In vitro analysis of the interaction between the small RNA SR1 and its primary target ahrC mRNA

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ABSTRACT

Small regulatory RNAs (sRNAs) from bacterial chromosomes became the focus of research over the past five years. However, relatively little is known in terms of structural requirements, kinetics of interaction with their targets and degradation in contrast to well-studied plasmid-encoded antisense RNAs. Here, we present a detailed in vitro analysis of SR1, a sRNA of Bacillus subtilis that is involved in regulation of arginine catabolism by basepairing with its target, ahrC mRNA. The secondary structures of SR1 species of different lengths and of the SR1/ahrC RNA complex were determined and functional segments required for complex formation narrowed down. The initial contact between SR1 and its target was shown to involve the 5’ part of the SR1 terminator stem and a region 100 bp downstream from the ahrC transcriptional start site. Toeprinting studies and secondary structure probing of the ahrC/SR1 complex indicated that SR1 inhibits translation initiation by inducing structural changes downstream from the ahrC RBS. Furthermore, it was demonstrated that Hfq, which binds both SR1 and ahrC RNA was not required to promote ahrC/SR1 complex formation but to enable the translation of ahrC mRNA. The intracellular concentrations of SR1 were calculated under different growth conditions.

INTRODUCTION

Small regulatory RNAs (sRNAs) are expressed in both prokaryotes and eukaryotes, primarily as posttranscriptional regulators. Over the past six years, about 70 sRNAs have been discovered in E. coli, and about 20 of them have been assigned a function. Many of these trans-encoded RNAs are involved in metabolic processes [e.g. Spot42, DsrA, RprA, RyhB, SgrS, GadY, reviewed in (1)] and at least eight sRNAs regulate the expression of membrane proteins [reviewed in (2)]. To date, relatively few systematic searches have been performed in Gram-positive bacteria. Among the recently discovered sRNAs in Gram-positive hosts are RatA from the Bacillus subtilis chromosome (3), which came up in a systematic search (4) together with 12 other sRNAs that proved to be sporulation-controlled, but still await the identification of their targets (5). Furthermore, in addition to the well-studied RNAIII from Staphylococcus aureus (6), 12 novel sRNAs from Staphylococcus aureus pathogenicity islands have been detected (7) as well as three Hfq-binding sRNAs of Listeria monocytogenes with still unknown function (8), and nine novel sRNAs from Listeria monocytogenes within intergenic regions found by in silico-based approaches (9). Additionally, more than 100 potential 6S RNA species have been identified by bioinformatics approaches, and many of them were verified experimentally, among them two 6S RNA species in B. subtilis (10,11). Still, the identification of mRNA targets of the recently discovered sRNAs is a challenging issue, and has been successful only in less than one-third of all cases.

One important hallmark of many trans-encoded regulatory RNAs from E. coli is their ability to bind the Sm-like abundant RNA chaperone Hfq (12). While several sRNAs have been found to require Hfq for their stability, some were shown to need Hfq for efficient complex formation with their target RNA (13,14). For DsrA/rpoS/Hfq, the pathway of complex formation has been investigated by biophysical techniques (15). However, for sRNAs from Gram-positive bacteria, the putative function of Hfq is still elusive. At least in one case, staphylococcal RNAIII/spa interaction, no influence of Hfq has been found (16).

In contrast to the cis-encoded sRNAs from accessory genetic elements like plasmids, phages, transposons that have been studied in detail over the past 25 years [reviewed in (17)], relatively little is known about structural requirements, binding kinetics and mechanisms or degradation pathways of these new trans-encoded regulatory sRNAs. Although complexes between sRNA and mRNA
have been detected in vitro in some instances, only in five cases secondary structures of such complexes predicted by Mfold have been confirmed by experimental secondary structure probing. These include MicF/ompF (18), Spol42/galK (19), RyhB/sodB (20), MicA/ompA (14,21) from Escherichia coli and RNAIII/spa from Staphylococcus aureus (22). So far, the region of initial contact between a trans-encoded sRNA and a target RNA sharing more than one complementary region has not been narrowed down. The mechanism of action has been proposed in some cases, but not always corroborated by a combination of in vivo and in vitro experiments.

The 205-nt untranslated RNA SR1 from the B. subtilis genome was found in our group by a combination of computer predictions and northern blotting (23). Recently, we have shown that SR1 is a bona fide antisense RNA that acts by basepairing with its primary target, ahrC mRNA, the transcriptional activator of the rocABC and rocDEF arginine catabolic operons (24). In vitro translation data and translational reporter gene fusions suggested that SR1 might inhibit ahrC translation at a post-initiation stage. Hfq was shown to be dispensable for the stability of SR1.

Here, we provide a detailed in vitro characterization of SR1 and the SR1/ahrC complex with and without Hfq. We determined the region of initial contact between SR1 and ahrC. Furthermore, a combination of toeprinting and SR1/ahrC complex probing studies demonstrated that SR1 inhibits translation initiation of ahrC mRNA by inducing structural changes between the ahrC 5′-SD sequence and the first complementary region G. In contrast to many Escherichia coli sense/antisense systems, Hfq was shown to be exclusively required for translation of ahrC RNA, but not for promoting the SR1/ahrC interaction. The intracellular concentration of SR1 in B. subtilis was calculated to be 30 nM in log phase and 315 nM in stationary phase in complex TY medium.

MATERIALS AND METHODS
Enzymes and chemicals
Chemicals used were of the highest purity available. Taq DNA polymerase was purchased from Roche or SphaeroQ, Netherlands, respectively, RNA ligase from New England Biolabs and Thermoscript reverse transcriptase and M-MuLV reverse transcriptase from Invitrogen and Fermentas, respectively. Firepol polymerase was purchased from Solis Biodyne, Estonia.

