{prpC} and \textit{ack} together with genes whose products exhibit methylcitrate dehydratase activity belong to a gene cluster which enables the bacteria to convert propionic acid to pyruvate via the methylcitrate cycle. This gene cluster is not present in the closely related \textit{Neisseria} species \textit{N. lactamica}, \textit{N. polysaccharaee} and \textit{N. cinerea} suggesting its acquisition by horizontal gene transfer \cite{46}. In contrast to \textit{N. gonorrhoeae} FA
1090, the prpB gene of strain MS11 is interrupted by a stop codon at position 97 and, therefore, is annotated as two consecutive ORFs, NGFG_02434 and NGFG_01403. BLAST analysis demonstrated that the prpB gene is interrupted in about one-tenth of N. gonorrhoeae strains represented in the NCBI taxid 485, while the prpB gene is intact in all meningococcal strains (taxid 487).

Deletion of the sibling sRNAs affects the mRNA levels of putative target genes

Since target mRNA levels typically increase due to protection of the translating ribosome when negative post-transcriptional regulation is abolished, we quantified the transcript amounts of the putative sibling sRNA targets in the double deletion mutant MS11 ΔΔ162/163 compared to the wild-type parent strain via qRT-PCR. In fact, in the case of prpB, prpC, ack, gdhR, NGFG_02049 and NGFG_01721 mRNA levels were 2- to 39-fold higher in the double deletion mutant (Fig. 4a, b), suggesting that the sibling sRNAs are indeed post-transcriptional regulators of the genes under investigation. The amounts of NGFG_00785- and NGFG_02025-specific transcripts changed only marginally (less than 1.5-fold; data not shown), therefore, these genes were not analysed further. To confirm that the deregulation of prpB, prpC, ack, gdhR, NGFG_02049 and NGFG_01721 in MS11 ΔΔ162/163 was caused by the deletion of the sibling sRNAs, the double mutant was complemented by integration of both sRNA genes into the unrelated iga-trpB locus. qRT-PCR analysis demonstrated that, as expected, the transcript amounts decreased in the complemented mutant, reaching almost wild-type levels (Fig. 4a, b). To investigate the relative contribution of the individual sRNAs to post-transcriptional regulation of the identified targets the double deletion mutant MS11 ΔΔ162/163 was also complemented with either NgncR_162 or NgncR_163. The amounts of ack- and NGFG_01721-specific transcripts were quantified in wild-type MS11, MS11 ΔΔ162, MS11 Δ163, the double deletion mutant and the three complemented mutants (Fig. 4a). While in the case of both targets a considerable upregulation was observed in the double deletion

Fig. 1. Sequence comparison and predicted secondary structure of the sibling sRNAs NgncR_162 and NgncR_163. (a) Alignment of the primary sequences of NgncR_162 and NgncR_163. Single-stranded region SSR1 and the loop of SL2 are highlighted in bold. The anti-Shine–Dalgarno sequences present in SSR1 and the loop sequence of SL2 are underlined. The alignment was generated using the HUSAR Sequence Analysis Package (http://genius.embnet.dkfz-heidelberg.de). (b) Prediction of the secondary structures of NgncR_162 and NgncR_163 using the RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). sRNA secondary structures were drawn using VARNAv3-93 [63]. Stem-loops SL1–SL3 and SSR1 are indicated.
mutant (ack: 7.5-fold; NGFG_01721: 39.5-fold), transcript levels increased only slightly in both MS11 ΔA162 (ack: 1.8-fold; NGFG_01721: 1.8-fold) and MS11 ΔA163 (ack: 2.1-fold; NGFG_01721: 2.4-fold). In the case of both targets complementation of the double mutant with NgncR_163 resulted in transcript levels close to those of the double complemented mutant MS11 ΔA162/163, whereas mRNA amounts remained modestly elevated after complementation with NgncR_162 (ack: 3.5-fold; NGFG_01721: 6.3-fold). These data indicate that NgncR_162 and NgncR_163 share a common set of targets and that expression of one of the sibling sRNAs is sufficient for extensive downregulation of the target, demonstrating their functional redundancy on these target genes. However, the complementation experiments suggest that the regulatory effect of NgncR_163 is somewhat more pronounced, possibly due to its higher abundance in the cell (Fig. 2).

