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molecular microbiology

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Journal:	*Molecular Microbiology*
Manuscript ID:	MMI-2009-09673.R2
Manuscript Type:	Research Article
Date Submitted by the Author:	25-Mar-2010
Complete List of Authors:	Gimpel, Matthias; Friedrich-Schiller-Universität Jena, AG Bakteriengenetik Heidrich, Nadja; Uppsala University, Institute of Cell and Molecular Biology Mäder, Ulrike; Ernst-Moritz-Arndt-Universität Greifswald, Interfaculty INstitute for Genetics and Functional Genomics Krügel, Hans; Leibniz Insitute for Natural Product Research and Infection Biology, Molecular Biology Brantl, Sabine; Friedrich-Schiller-Universität Jena, Biol-Pharm. Fakultät, AG Bakteriengenetik
Key Words:	small regulatory RNA, sRNA, Bacillus subtilis, dual-function sRNA, carbon metabolism

A dual-function sRNA from *B. subtilis*: SR1 acts as a peptide encoding mRNA on the *gapA* operon

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Running title: Dual-function sRNA SR1 controls *gapA* operon
Key words: small regulatory RNA/SR1/dual function sRNA/*Bacillus subtilis*/carbon metabolism

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SUMMARY

Small noncoding RNAs (sRNAs) have been found to regulate gene expression in all three kingdoms of life. So far, relatively little is known about sRNAs from Gram-positive bacteria. SR1 is a regulatory sRNA from the *B. subtilis* chromosome that inhibits by basepairing translation initiation of *ahrC* mRNA encoding a transcriptional activator of the arginine catabolic operons.

Here we present a novel target of SR1, the glycolytic *gapA* operon. Both microarray and Northern blot analyses show that the amount of *gapA*-operon mRNA is significantly higher in the presence of SR1 when cells were grown in complex medium till stationary phase. Translational *lacZ* fusions and toeprinting analyses demonstrate that SR1 does not promote translation of *gapA* mRNA. By contrast, the half-life of *gapA*-operon mRNA is strongly reduced in the *sr1* knockout strain. SR1 does not act as a basepairing sRNA on *gapA* operon mRNA. Instead, we demonstrate that the 39 aa peptide encoded by SR1, SR1P, is responsible for the effect of SR1 on the *gapA* operon. We show that SR1P binds GapA, thereby stabilizing the *gapA*-operon mRNA by a hitherto unknown mechanism. SR1 is the first dual-function sRNA found in *B. subtilis*.

INTRODUCTION

Small regulatory RNAs (sRNAs) are expressed in all three kingdoms of life, primarily as posttranscriptional regulators. Over the past 8 years, more than 100 sRNAs have been discovered in *E. coli*, and about a fourth of them have been assigned a function. It has been estimated that 200-300 such sRNAs are present in an average bacterial genome (e.g. Hershberg *et al*., 2003).

The majority of these trans-encoded sRNAs are stress regulators that allow the bacteria to adjust their physiology to environmental changes (e.g. Spot42, DsrA, RprA, RyhB, SgrS, GadY, rev. in Storz *et al.*, 2005; Waters and Storz, 2009) or virulence gene regulators (rev. in Toledo-Arana *et al*., 2007). Bacterial sRNAs can be grouped into two classes: *bona fide* antisense RNAs that act by basepairing on their targets, and sRNAs that act by protein binding (rev. in Brantl, 2009; Repoila and Darfeuille, 2009).

Although it can be assumed that many trans-encoded sRNAs have multiple targets, only in a few cases experimental proof for more than one target has been provided. Examples include the *E. coli* sRNAs DsrA (at least two targets; Lease *et al*., 2004), RyhB (18 targets, Massé *et al*., 2007), GcvB (seven targets, all ABC transporter mRNAs; Sharma *et al*., 2007) and CyaR (at least five targets, De Lay and Gottesman, 2009) as well as *S. aureus* RNAIII essential for virulence (at least five targets, e.g. Boisset *et al*., 2007). The mechanism of action of many sRNAs is blockage of translation initiation by directly binding to the ribosome binding site (as MicC on *ompC*, rev. in Vogel and Papenfort, 2006), upstream of it (as GcvB on *gltI*, Sharma *et al*., 2007) or within the 5′ part of the coding region (as RybB on *ompN*, Bouvier *et al*., 2008). In Gram-negative bacteria, translation inhibition by sRNAs is associated with rapid degradation of the target mRNA by RNase E (Morita *et al*., 2005) or RNase III (Afonyushkin *et al*., 2005). Other sRNAs act by directly promoting mRNA decay (as RNAIII, Huntzinger *et al*., 2005) or by stabilizing mRNAs (as GadY, Opdyke *et al*., 2004).