Strains, media and growth conditions
Escherichia coli strains DH10B and ER2566(Δhfq::kan) were used for cloning and for expression of the B. subtilis hfq gene, respectively. Bacillus subtilis strains DB104 (25) and E. coli strains were grown in complex TY medium (26).

In vitro transcription and secondary structure analysis of SR1, ahrC and SR1/ahrC complexes
In vitro transcription and partial digestions of in vitro synthesized, 5′-end-labelled SR1 and ahrC RNA species with ribonucleases T1, T2 and V were carried out as described (26). For the analysis of SR1/ahrC complexes with T1, T2 and V, either SR1 or ahrC were 5′ end-labelled and a 6- to 60-fold excess of the cold complementary RNA was added prior to RNase digestion.

Analysis of RNA–RNA complex formation
Both ahrC RNA and SR1 were synthesized in vitro from PCR-generated template fragments with primer pairs indicated in Table 1 Supplementary Data. SR1/ahrC complex formation studies were performed as described previously (24). Complex formation in the presence of Hfq was assayed in TMN buffer (24) using purified Hfq from B. subtilis.

Purification of B. subtilis Hfq
For the purification of B. subtilis Hfq, the IMPACTTM-CN system from New England Biolabs was used. To prevent the purification of E. coli/B. subtilis Hfq-heterohexamers, E. coli strain ER2566(hfq::kan) was transformed with plasmid pTYB11-BsHfq. (All plasmids used in this study are summarized in Table 1). The resulting strain was grown at 37°C till OD$_{560}$ = 0.7, induced with 0.25 mM IPTG, and grown at 18°C for further 18 h. The fusion protein was purified by affinity chromatography on a chitin column as described by the manufacturer. On-column cleavage was performed with 20 mM Tris-HCl pH 8.0, 500 mM NaCl and 50 mM DTT for 20 h at room temperature. Millipore microcon columns were used to concentrate the eluted Hfq protein and to exchange the buffer for 50 mM Tris-HCl pH 8.0. The purified protein was stored at 4°C.

Construction of plasmids for the in vivo reporter gene test system
For the construction of the three translational fusions, chromosomal DNA from B. subtilis DB104 was used as

| Plasmid         | Description                                                                 | Reference                |
|-----------------|------------------------------------------------------------------------------|--------------------------|
| pTYB11-BsHfq    | pTYB11 vector with B. subtilis hfq gene                                      | P. Valentin-Hansen       |
| pGF-BgaB        | Integration vector for amyE gene, heat-stable                               | 27                       |
|                 | β-galactosidase from B. stearothermophilus without SD for translational fusions, Km$^R$, Ap$^R$ | this study               |
| pGGA4           | pGF-BgaB with nt 1 to 119 of ahrC                                           | this study               |
| pGGA6           | pGF-BgaB with nt 1 to 113 ahrC                                              | this study               |
| pGGA7           | pGF-BgaB with nt 1 to 279 of ahrC but lacking nt 102-112                     | this study               |
| pGGA8           | pGF-BgaB with nt 1 to 119 of ahrC, but 2 nt exchange                         | this study               |

Table 1. Plasmids used in this study
template in three PCR reactions with upstream primer SB979 and the corresponding downstream primers SB980 (pGGA4), SB987 (pGGA6) and SB1065 (pGGA8). All fragments were digested with BamHI and EcoRI and inserted into the BamHI/EcoRI vector pGF-BgaB (27) encoding the promoterless heat-stable β-galactosidase from B. stearothermophilus. For the construction of plasmid pGGA7 carrying an internal deletion of 11 bp (nt 102 to 112) of ahrC, a two-step PCR with outer primers SB979 and SB976 and internal primers SB989 and SB988 was performed on chromosomal DNA as template, the third PCR product obtained with SB979 and SB976 cleaved with BamHI and EcoRI and inserted into the BamHI/EcoRI vector pGF-BgaB.

Toeprinting analysis

The toeprinting assays were carried out using 30S ribosomal subunits, ahrC483 mRNA and tRNA fMet basically according to (28). The 30S ribosomal subunits devoid of initiation factors were prepared from E. coli strain MRE600 essentially as described by Spedding (29). The 5'-32P-labelled ahrC-specific oligonucleotide SB1068 (5'-TAC CGT GGC CTG CGT TAC) complementary to ahrC mRNA was used as a primer for cDNA synthesis in the toeprinting reactions. An aliquot of 0.04 pmol of ahrC mRNA annealed to primer SB1068 was incubated at 37°C without or with 0.4 pmol of 30S subunits and 8 pmol of uncharged tRNAfMet (Sigma) before supplementing with 1 μl M-MuLV-RT (80 units). cDNA synthesis was performed at 37°C. Reactions were stopped after 10 min by adding formamide loading dye. The samples were separated on a denaturing 8% polyacrylamide gel. For the analysis of the effect of sRNAs on 30S complex formation, ahrC483 mRNA and the corresponding sRNA were incubated for 15 min at 37°C before the addition of 30S ribosomes and initiator tRNA. Toeprint efficiency was determined by PhosphorImaging using the Image-quant software package (PC-BAS 2.0).

Preparation of total RNA and northern blotting

Preparation of total RNA and northern blotting were carried out as described previously (23).