Recently, target genes of the meningococcal homologues of NgncR_162 and NgncR_163 have been described [32, 33]. Besides prpB and prpC, citric acid cycle genes gltA (encoding citrate synthase), sdhC (encoding succinate dehydrogenase cytochrome b556 subunit), sucC (encoding succinyl-CoA synthetase subunit beta), and fumC (encoding fumarate hydratase) were validated as being post-transcriptionally controlled by the meningococcal sibling sRNAs [33]. Therefore, qRT-PCR experiments were performed to determine the ratios of the respective mRNAs in MS11 ΔA162/163 compared to the wild-type. We observed a twofold increase of fumC, sdhC and sucC mRNAs, as well as a fivefold upregulation of the gltA-specific transcript (Fig. 4c), suggesting that these genes are also under control of NgncR_162 and NgncR_163 in N. gonorrhoeae.

Validation of sibling sRNA/mRNA interactions in E. coli

To validate post-transcriptional regulation of prpB, prpC, ack, gdhR, NGFG_01721 and NGFG_02049 by the sRNA we used the E. coli two-plasmid gfp reporter system developed by Urban and Vogel [39]. The region covering the 5'-UTR and the first 13 to 45 codons of the putative target genes were fused in frame to gfp in a plasmid vector under control of the constitutive PracO promoter. prpC, ack and NGFG_02049 which were assumed to be intercistronic targets [9, 46] were analysed in the operonic gfp fusion vector pXG-305F [41]. The sRNA genes NgncR_162 and NgncR_163 were cloned into a vector derived from plasmid pJV300 [40], allowing their constitutive expression from the PracO promoter. The empty pJV300 plasmid expressing a nonsense sRNA was used as a negative control. In addition, plasmid pJV152 [31], expressing the gonococcal homologue (NgncR_152) of the meningococcal sRNA Bns1 was included in the analysis, since Bns1 was reported to be a positive regulator of the prpB operon [12]. sRNA-expressing plasmids and target-gfp fusion plasmids were transformed pairwise into E. coli and gfp expression in the respective strains was monitored via Western blot analysis. In the presence of sRNA NgncR_162, the amount of GFP was clearly reduced in comparison to E. coli strains harbouring pJV300 in the case of prpB, prpC, ack, NGFG_01721 and NGFG_02049 (Fig. 5a–e, compare lanes 3 and 1), confirming the specific inhibitory interaction between the sRNA and 5'-UTR of the respective mRNAs which was suggested by the in silico analysis. In contrast, NgncR_162 caused no apparent changes in gfp expression in the case of gdhR (data not shown). In accordance with the predicted lack of complementarity between NgncR_152 and the 5'-UTRs of the investigated mRNAs GFP levels were not altered in the presence of the gonococcal Bns1 homologue (Fig. 5a–e, compare lanes 2 and 1).