Meanwhile, systematic searches for sRNAs have been performed in some Gram-positive bacteria, too. Currently, in *Bacillus subtilis*, 24 sRNAs are known (Silvaggi *et al*., 2005 and 2006; Lee *et al*., 2001, Licht *et al*., 2005; Gaballa *et al*., 2008; Eiamphungporn and Helmann, 2009; Pichon *et al*., 2005; Saito *et al*., 2009, Barrick *et al.*, 2005) and for six of them targets have been identified: RatA controls the toxin TxpA (Silvaggi *et al*., 2005), SR1 regulates transcription activator AhrC (Heidrich *et al*., 2006, see below), FsrA controls *sdhCAB, citB, yvfW* and *leuCD* (Gaballa *et al*., 2008), a 750 nt cis-encoded antisense-RNA regulates *yabE* (Eiamphungporn and Helmann, 2009), whereas BsrA and BsrB are two 6S RNAs (Barrick *et al*., 2005). In *Staphylococcus aureus*, in addition to RNAIII (e.g. Boisset *et al*., 2007), and the 7 RNAs SprA-G (Pichon and Felden, 2005), 11 novel noncoding sRNAs (RsaA-K) have been discovered (Geissmann *et al.*, 2009), among them one – RsaE – that is also expressed in *Bacillus subtilis*. Three Hfq-binding sRNAs and a σ^B-controlled sRNA with still unknown function (Christiansen *et al*., 2006, Nielsen *et al*., 2008) as well as 9 novel sRNAs within intergenic regions have been identified in *Listeria monocytogenes* (Mandin *et al*., 2007). Recently, five small RNAs were found in *Streptococcus pneumoniae* that are controlled by the response regulator CiaR (Halfmann *et al.*, 2007). However, identification of mRNA targets for the newly discovered sRNAs is still a

challenging issue, and has been successful only in few cases. Recently, all currently known chromosome-encoded sRNAs for which targets were identified, have been summarized (Brantl, 2009).

The majority of sRNAs from *E. coli* bind the abundant RNA chaperone Hfq (Valentin-Hansen *et al*., 2004; Zhang *et al*., 2002). Thereby, Hfq is required for stabilization of the sRNAs and/or to promote complex formation with the target RNAs. In two cases from Gram-positive bacteria, staphylococcal RNAIII/*spa* interaction (Huntzinger *et al.,* 2005), and in the SR1/*ahrC* interaction (Heidrich *et al*., 2006 and 2007), no influence of Hfq on either the sRNA or the sRNA/target interaction has been found. However, the recent example of *Listeria monocytogenes* sRNA LhrA, whose stability and interaction with its target *lmo0850* were Hfq-dependent showed that in Gram-positive bacteria both Hfq-dependent and -independent mechanisms might exist (Nielsen *et al*., 2010).

Recently, we have demonstrated that SR1 is a *trans*-encoded antisense RNA that acts by basepairing with its primary target, *ahrC* mRNA encoding the transcriptional activator of the *rocABC* and *rocDEF* arginine catabolic operons (Heidrich *et al*., 2006). Seven complementary regions between SR1 and *ahrC* were identified, of which the most 5′ region, G, is located 97 nt downstream from the *ahrC* RBS. SR1 inhibits translation initiation of *ahrC* mRNA by a novel mechanism: induction of structural changes downstream from the ribosome binding site (Heidrich *et al.,* 2007). Interestingly, SR1 is expressed under gluconeogenic conditions and repressed under glycolytic conditions, and this repression is mediated mainly by CcpN and, to a minor extent, by CcpA (Licht *et al*., 2005). This sugar-dependent regulation prompted us to search for additional targets of SR1 involved in central carbon metabolism.