RESULTS

Secondary structures of SR1 and truncated SR1 species

So far, only for a few chromosomally encoded regulatory sRNAs, secondary structures have been determined experimentally. Examples include MicF (18), OxyS (30), RNAIII of S. aureus (31), DsrA (32), Spot42 (19), RyhB (20) and MicA (14,21). Since computer-predicted RNA structures often deviate from experimentally determined ones [e.g. RNAIII of pIP501 (33) or RNAI/RNAII of pT181 (34)], we performed limited digestions with structure-specific ribonucleases in vitro to determine the secondary structure of SR1. The wild-type SR1 (205 nt) as well as the 3' truncated species SR1132, the 5' truncated species SR198 and the 5' and 3' truncated species SR178 were 5'-end labelled, gel-purified and treated with RNases T1 (cleaves 3' of unpaired G residues), T2 (unpaired nucleotides with a slight preference for A residues) and V1 (double-stranded or stacked regions). Figure 1A shows an analysis of SR1205 and the truncated species SR1132.

Figure 1. Secondary structures of SR1 species of different lengths. (A) Secondary structure probing of wild-type SR1 (205 nt) and truncated species SR1132 with RNases. Purified, 5’-end-labelled SR1 was subjected to limited cleavage with the RNAses indicated. The digested RNAs were separated on 8% denaturing gels. Autoradiograms are shown. RNase concentrations used were: T1: 10^{-12} U/μl (1:50), T2: 10^{-14} U/μl (1:500), V1: 10^{-12} U/μl (1:10), C, control without RNase treatment, L, alkaline ladder. (B) Proposed secondary structure of SR1. A structure consistent with the cleavage data in Figure 1A and additional experiments (data not shown) is depicted. Major and minor cuts are indicated by symbols (see box). The three main stem-loops SL1, SL2 and SL3 are indicated.
whereas Figure 2B contains the schematic representation of the structure of SR1205 derived from the cleavage data. The experimentally determined structure for wild-type SR1 comprises three main stem-loops: SL1 (nt 1 to 112), SL2 (nt 138 to 154) and the terminator stem-loop SL3 (nt 173 to 203) interrupted by two single-stranded regions SSR1 (nt 113 to 137) and SSR2 (nt 155 to 172). It deviates from the structure predicted with Mfold in the 5' portion: The 5' part was found to be single-stranded between nt 38 and 51, and the double-stranded stem proved to be much longer than predicted and comprises 20 paired nucleotides (nt) interrupted by three internal loops or bulged-out bases, respectively, compared to only 10 paired nt in the predicted structure. For the 3' part, two stem-loops and the terminator stem-loop were predicted by Mfold, whereas the structure probing data support in addition to the terminator stem-loop only the second stem-loop SL2 in the centre of a long single-stranded region.

Structure probing of the 5' 132 nt of SR1 (Figure 1A, right part) showed that this portion of the molecule folded independently and exactly as in the full-length sRNA. The secondary structure for the 3' 98 nt of SR1 contained exactly the terminator stem-loop as in wild-type SR1 (not shown) and the secondary structure for SR178 comprising nt 109 to 186 revealed the single stem-loop SL2 surrounded by single-stranded regions as expected (not shown).

The information on the secondary structures of the truncated derivatives was necessary to assess the data on complex formation between different SR1 species and its target, ahrC mRNA.
Previously, we have shown that SR1 binds to the 3′76 nt of ahrC mRNA (ahrC376, Figure 2C) with an equilibrium dissociation rate constant $K_D$ of 3.21 $\times 10^{-7}$ M (24). Since seven regions of complementarity have been predicted between SR1 and ahrC mRNA [(24) and Figure 2C], we intended to narrow down the segment of SR1 that is required for the initial contact with its target. To this end, SR1 species of different lengths were generated by in vitro transcription with T7 RNA polymerase, 5′ end-labelled, gel-purified and used for binding assays with the ahrC376 RNA. The results are shown in Figure 2A: 3′ truncated species SR1132 and SR1104 comprising only stem-loop SL1 and lacking SL2 and the terminator stem-loop, were not able to form complexes with ahrC mRNA even at 400 nM. In contrast, 5′ truncated species SR1169 comprising only the single-stranded region, SL2 and the 5′ half of the terminator stem-loop, was as efficient in complex formation as SR1186, a species that only lacked the 3′ half of the terminator stem-loop, but otherwise contained the complete wild-type sequences and structures. In accordance with these data, both SR1169 lacking SL3 completely and SR1169, lacking SL1 and SL3, were significantly impaired in the interaction with their target and only at 400 nM ahrC mRNA, a weak complex was observed.

From these results we can conclude that for efficient complex formation between SR1 and ahrC mRNA, SL1 and the 3′ half of SL3 are not required. Furthermore, the opening of the terminator stem-loop SL3 seems to be essential for an efficient interaction and a sequence located in the 5′ half of SL3 proved to be important for the contact between antisense-RNA and target.

To analyse the regions of ahrC required for efficient pairing with SR1, five 5′ labelled ahrC RNA species (shown schematically in Figure 2C) were used in complex formation experiments with SR1186 (Figure 2B). As expected, labelled ahrC376 comprising nt 108 to 483 of ahrC RNA, but lacking the 5′ part and the SD sequence of ahrC mRNA with nt 113 to 483 was used in all cases. Above, the schematic representation of the SR1 species is shown.
ahrC formed a complex with unlabelled SR1\textsubscript{186} with the same $K_D$ as determined previously for the labelled SR1/unlabelled ahrC\textsubscript{376} pair. The same efficiency for complex formation was observed for ahrC\textsubscript{88} containing region G' but lacking the SD sequence. By contrast, labelled ahrC\textsubscript{136} and ahrC\textsubscript{196} comprising the 5' 136 and 196 nt of ahrC mRNA, respectively, including SD sequence and region G', were significantly impaired in complex formation with unlabelled SR1\textsubscript{186}. The complete ahrC\textsubscript{383} mRNA including 5' end, SD and all complementary regions to SR1 formed a weak complex with SR1 only at 400 nM concentration. These results suggest that the SD sequence of ahrC mRNA might be sequestered by intramolecular basepairing and that a factor might be needed to facilitate ribosome binding.