To confirm the SL2 loop of the sibling sRNAs as the region binding to the target mRNAs, the loop sequence TTCTCCTTT was mutagenized to TTCAAGCT in plasmid pJV300m (Fig. S4). These mutations abolished complementarity to the RBS of the target mRNAs, but did not change the secondary structure of the mRNA according to RNAfold prediction. As expected, post-transcriptional regulation of prpB, prpC, ack, NGFG_01721 and NGFG_02049 was not observed in the presence of pJV162m (Fig. 5a–e, compare lanes 4 and 1). In plasmid pXG-prpBm2 we mutated nucleotides flanking the RBS which are part of the predicted region of complementarity with the SL2 loop (from AAAGGAGA to GGAGGAGT) and introduced the complementary mutations into the sRNA gene present in pJV162m (TTCTCCTTT to TACTCCTCC) (Fig. S4). Compared to pXG-prpB, GFP levels were marginally decreased in the presence of both sibling sRNAs.
increased in *E. coli* harbouring pXG-prpBm2 (Fig. 4a, compare lanes 8 and 1). NgncR_162m2 affected *gfp* expression from pXG-prpB harbouring the wild-type 5'-UTR of *prpB* only moderately (Fig. 5a, compare lanes 5 and 1), and vice versa – there was only a modest decrease in the amount of GFP, when *E. coli* was co-transformed with pXG-prpBm2 and pJV-162 (Fig. 5a, compare lanes 9 and 8). However, when plasmids pXG-prpBm2 and pJV-162m2 harbouring complementary mutations were combined, *gfp* expression decreased to about 30% of the amount observed in the absence of sRNA (Fig. 5a, compare lanes 10 and 8).

Furthermore, we investigated whether SL2 is sufficient to downregulate target gene expression in *E. coli*. For that purpose the SL2 stem was extended by a GC pair to increase its stability and a poly-T sequence was added to its 3'-end in plasmid pJV-162SL2 to ensure transcription termination. SL2 expression strongly downregulated the *prpC-gfp* fusion (Fig. 5c, compare lanes 6 and 1) and an inhibitory effect was also detected in the case of NGFG_01721-*gfp* (Fig. 5b, compare lanes 6 and 1), though, GFP levels were not affected in the case of *prpB*, *ack* and NGFG_02049 (Fig. 5a, compare lanes 7 and 1; Fig. 5d, e, compare lanes 6 and 1). Taken together, these data provide evidence that NgncR_162 targets are controlled via the interaction of the SL2 loop with complementary sequences in the 5'-UTR of the target.

Recently, Pannekoek *et al.* [33] reported that in the case of the meningococcal NgncR_162 homologue NmsR_A also the single-stranded region separating SL1 and SL2 (SSR1) which comprises the SL2 loop sequence motif UUCUCC is employed for target regulation. Therefore, this region was mutated from TTTTCTCC to TTTTGCTA in plasmid pJV-162m3 and the effect of the mutation on expression of the target-*gfp* fusions was analysed. While downregulation of *prpC-gfp* and NGFG_01721-*gfp* in the presence of NgncR_162m3 was almost as efficient as in the presence of the wild-type sRNA (Fig. 5b, c, compare lanes 5 and 1), downregulation of the remaining targets occurred to a lesser extent, when SSR1 was mutated (Fig. 5a, d, e, compare lanes 6 (5a) or 5 (5d, e) and 1). We conclude that – depending on the target – SSR1 contributes to the mRNA/sRNA interaction, but that the SL2 loop predominates in mRNA binding.

Post-transcriptional regulation of target-*gfp* fusions by sRNA NgncR_163 was investigated in the case of *prpB*, *ack* and NGFG_01721 (Fig. 6). Although the SL2 sequences are identical in the sibling sRNAs and the SSR1 regions differ only in two nucleotides adjacent to SL1 (Fig. 1a; note that the UUCUCC motif of SSR1 is conserved in both sRNAs), downregulation of *gfp* expression was much less pronounced than observed with NgncR_162 (Fig. 5). Nevertheless, these results confirm the function of NgncR_163 as a
post-transcriptional regulator of the in silico identified target genes.