Glycolysis is one of the main pathways of carbon catabolism in *Bacillus subtilis*. The key enzyme, glyceraldehyde-3-phosphate dehydrogenase (GapA), is encoded by the hexacistronic *gapA* operon. Under non-inducing conditions, the CggR protein, encoded in the 5′ proximal part of the operon, represses expression of the *gap* operon (Ludwig *et al*., 2001). Genetic and *in vitro* studies have demonstrated that fructose-1,6-bis-phosphate (FBP) is the negative effector of CggR activity (Doan and Aymerich, 2003). Several mRNA transcripts are found in Northern blots, which result from either processing or premature transcription termination: the hexacistronic *cggR-gapA-pgk-tpi-pgm-eno*-transcript, a pentacistronic *gapA-pgk-tpi-pgm-eno*-transcript, a tetracistronic *pgk-tpi-pgm-eno*-transcript, a bicistronic *cggR-gapA* transcript, and the monocistronic *cggR* and *gapA* transcripts (for a schematic overview see Fig. 1). A specific processing event takes place 65 nt upstream of the *gapA* start codon resulting in a more stable monocistronic *gapA* mRNA. This RNA is translated more efficiently, which is reflected in an about 100-fold excess of GapA over CggR protein in Western blots (Meinken *et al*., 2003). Recently, the RNase responsible for this processing has been identified and designated RNase Y. This RNase was found to interact with enolase, phosphofructokinase, PNPase and RNase J1 in the *B. subtilis* degradosome (Commichau *et al*., 2009). The five genes *gapA, pgk, tpi, pgm* and *eno* are essential in *B. subtilis* (Kobayashi *et al*., 2003).

Here, we demonstrate that the *gapA*-operon mRNA is a new target of SR1. In the presence of SR1, about 10- to 30-fold higher amounts of the *gapA* operon mRNAs were found in both microarrays and Northern blots when cells were grown in complex medium till stationary phase. The half-life of *gapA*

mRNA is higher in wild-type compared to *sr1*-knockout strains. We show that SR1 does not act as basepairing sRNA on *gapA*, but that the highly conserved small peptide SR1P encoded in SR1 is responsible for the effect on *gapA* mRNA. The positive effect of SR1P is not due to promotion of *gapA* translation, but to stabilization of the *gapA*-operon mRNAs. We show that SR1P binds GapA, and this interaction stabilizes *gapA* operon mRNA by an unkown mechanism. So far, SR1 is the first dual-function sRNA discovered in *Bacillus subtilis*.

RESULTS

Transcriptome and Northern blot analyses demonstrate that the amount of gapA*-operon mRNAs is significantly higher in the presence of SR1*

Four independent microarray-analyses were carried out with two sets of RNA samples prepared from *B. subtilis* strain DB104 grown in complex medium till OD_{560} = 5.0 (maximum expression of SR1). The first set involved the wild-type and the *sr1*-knockout strain which were not treated with anhydro-tetracycline. The second set comprised the *Δsr1*-strain with and without overexpression plasmid pWSR1 (containing the TetR repressor system) which were induced with sublethal anhydro-tetracycline concentrations for 15 min to obtain a pulse expression of SR1 from high-copy vector pWSR1. Table 2 shows the results. The most prominent difference was found for the *gapA* operon mRNA and the mRNA encoding its regulator CggR. Here, 100- and 25-fold lower levels for *gapA*-mRNA and *cggR*-mRNA and about 3- to 5-fold lower levels for the other four mRNAs (*pgm, tpi, eno* and *pgk*-RNA) were observed in the absence of SR1. The results for the induced overexpression of SR1 corroborated these findings: 46- and 33-fold higher amounts of *gapA*- and *cggR*-mRNA, respectively, were found upon a short-time overexpression of SR1, and •3-fold higher amounts were measured for the other four RNAs.