Secondary structure of the SR1/ahrC complex

The results from the binding assays indicate that SR1\textsubscript{78} is sufficient for efficient complex formation with ahrC mRNA and that without opening of the 5' half of the terminator stem-loop no efficient complex can form. To investigate the alterations in the secondary structures of SR1 and ahrC upon pairing, the secondary structure of the SR1\textsubscript{186}/ahrC\textsubscript{376} complex was determined. To ascertain alterations in the SR1 structure, labelled SR1\textsubscript{186} was incubated with a 6- to 60-fold excess of unlabelled ahrC RNA, the complex was allowed to form for 5 min at 37°C, and, subsequently, partially digested with RNases T1, T2 and V1. In parallel, free SR1\textsubscript{186} was treated in the same way. Figure 3A shows the result. As expected, no significant alterations were observed within the 5' 112 nt of SR1 that contain only region A (nt 15 to 19) complementary to ahrC. By contrast, significant alterations in the T1, T2 and V cleavage pattern were observed within the other six complementary regions B, C, D, E, F, and G (Figure 3A, right half). The data are summarized in Figure 3C: Whereas in region B, only one reduced T1 cut was detected at G\textsubscript{113}, drastic alterations were observed in both regions C and G: In C, all 9 nt complementary to ahrC showed reduced T2 cleavages, G\textsubscript{126} and G\textsubscript{127} exhibited reduced T1 cleavage and at U\textsubscript{123} and U\textsubscript{125}, an induction of V1 cleavage was detected indicating that this region became double-stranded upon pairing with ahrC. The same was true for region G, where the cleavage pattern at all positions was altered compared to free SR1: nt 175 to 181 showed a decreased T2 cleavage, among them G\textsubscript{176} and G\textsubscript{181} a reduced T1 cleavage, whereas at U\textsubscript{180} and G\textsubscript{181} new V cuts appeared. Fewer changes were found in regions D, E and F, where G\textsubscript{133} (region D), U\textsubscript{146} and A\textsubscript{147} (region E) and G\textsubscript{156}, U\textsubscript{157} and U\textsubscript{158} (region F) were not single-stranded anymore and, instead, U\textsubscript{132} and U\textsubscript{133} (region D), A\textsubscript{148} and A\textsubscript{149} (region E) as well as U\textsubscript{155} and G\textsubscript{156} (region F) showed induced V cleavages, i.e. became double-stranded.

To further substantiate these results, secondary structure probing was performed with a complex formed between labelled ahrC and a 6- to 60-fold excess of unlabelled SR1. To corroborate our previous hypothesis that SR1 does not inhibit the translation initiation at the ahrC SD sequence, both the complex between ahrC\textsubscript{136} (5' 136 nt of ahrC including SD sequence and region G') and the complex between ahrC\textsubscript{376} (lacking the 5' 112 nt of ahrC including SD, but comprising all regions complementary to SR1) were probed with RNases T1, T2 and V. The results are shown in Figure 3B and are summarized in Figure 3D: In the case of ahrC\textsubscript{376}, induced V cuts were visible in regions E and G. Furthermore, between region D and in region C, T2 cuts were induced which is expected when one strand of a double-stranded region interacts with SR1, and the other half becomes, consequently, single-stranded. The same holds true for the induced T1 cuts in region B and the induced T2 cut in the region upstream of B.

The lower part of Figure 3B presenting the results of SR1/ahrC\textsubscript{136} interaction clearly shows that the ahrC SD sequence itself was not affected upon addition of increasing amounts of unlabelled SR1. Surprisingly, a number of alterations could be observed further downstream from it and upstream of complementary region G'. In particular, prominent V cuts were induced at nt 40, nt 46 to 48, nt 52, nt 56, nt 71 and nt 90, accompanied by induced T2 cuts around nt 56 and 74, 75, 77 and 78 (Figure 3B left and Figure 3D). These data suggest that binding of SR1 causes structural changes in the 5' part of ahrC mRNA between the SD sequence and region G'.

The initial contact between SR1 and ahrC RNA requires complementary region G

As published previously (24), one out of seven regions of complementarity between SR1 and ahrC RNA comprises nt 176 to 181 within the 5' half of the SR1 terminator stem-loop SL3 (designated G) and nt 113 to 118 of ahrC mRNA (designated G'). If these two regions were involved in a first contact between SR1 and ahrC RNA, nucleotide exchanges in either SR1 or ahrC RNA should impair or abolish complex formation, and compensatory mutations should, at least partially, restore binding. To test this hypothesis, three mutated SR1\textsubscript{186} species with either a 10 nt exchange (5'AGCAUGCGCCG to 5'UCGUACGCCG) between nt 176 and 185 denoted SR1\textsubscript{186_G10}, a 6 nt exchange (5'AGCAUG to 5'UCGUAC), denoted SR1\textsubscript{186_G6} or a 2 nt exchange (G\textsubscript{177}C\textsubscript{178} to T\textsubscript{177}T\textsubscript{178}) denoted SR1\textsubscript{186_G2}, were assayed in complex formation with wild-type ahrC\textsubscript{88} comprising nt 109 to 196 of ahrC mRNA (region G'). The 6 and 10 nt exchanges were designed such that the GC/AU content of the region was not altered compared with the wild-type. As shown in Figure 4A, no interaction between these three mutated SR1 species and wild-type ahrC RNA was observed. By contrast, the exchange of only C\textsubscript{178} to G (SR1\textsubscript{186_G1}) did not impede complex formation, suggesting that either G\textsubscript{177} is most important for the initial contact or that substitution of one nucleotide is not sufficient to cause an effect. Interestingly, when ahrC RNA\textsubscript{88_G2}, a derivative of the same length carrying the compensatory mutations to SR1\textsubscript{186_G2} was used, binding could be restored (Figure 4A and B) confirming a specific basepairing interaction between SR1 and ahrC mRNA. When a longer ahrC\textsubscript{376} RNA comprising all seven
complementary regions G’ to A’ was analysed, binding was abolished by the above-mentioned mutations too, and partially restored with the compensatory mutation ahrC376_G2 mRNA (not shown). These data indicate that the complementary region G of SR1 (nt 176 to 181) plays an important role for the recognition of ahrC mRNA.