**Deletion of the sibling sRNAs causes target deregulation on the protein level**

To prove post-transcriptional regulation by the sibling sRNAs on the protein level, target-gfp fusions were constructed in N. gonorrhoeae MS11 and MS11 ΔΔ162/163. In the case of *prpB*, *prpC* and *ack* the respective genes were replaced by the gfp fusions covering the first 13 to 30 codons of the target gene. It should be noted that due to the stop codon that splits the *prpB* ORF in N. gonorrhoeae MS11, gfp was fused to ORF NGFG_02434. The NGFG_01721-gfp fusion (covering the first 20 codons) under control of the P*NGFG_01721* promoter was integrated into the iga-trpB locus. Despite the fact that the presence of NgncR_162 did not affect gfp expression of the *gdhR*-fusion in *E. coli*, we decided to investigate expression of a translational reporter gene fusion of this putative target in N. gonorrhoeae, since we had observed a minor deregulation of *gdhR* mRNA in the sibling sRNA double deletion mutant (Fig. 4b). The *gdhR*-gfp fusion (covering the first 18 codons) placed under control of the P*gdhR* promoter was also inserted in the iga-trpB locus in order to keep the transcriptional regulator GdhR intact. GFP could not be detected by Western blot analysis in protein lysates from MS11 derived strains harbouring the *prpB*- and *ack*-gfp fusions (Fig. 7a, c). In the absence of NgncR_162 and NgncR_163 GFP-specific signals could be observed which were very weak in the case of MS11 prpB-gfp/ΔΔ and MS11 ack-gfp/
DD (Fig. 7a). Also in the case of the gdhR-gfp fusion, which was expressed in the MS11 background, we observed a marked upregulation of GFP when the sibling sRNAs were deleted (Fig. 7b). To complement sRNA deficiency in the double deletion mutants a copy of NgncR_162 was inserted downstream of the target-gfp fusion to yield strains MS11 1721-gfp/DD c162 and MS11 gdhR-gfp/DD c162. As shown in Fig. 7(b, c), GFP expression returned to wild-type levels in the mutants complemented with a single sRNA gene copy and the control plasmid pJV300 is indicated. In panel (a) relative gfp expression (%) in E. coli TOP10 (pXG-prpBm2, pJV300) compared to E. coli TOP10 (pXG-prpB, pJV300) is also presented. Values represent the means from three independent replicates. Statistical significance was determined by using Student’s t-test analysis. *=P<0.05, **=P<0.01, ***=P<0.001.

**DISCUSSION**

In this work we describe the initial characterization of a pair of paralogous sRNAs of *N. gonorrhoeae* which are conserved in both the pathogenic and commensal members of the genus *Neisseria* and seem to be involved in the control of basic metabolic processes. *In silico* predicted target genes whose post-transcriptional regulation by the sibling sRNAs could be validated both in *E. coli* and in *N. gonorrhoeae* (Figs 5, 6 and 7) include prpB, prpC and ack belonging to the methylocitrinate cycle, 3-hydroxy isobutyrate dehydrogenase (NGFG_02049) involved in the degradation of valine, an aa transporter (NGFG_01721) and the transcriptional regulator GdhR. NGFG_01721 is annotated as an AGCS family alanine or glycine:cation symporter, but might also be involved in the uptake of valine. GdhR belongs to the GntR family comprising transcriptional regulators whose activities are controlled allosterically by the binding of a metabolite [45, 47]. prpB, prpC, ack and the respective NGFG_02049 and NGFG_01721 homologues have also been suggested as targets of the homologous sRNAs of meningococci due to their upregulation in the proteome of a sRNA double deletion mutant (*prpB*, *prpC*, *ack*, NMB1584) [33] or a combined evaluation of *in silico* analysis and RNA co-immunoprecipitation with Hfq (*prpB*, NMV_0194) [32]. However, experimental validation of post-transcriptional regulation of these targets by RcoF2/F1 or NmsR_A was provided only in the case of *prpB* and *prpC* [32, 33]. Moreover, the mRNAs of the citric acid cycle genes sucC, sdhC, fumC and *gltA* were shown to interact with the meningococcal sRNA NmsR_A [33]. We also observed deregulation of these mRNAs in MS11 ΔA162/163 indicating
post-transcriptional regulation by the sibling sRNAs in gonococci as well (Fig. 4c). In fact the 5'UTRs of the respective gonococcal mRNAs exhibit complementarity to the SL2 loop sequence of NgncR_162/163 (Fig. S3) which, however, was not detected by target RNA using standard settings. The sibling sRNAs of both gonococci and meningococci show a strong association with the RNA chaperone Hfq (Heinrichs and Rudel, unpublished) [33]. Hence, a subset of the abovementioned targets was also found to be deregulated in hfq deletion mutants of N. gonorrhoeae MS11 (prpC, ack, NGFG_01721) and N. meningitidis MC58 (prpC, ack, gltA, fumC, NMB1584, NMB0177) [12, 48]. Expression of the sibling sRNAs which is high when gonococci are grown on rich media might respond to nutrient availability and might thereby adjust the activity of central metabolic pathways to carbon source supply. The fact that an allosterically regulated transcription factor is controlled by NgncR_162/163 enlarges the regulatory network of the gonococcal sibling sRNAs. Transcription factors have previously been recognized as being subject to post-transcriptional regulation. Notably, in E.coli Lrp which controls genes involved in aa biosynthesis and catabolism, nutrient transport and pilus synthesis is repressed by three different sRNAs, i.e. MicF, GcvB and DsrA [49–51]. Interestingly, NgncR_163 was found to be upregulated during infection of the lower genital tract of women [52] suggesting an important role for riboregulation in metabolic adaptation during host colonization.

It has been suggested that the prpB gene cluster, enabling the bacteria to use propionic acid as a carbon and energy source, provides a metabolic advantage to N. meningitidis in the oral cavity and nasopharynx of adolescents and adults which is colonized by propionic acid generating bacteria [46]. Of note, three enzymes from this metabolic pathway are controlled by the sibling sRNAs in both meningococci and gonococci. In a considerable number of gonococcal strains, including MS11, prpB is split into two ORFs by a stop codon in the 5' region of the gene, while it is intact in virtually all sequenced strains of N. meningitidis. It is unclear whether 2-methylcitrate lyase activity is retained in the N-terminally truncated protein, but it is tempting to speculate that a prpB allele with a detrimental mutation persisted in certain N. gonorrhoeae strains because propionate utilization is less crucial for colonization of the urogenital tract. Recently, it was reported that a polycistronic mRNA covering prpB, prpC and the upstream and downstream genes NMB0429 and NMB0432 is positively regulated by another meningococcal sRNA named Bns1 which shows complementarity to a region upstream of prpB and overlapping the 3'-end of ORF NMB0429. Bns1 is repressed by the GntR family transcriptional regulator NMB1563 (N. gonorrhoeae homologue: NGFG_02027) under carbon source limitation [12]. A Bns1 homologue, NgncR_152, is also expressed in gonococci [9], however, a regulatory effect of NgncR_152 on mRNA derived from the prpB locus could not be addressed since repeated attempts to delete NgncR_152 in N. gonorrhoeae MS11 failed. Currently it is not clear whether the NMB0429 homologue is co-transcribed with prpB in gonococci. In N. gonorrhoeae MS11, the transcriptional start site of ORF NGFG_02434 was mapped immediately upstream of the start codon by RNA-seq [9], therefore, the region of complementarity with NgncR_162/163 would not be expected to be part of the mRNA. However, the fact that the NGFG_02434-gfp fusion

**Fig. 6.** Analysis of post-transcriptional regulation of selected sibling sRNA targets by NgncR_163 in E. coli. E. coli TOP10 were co-transformed with plasmids expressing either a translational prpB-gfp-fusion (lanes 1–3), translational ack-gfp-fusion (lanes 4–6), or a translational NGFG_01721-gfp-fusion (lanes 7–9) and plasmid pJV-163 expressing sRNA NgncR_163 (lanes 3, 6 and 9). Plasmids pJV300 and pJV-162, expressing a nonsense sRNA (lanes 1, 4 and 7) and NgncR_162 (lanes 2, 5 and 8), respectively, were included as negative and positive controls. Bacteria from 2 ml of liquid culture were harvested at an OD600=1.0 and used for lysis preparation. Immunoblot analysis using a monoclonal antibody against GFP was performed on samples separated on a 12% polyacrylamide-SDS gel. Hsp60 was probed as the loading control. The results from a representative experiment are shown. GFP expression (%) relative to E. coli cells harbouring the respective gfp-fusion plasmid and the control plasmid pJV300 is indicated. Values represent the means from three independent replicates. Statistical significance was determined by using Student’s t-test analysis. *P<0.05, **P<0.005.
was upregulated in MS11 ΔΔ162/163 (Fig. 7) clearly demonstrates that another transcript must be generated with a sufficiently long 5′-UTR to cover the sRNA interacting site. As expected, due to the lack of complementarity to the immediate 5′-UTR of the targets of the sibling sRNAs, NgncR_152 did not affect the initiation of translation of the respective mRNAs in *E. coli* (Fig. 5).