To investigate the contribution of the other regions of SR1 complementary to ahrC RNA to efficient binding with its target, two SR1186 species carrying 9 nt exchanges each in either region C (nt 119 to 127)—SR1S5—or region E and the first 2 nt of region F (comprising nt 146 to 154)—SR1S6—were analysed for complex formation with ahrC RNA carrying the wild-type or mutated regions (Figure 4C). Complex formation was significantly impaired in both cases: SR1S5 exhibited about 10-fold and SR1S6 about 30-fold decreased efficiency to pair with ahrC RNA. A combined substitution of regions C, E, and F (SR1S7) or a combined exchange of regions C, D, E, and F (SR1S8) resulted in a complete loss of pairing. Figure 4D shows a schematic representation of the four mutated SR1186 species.
These data indicate that, although region G is crucial, regions C, D, E and F contribute to efficient pairing.

An in vivo reporter gene test system confirmed the importance of region G for the interaction between SR1 and ahrC mRNA.

To test the importance of region G (nt 113 to 118 of ahrC-mRNA complementary to nt 176–181 of SR1) for the interaction with SR1 in vivo in B. subtilis, the following three translational ahrC-BgaB fusions were constructed: pGGA6 containing nt 1 to 113 but lacking all but one nt of region G, pGGA4 comprising nt 1 to 119, i.e. the entire region G + one additional nt, and, hence, no other complementary region, and pGGA7 identical to pGGA3 (comprising G, F and E, 24) but lacking nt 102 to 112 upstream of G. All fusions were integrated into the amyE locus of the B. subtilis DB104 chromosome, grown till OD$_{560}$ ~ 5 (maximal expression of SR1) and β-galactosidase activities measured. As shown in Table 2, β-galactosidase activities measured with pGGA4 and pGGA7 were, in both cases, about 30-fold lower than that of the pGGA6-integration strain lacking any complementary region to SR1. Since pGGA4 yielded the same decrease in β-galactosidase activity compared to a construct lacking any complementarity with SR1 as our previous construct pGGA3 that encompassed regions G, E and F, it can be concluded that region G alone is sufficient to inhibit ahrC translation almost completely. The results obtained with pGGA7 and pGGA4 exclude the possibility that the sequences immediately adjacent to region G are involved in the observed decrease of β-galactosidase activity, e.g. by providing a cleavage site for an RNase.
To test whether point mutations in region G' abolish the effect of SR1 on ahrC translation, pGGA8 was constructed carrying the same 2 nt exchange as SR1\textsuperscript{186-2} analysed in the binding assay (Figure 4A), but lacking any sequences downstream from nt 118 (3' end of region G') and integrated into the amyE locus of \textit{B. subtilis}. The \(\beta\)-galactosidase activity measured with pGGA8 was nearly the same as with pGGA6 (Table 2), confirming the \textit{in vitro} result that the 2-nt exchange in region G prevented the interaction between SR1 and ahrC.
Hfq does not promote the interaction between SR1 and ahrC mRNA, but is required for the translation of ahrC mRNA

Many small RNAs from E. coli need Hfq for either stability or their interaction with their targets (see Introduction section). Previously, we have shown that Hfq is neither required for the stabilization of SR1 nor that of ahrC (24). However, in the absence of Hfq, but presence of SR1, the expression of the downstream SR1 targets, rocABC mRNA and rocDEF mRNA, was about 3- and 6-fold, respectively, increased. Therefore, we wanted to investigate, whether Hfq is required for the stabilization of SR1 and ahrC RNA. (A) Analysis of mutations in region G. (B) Schematic representation of SR1 with the mutations introduced into region G. (C) Analysis of mutations in regions C, D, E and F. (D) Schematic representation of the mutated SR1 species. Grey boxes denote the substituted regions.

Table 2. β-Galactosidase activities

| Strain            | 5' ahrC Sequence | β-Galactosidase activity (Miller units) |
|-------------------|------------------|----------------------------------------|
| DB104::pGGA6      | 113 nt (no)      | 251 ± 28                                |
| DB104::pGGA4      | 119 nt (G)       | 7.6 ± 2                                 |
| DB104::pGGA7      | 280 nt (G, F, E, but Ant102-112) | 3.5 ± 1.4                      |
| DB104::pGGA8      | 119 nt (G, but 2 nt exchange) | 240 ± 35                             |
| DB104::pGF-BgaB   | no               | 2.9 ± 0.5                               |
| DB104::pGGA6 (Δhfq::cat) | 113 nt (no) | 1.3 ± 0.5                               |

All values represent averages of at least three independent determinations. Plasmid pGF-BgaB is the empty vector. All plasmids contain ahrC sequences fused in frame to the promotorless, SD less gaba gene encoding the heat-stable β-galactosidase of B. stearothermophilus and were inserted into the amyE locus of the B. subtilis chromosome. β-Galactosidase activities were measured at 55°C. In brackets, the presence of complementary regions to SR1 is denoted.

Although a ternary SR1/ahrC/Hfq complex formed, this complex was not observed at lower ahrC concentrations compared to the binary SR1/ahrC complex, and the amount of this complex did not increase with increasing of 10 μM (amount required to bind 50% SR1), to the mixture of 1.0 nM labelled SR1 and different amounts of unlabelled ahrC mRNA, incubated for 15 min at 37°C and complexes were separated on 6% native PAA gels. Although a ternary SR1/ahrC/Hfq complex formed, this complex was not observed at lower ahrC concentrations compared to the binary SR1/ahrC complex, and the amount of this complex did not increase with increasing
concentrations of unlabelled *ahrC* RNA (Figure 5C). In contrast, upon higher concentrations of unlabelled *ahrC* RNA (≥100 nM), this RNA, apparently, successfully competed with SR1 for Hfq binding, so that the amount of unbound labelled SR1 increased again (Figure 5C).

In summary, all these data clearly prove that the RNA chaperone Hfq does not facilitate the interaction between SR1 and its target *ahrC* mRNA.
To reconcile these observations as well as the lacking effect of Hfq on SR1 stability with the increase of the rocABC and rocDEF mRNA levels in the hfq knockout strain, we tested whether the translation of ahrC is affected by Hfq. For this purpose, the ahrC-Bgab translational fusion pGGA6 was integrated into the amyE locus of DB104Δhfq::cat, and β-galactosidase activity was measured and compared to that determined in the presence of Hfq in DB104. A 250-fold lower β-galactosidase activity was detected in the absence of Hfq, indicating that this RNA chaperone is required for efficient translation of ahrC mRNA in vivo (Table 2).

To substantiate the role of Hfq in promoting translation of ahrC mRNA, the secondary structures of ahrC mRNA and SR1 were probed with RNases T1 and T2 in the presence and absence of Hfq. As shown in Figure 5D, one binding site of Hfq on ahrC mRNA (5′ AAAUA) is located immediately upstream of the SD sequence. The same assay was used to determine the binding site(s) of Hfq on SR1. Here, one binding site around nt 9–13 in the 5′ part of SR1 and a second in the bulge of stem-loop SL1 (nt 43 to 47) were found (gel not shown). The facts that Hfq gel-shifts with wild-type SR1 and SR1104 comprising only the 5′ stem-loop were identical (Figure 5A), support the absence of Hfq-binding sites on SR1 downstream from nt 104.

**SR1 blocks ribosome binding to the ahrC mRNA translation initiation region**

Although the first complementary region between ahrC and SR1 is located 87 nt downstream from the ahrC SD sequence, we performed a toeprinting analysis (28) to examine the effect of SR1 on formation of the translation initiation complex at ahrC mRNA. Figure 6A shows that in the presence of initiator tRNA^{Met}, 30S ribosomal subunits bind to the ahrC translation initiation region and block reverse transcription of a labelled primer, annealed downstream, at the characteristic position +15 (start codon A is +1). This signal provides a measure for the formation of the ternary complex, since it is dependent on both 30S subunits and initiator tRNA^{Met}. Addition of increasing amounts of SR1WT or SR1186 prior to addition of 30S subunits and initiator tRNA^{Met} interfered with ternary complex formation, resulting in a weaker toeprint signal.
(Figure 6A and C). Thereby, the inhibitory activity of SR1\textsubscript{186} was higher than that of SR1\textsubscript{WT} which correlates with its more efficient binding activity to ahrC mRNA (Figure 2). By contrast, both the addition of a noncognate small RNA, SR2 from \textit{B. subtilis} or RyhB from \textit{E. coli}, failed to decrease the toeprint signal on ahrC mRNA (summarized in Figure 6D) indicating that SR1-dependent inhibition of ribosome binding was specific. To support the specificity of the SR1 inhibitory action on ternary complex formation on ahrC mRNA, a control toeprint was performed with SR1 and sodB mRNA (target of RyhB). Since SR1 did not affect ternary complex formation on sodB mRNA (Figure 6B), whereas RyhB did as expected, it can be excluded that the effect of SR1 on ahrC mRNA is simply due to binding to the ribosome. To corroborate the importance of complementary region G for the interaction between SR1 and ahrC mRNA, an additional toeprinting assay was carried out with the G-region mutant SR1\textsubscript{186,G2} compared to SR1\textsubscript{186} (Figure 6C). The autoradiogram and the quantification (Figure 6D) show that this mutant is clearly impaired in blocking the binding of the 30S initiation complex, although it has still some residual activity. This result confirms both the specificity of the SR1/ahrC interaction and substantiates our conclusion from the binding assays (Figures 2 and 4) that G is required for the initial contact between SR1 and ahrC mRNA. In summary, these data demonstrate that binding of SR1 to ahrC mRNA prevents the formation of translation initiation complexes.

The intracellular concentration of SR1 increases about 10-fold in stationary phase

To determine the intracellular concentration of SR1 in \textit{B. subtilis} in logarithmic and stationary growth phase, strain DB104 was grown in complex medium, and samples were withdrawn at OD 2 (log phase) and OD 4.5 (onset of stationary phase). Cell numbers were determined upon plating of appropriate dilutions of the harvested cultures on agar plates. Total RNA was prepared, separated on a denaturing polyacrylamide gel alongside defined amounts of \textit{in vitro} synthesized SR1 and subsequently, subjected to northern blotting (Figure 7). Losses during RNA preparation were calculated using \textit{in vitro} synthesized SR1, lanes 3 and 4, DB104 (sr1::cat) with 6.6 and 33.3 fmol of \textit{in vitro} synthesized, purified SR1 mixed at the beginning of the RNA preparation, lanes 5, two and three parallels of RNA isolated from DB104. An autoradiogram of the northern blot is shown.