The sibling sRNAs engage the same single-stranded domain(s) to control all target mRNAs which is most common among sRNAs [53]. They bind their target mRNAs via the loop region of SL2 which contains an anti-Shine–Dalgarno sequence and probably acts as the seed region for more extensive base-pairing involving nucleotides from the stem and – in the case of *prpB* – also the adjacent single-stranded regions (Fig. 3). Analysis of sRNA-target interactions in *E. coli* demonstrated that mutagenesis of the SL2 loop sequence of NgncR_162 abolished post-transcriptional regulation, underlining its predominant role in target recognition. Specificity of the interaction was confirmed by the observation that mutagenesis of the nucleotides flanking the anti-Shine–Dalgarno sequence of NgncR_162 and the introduction of complementary mutations in the 5′-UTR of *prpB* restored post-transcriptional regulation. In-line probing experiments performed with the *N. meningitidis* sibling sRNAs had also confirmed the sRNA/mRNA interaction via the SL2 loop sequence [32]. Pannekoek *et al.* [33] reported that in addition to the SL2 loop sequence SSR1 of NmsR, the SL2 loop sequence SSR1 of NmsR is crucial in target mRNA recognition. We also detected an influence of SSR1 on the control of a subset of targets, however, the SSR1 sequence was dispensable for sRNA interaction with the NGFG_01721 and *prpC* mRNAs. Accordingly, the expression of SL2 was found to be sufficient for post-transcriptional control of the latter mRNAs. The *Neisseria* sibling sRNAs therefore add to a new class of sRNAs composed of the members of the *Listeria monocytogenes* LhrC family which contain multiple binding sites targeting the same region of the mRNA [54].

Unexpectedly, NgncR_163 showed only a limited inhibitory effect on target gene expression in *E. coli* (Fig. 6). In contrast, the analysis of single sRNA deletion mutants and complementation of MS11 ΔΔ162/163 (Figs 6a and 7b, c) clearly demonstrated functional redundancy of the sibling sRNAs in *N. gonorrhoeae* MS11. Inefficient target regulation by NgncR_163 in *E. coli* might be due to reduced RNA
stability compared to NgncR_162 or less efficient binding to E. coli Hfq which might be indispensable for the regulation of certain target genes. In fact, NGFG_01721 was efficiently downregulated by NgncR_163 as well as SL2 which is unlikely to have retained Hfq binding capability. Since we experienced difficulties in the cloning of NgncR_163 it is also conceivable that replicating E. coli populations accumulate deleterious mutations in pJV-163, resulting in a diminished number of cells harbouring functional sRNA molecules. In this context it should be noted that E. coli harbouring NgncR_162 or NmsR_A exhibit a modest growth retardation and that attempts to clone NmsR_B failed [33].