For all recently discovered trans-encoded sRNAs the targets of which have been identified, only one or two complementary regions were found. In the majority of cases, these regions covered the 5′ part of the target RNA, mostly including the SD sequence, and the mechanism of action was found to be inhibition or activation of translation initiation. Rather unusually, SR1 and ahrC mRNA contain seven regions of complementarity that comprise the 3′ half of SR1 and the central and 3′ portion of ahrC mRNA (24). This prompted us to determine the secondary structures of SR1 and the ahrC/SR1 complex and to investigate the structural requirements for efficient ahrC/SR1 pairing.

Figure 1B shows that SR1 is composed of one large 5′ stem-loop (SL1) structure with a prominent bulge, a central small stem-loop SL2 and the terminator stem-loop SL3 separated by two single-stranded regions. Six out of seven regions of complementarity to ahrC RNA (B to G) are located in the 3′ 100 nt of SR1. Secondary structure probing of labelled SR1 in complex with increasing concentrations of unlabelled ahrC and vice versa (Figure 3A and B) revealed structural alterations in six of the seven complementary regions. In SR1, all positions in region C and G as well as a few positions in B, D, E and F were affected (summarized in Figure 3C). In ahrC, alterations in regions C, E, F and G as well as additional alterations between regions D and E were found. Interestingly, structural changes over a stretch of ~50 nt were also observed upstream of region G (Figure 3B left), although the ahrC SD sequence (nt 21 to 25) and the start codon remained unaffected indicating that binding of SR1 causes structural changes in the 5′ part of ahrC-mRNA, too.

Whereas for cis-encoded antisense RNAs from plasmids, phages and transposons, a number of studies have been performed to elucidate binding pathways and to determine structural requirements for the two contacting RNA molecules (17), little is known, so far, about the formation of initial contacts between trans-encoded sRNAs and their targets. Here, we show that a solely 78-nt long SR1 species spanning nt 109 to 186 is sufficient for efficient complex formation with ahrC mRNA, i.e. the 5′ portion of SR1 is not needed (Figure 2A). Generally, all SR1 species lacking the 5′ half of SL3 with region G or
comprising a complete SL3 were significantly impaired in pairing with ahrC RNA. This might indicate that in vivo some factor — most likely a protein or an RNAse cutting within the loop of SL3 — opens the terminator stem-loop to promote complex formation. Since in vivo only full-length SR1\textsubscript{205} can be observed (northern blots and 3' RACE, 23), the involvement of an endoribonu- 
clease is highly unlikely. The possibility that the RNA chaperone Hfq that binds upstream of the terminator 
stem-loop of SR1 is responsible for opening up this structure, can be eliminated, too (see below). Most 
probably, another, yet unknown RNA-binding protein is needed to open SL3.

Two lines of evidence show that the initial contact between SR1 and ahrC RNA occurs at complementary 
region G of SR1: complex formation assays of truncated SR1/ahrC pairs containing mutations and compensatory 
mutations in region G (Figure 4) and translational ahrC-
lacZ reporter gene fusions with the same point mutations 
(Table 2). Furthermore, complex formation assays with 
SR1 mutants affected in regions C, D, E/F or a combination thereof and a lacZ fusion with regions E', 
F' and G' revealed a contribution of the other complementary 
regions to SR1/ahrC pairing. In summary, since, 
(i) in the absence of region G, no efficient complex could 
form, (ii) in the presence of wild-type regions A to E, a 
2-nt exchange within G inhibits pairing and (iii) in the presence of G, significant simultaneous alterations in 
regions C, E and F did affect complex formation, we can 
conclude, that region G is responsible for the initial 
contact between SR1 and ahrC RNA, but the other 
complementary regions add to efficient antisense/target 
RNA pairing.

Region G' in unpaired ahrC mRNA is double-stranded 
with a bulged-out G at position 116 (Figure 3B left). Interestingly, only when this G and the neighboured C 
were replaced by a C and G (ahrC\textsubscript{88_G2}, see Figure 4), the interaction with SR\textsubscript{186_G2} was restored indicating that it is 
crucial for the initial contact. As proposed above, some 
factor is needed to melt or open up region G in SR1, so 
that the two regions can interact. Our data suggest that 
pairing initiates at G, but for subsequent steps and stable 
complex formation, a contribution of the other complementary 
regions B to F is needed. This is reminiscent of the binding pathway of the antisense/sense RNA pair 
CopA/CopT involved in regulation of plasmid R1 replication [reviewed in (35)]. Here, binding starts with 
the interaction of two single-stranded kissing loops and, 
afterwards, a second region is needed to overcome the 
torsional stress and to propagate the helix. By contrast, 
for the antisense/sense RNA pair RNAIII/RNAII of 
plasmid pIP501, the simultaneous interaction of two 
complementary loop pairs was found to be required 
(36). In other cases, a single-stranded region and a loop 
form the first complex [e.g. Sok/hok of plasmid R1 or 
RNA-OUT/RNA-IN of transposon IS10, reviewed in (17)].

For many trans-encoded sRNAs in E. coli, the RNA 
chaperone Hfq has been shown to be required for either stabilization of the sRNA or/and efficient duplex 
formation with the target RNA (see the Introduction 
section). Previous experiments have demonstrated that 
Hfq does not stabilize SR1 (24). This report shows that 
although B. subtilis Hfq binds both SR1 and ahrC RNA, it 
is not able to promote complex formation between SR1 
and ahrC (Figure 5). This is in agreement with data 
obtained for the RNAIII/spa interaction in S. aureus, for 
which Hfq was found to be dispensable for RNAIII/spa 
complex formation (22,16). The fact that no requirement 
for Hfq was observed in the RatA/txpA system of 
B. subtilis (3), too, suggests that in Gram-positive bacteria 
Hfq might not be needed for sRNA/target RNA interaction or, alternatively, that another RNA chaperone may 
fulfil the function of Hfq. One candidate might be HBsu, 
for which RNA-binding activity was demonstrated (37).

However, our previous observation that the levels of the 
secondary targets of SR1, rocABC and rocDEF mRNA, 
were increased 3- to 6-fold in an hfq knockout strain (24) 
raised the question on the role of this chaperone in the 
SR1/ahrC system. Suprisingly, ahrC mRNA proved to be 
not translated in a B. subtilis hfq knockout strain 
(Table 2). This indicates that Hfq is required for efficient 
translation of ahrC, possibly by opening up some 
secondary structures that otherwise inhibit binding of 
the 30S initiation complex. This is supported by the 
finding of one Hfq-binding site (5' AAAUA) immediately 
upstream of the ahrC ribosome-binding site (RBS). Interestingly, for E. coli rpoS mRNA it has been also 
shown that Hfq is essential for efficient translation (38). 
In contrast to ahrC, the binding of Hfq to SR1 does not 
seem to play a role in this context. The fact that Hfq binds 
upstream of six out of seven SR1 regions complementary 
to ahrC mRNA supports the failure of Hfq to promote 
complex SR1/ahrC formation. However, we cannot 
exclude that Hfq binding might be important for the 
interaction of SR1 with other, still unidentified target 
mRNAs.

Based on a series of translational ahrC-lacZ fusions, the 
dispensability of the ahrC SD sequence for pairing with 
SR1 and in vitro translation data with chimeric ahrC/sodB 
RNAs, we suggested previously that SR1 might affect 
ahrC translation at a post-initiation stage (24). However, 
the structural alterations found in the ahrC mRNA 
downstream from the SD sequence in the presence of 
increasing amounts of SR1 prompted us to re-evaluate 
our previous data using a toeprinting analysis (Figure 6). 
Both SR1\textsubscript{WT} and SR1\textsubscript{186}, but not two heterologous 
RNAs, were able to inhibit binding of the 30S ribosomal 
subunit and formation of a ternary complex with 30S and 
tRNA\textsuperscript{Me} on full-length ahrC mRNA. These results — 
together with the structure probing data — demonstrate 
that binding of SR1 induces structural changes in a ~65-nt 
long stretch of ahrC RNA between SD sequence and 
complementary region G that eventually inhibit formation 
of the 30S initiation complex. Since the 30S ribosomal 
subunit covers 54 nt, i.e. 35 (±2) nt upstream and 19 nt 
downstream from the start codon (39), the 5' part of the 
SR1-induced structural alterations of ahrC mRNA coincides 
exactly with this region. The analysis of the G region 
mutant SR1\textsubscript{186_G2} in the toeprinting assay (Figure 6C) 
corroborated that this region is involved in the first 
contact between SR1 and ahrC mRNA and supported
the specific basepairing interaction between both RNA molecules. The toeprinting results are not opposed to the previously observed translation inhibition of ahrC-lacZ fusions (24), as this inhibition can be explained by SR1-induced structural changes in the 5′ part of ahrC RNA, too. Therefore, we can conclude that the mechanism of action employed by SR1 is inhibition of translation initiation. This is the first case of a small regulatory RNA that binds ∼90 nt downstream from the ribosome-binding site and interferes with translation initiation. In contrast, in the well-studied E. coli systems like RyhB/sodB (20) or MicA/ompA (14,21), the complementarity regions between small RNA and mRNA are located upstream of or overlap the target SD sequence, making an effect on ribosome binding and hence, translation initiation, more plausible. Our results raise the question on the maximal distance between SD sequence and a binding region for a small RNA permitting to affect 30S subunit binding. Furthermore, in many E. coli cases the inhibition of translation initiation was accompanied by significantly decreased amounts of the target mRNA(s) [e.g. RyhB/sodB (40) or SgrS/ptsG (41)] that was attributed to degradation of the unprotected target RNA by RNase E or of the complex by RNase III (42).

Surprisingly, ahrC levels were found to be independent of the presence or absence of SR1 (24). To date, no RNase E has been found in B. subtilis. Although two novel endoribonucleases with homology to RNase E, RNase J1 and J2, were recently discovered (43), it is unclear, whether they fulfil the role of the main endoribonucleases as it does RNase E in Gram-negative bacteria.

In the few sense/antisense RNA systems, where calculations of the amount of both interacting species were performed (44,45), an at least 10-fold excess of the inhibitory small RNA over its target was determined. Here, the amount of SR1 in B. subtilis grown in complex medium was found to increase upon entry into stationary phase from 15–20 to 250 molecules per cell. This is much lower than the 4500 molecules measured for OxyS under oxidative stress conditions (30), but still in the range of RNAIII of plasmid pIP501 (∼1000 molecules). Since we could not detect ahrC mRNA in northern blots under any growth condition, its amount must be significantly lower than 15 molecules/cell ensuring at least a 15-fold excess of SR1.

The analysis of the SR1/ahrC mRNA interaction yielded three major issues, which might be important for sRNA/target RNA systems in general: First, whereas the major mechanism of action of trans-encoded sRNAs reported in Gram-negative bacteria is inhibition of translation initiation by direct binding to the RBS or 5′ of it, the B. subtilis SR1/ahrC pair is first case, where translation initiation is prevented by binding of the sRNA ∼90 nt downstream from the RBS. Second, while all sRNA/target RNA pairs studied so far comprise at the most two complementary regions, the SR1/ahrC pair is the first case with seven complementary regions between inhibitor and target RNA, and the major contribution of one region as well as the minor, but measurable contribution of five of the other regions has been demonstrated. Third, whereas in E. coli, Hfq was required for either sRNA stabilization or promotion of complex formation with the target RNA, at least complex formation in Gram-positive bacteria does not seem to depend on Hfq. The search for and analysis of other SR1 targets will reveal whether this sRNA exerts its function(s) by the same or alternative mechanisms.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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