Sibling sRNAs exhibiting a high degree of sequence relatedness have been identified in an increasing number of bacteria including E. coli, Salmonella enterica, Shigella dysenteriae, Yersinia sp., Vibrio sp., Pseudomonas sp., members of the order Rhizobiales and Streptococcus sp. [55, 56]. The largest number of multicopy sRNAs has been found in L. monocytogenes LO28. The LhrC family comprises seven members, LhrC1-5, Rli22-1 and Rli33-1 which target the same mRNAs encoding cell envelope-associated proteins, but respond to different environmental stimuli [54, 57]. A differential expression allowing for the integration of different input signals in the control of the same regulon has frequently been observed for sibling sRNAs [58–60]. When gonococci are grown under standard conditions on rich media, NgncR_162/163 perform redundant regulatory functions on the target genes investigated in this study (Figs 4a and 7b, c), however, we cannot rule out the possibility that the individual sRNAs control unique sets of target genes via the less well conserved SL1 region. Comparison of target mRNA levels in MS11 ΔA162/163 complemented with the individual sRNA genes suggested a slightly more pronounced contribution of NgncR_163 to target downregulation (Fig. 4a) which is in accordance with the higher abundance of NgncR_163 observed under standard growth conditions (Fig. 2).

Similar differences in the levels of the sibling sRNAs were also observed in N. meningitidis [32, 33]. The reason for the differences in sRNA abundance in gonococci remains unclear for the moment. Fusions of the individual sRNA promoters to the reporter gene gfp revealed no clue to differential transcription (Fig. S2). Differences in stability of the sRNA siblings must be considered, but seem unlikely, since identical half-lives were reported for the meningococcal counterparts [32]. Pannenkoek et al. [33] suggested a connection of sibling sRNA expression to the stringent response due to the upregulation of the sRNAs in a relA deletion mutant of N. meningitidis which was more pronounced in the case of NmsR_A. They reasoned that the promoter of nmsR_A contains a GC-rich discriminator sequence downstream of the −10 box which is a hallmark of promoters being repressed by (p)ppGpp and DskA [61], while the nmsR_B discriminator shows a lower GC content resulting in less restricted transcription of this sRNA gene in the presence of (p)ppGpp. Surprisingly, the effect of relA deletion was observed in meningococci grown on a nutrient-rich medium, although it was reported that (p)ppGpp was not produced in N. gonorrhoeae grown in a rich medium, but merely upon a shift to a minimal medium [62]. The influence of RelA and (p)ppGpp on the transcription of the sibling sRNAs in gonococci is currently addressed, however, it should be noted that the differences in the GC-content of the discriminators of the NgncR_162 and NgncR_163 promoters are less pronounced than in the case of NmsR_A and NmsR_B (Fig. S1). Furthermore, it has to be taken into account that the sibling sRNA genes might be differentially regulated in response to specific stimuli resulting in their non-redundant function under certain environmental conditions. The fact that the influence of the Lrp family regulator NGFG_02170 on the transcription of the sibling sRNAs was not apparent does not necessarily imply that it is not involved in sRNA expression control since the intracellular concentration of a ligand required for the activation of the regulator might not be appropriate in standard culture. The fact that targets involved in basic metabolic processes are controlled by the sibling sRNAs strongly suggests a connection between riboregulation and carbon source availability which will be investigated further.

Funding information
This work was funded by the Deutsche Forschungsgemeinschaft (DFG) grant RU 631/12-1 to T. R.

Acknowledgements
We thank Jörg Vogel for providing plasmids pXG10-SF, pXG30-SF and pJV300 and Maximilian Klepsch for the introduction to VARNAv3-93. Roy Gross is acknowledged for critical reading of the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References


33. Pannekoek Y, Huis In’t Veld RA, Schipper K, Bovenkerk S, Kramer G et al. Neisseria meningitidis uses sibling small regulatory RNAs to switch from cataplerotic to anaplerotic metabolism. MBio 2017;8:e00293-16.


45. Rouquette-Loughlin CE, Zalunci YM, Dhulipala VL, Balthazar JT, Doyle RG et al. Control of gdhR expression in Neisseria gonorrhoeae via autoregulation and a master repressor (MtrR) of a drug efflux pump operon. MBio 2017;8:e00449-17.


Edited by: P. Langford and M. Whiteley

Five reasons to publish your next article with a Microbiology Society journal

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as ‘excellent’ or ‘very good’.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.