To substantiate these results, Northern blot analyses with RNA obtained from DB104 wild-type and the *sr1*-knockout strain DB104[*Δsr1::cat*] grown till OD_{560} = 5.0 (250 copies of SR1/cell) and OD_{560} = 2.0 (25 copies of SR1/cell), respectively, were performed (Figure 2A). As expected, distinct bands were observed for *gapA* (mono-, bi- and pentacistronic transcripts) and *cggR* (mono- and bicistronic transcripts) in the wild-type strain, but almost no mRNA was observed in the *Δsr1* strain at OD_{560} = 5.0. The hexacistronic transcript is not visible in TY medium at stationary growth phase. No significant differences were observed in the logarithmic growth phase at OD_{560} = 2.0, where the SR1 expression is very low. Furthermore, the induction of SR1 transcription for 15 min from DB104(*Δsr1::cat*, pWSR1) by sublethal anhydro-tetracycline concentrations compensated for the loss of the *gapA* and *cggR* transcripts in the DB104(*Δsr1::cat*) strain (Fig. 2B). Therefore, the data from wild-type, *Δsr1* and the overexpression strain at OD_{560} = 5.0 and from wild-type and *Δsr1* strain at OD_{560} = 2.0 confirm that the observed effect is indeed due to the presence of SR1. The pattern for both *gapA* and *cggR* transcripts in the Hfq-knockout strain at OD_{560} = 2.0 or 5.0 revealed no significant differences compared to the wild-type indicating that Hfq does not promote the effect of SR1.

The amount of GapA and CggR is not altered in the absence of SR1

To investigate whether the lower *gapA*- and *cggR*-mRNA levels in the *sr1*-knockout strain are reflected in lower protein levels, we performed Western blot analyses with crude protein extracts prepared from *B. subtilis* wild-type, *Δsr1* and *Δhfq* strains using polyclonal antisera raised against GapA and CggR. As control, a polyclonal antiserum against the constitutively expressed CcpN was applied in

the same buffer. Surprisingly, the deletion of the *sr1* gene did not result in alterations of the GapA- and CggR-levels compared to the wild-type (Fig. 3). The same holds true for the deletion of the *hfq* gene.

In the Northern blots, no alteration of the amount of the 2.2 kb bicistronic *gapA-cggR* transcript compared to the amount of the monocistronic 1.2 kb *gapA* or 1.0 kb *cggR* transcripts was observed in the *Δsr1* strain (Fig. 2). This indicates that the processing pattern of the 2.2 kb bicistronic transcript does not change in the absence of SR1 (the hexacistronic transcript is not visible at stationary growth phase). Therefore, an effect of SR1 on the processing of the *gapA*-operon mRNA can be ruled out.

Three possible hypotheses for the higher amounts of *gapA*-operon mRNA in the presence of SR1 in complex medium at OD_{560}= 5.0 are conceivable: First, SR1 could positively affect transcription of the *gapA* operon by influencing a transcription factor for this operon, second SR1 could stabilize the *gapA*-operon-mRNAs directly or third, it could promote *gapA* translation, which, in turn, might prevent mRNA degradation.

SR1 does not influence transcription of the* gapA *operon

The only known factors that regulate the well-characterized *gapA* operon are the catabolite control regulator CcpA that binds to a *cre* site upstream of the *cggR* promoter and the transcriptional repressor CggR that is transcribed at the 5′ end of the *gapA* operon mRNA. To analyse an effect of SR1 on transcription of the *gapA* operon, plasmid pACC1 carrying a *cggR-lacZ* transcriptional fusion was constructed that contained the *cggR* promoter and the *cre* site upstream of it. Integration of pACC1 into the *amyE* locus of strains DB104 and DB104(*Δsr1::phleo*) and measurement of β-galactosidase activities revealed no differences in the presence and absence of SR1 (Table 3). Therefore, we can conclude that SR1 does not directly influence transcription of the *gapA* operon, neither by acting on CggR or CcpA nor on any other unknown transcriptional regulator.

LacZ ***fusions and toeprinting analyses show that SR1 does not promote translation of*** **gapA** ***mRNA***

To explain the effect of SR1 on the amounts of *gapA*- and *cggR*-RNA, we searched for complementarity between SR1 and the *gapA*-operon mRNA. Indeed, we found two adjacent stretches of complementarity – interrupted each by a bulged-out nucleotide – between the 5′ stem-loop of SR1 and a region overlapping the start codon and the following 6 codons of the *gapA*-ORF (shown schematically in Fig. 4). This region is located >60 nt downstream from the previously published processing site at the 3′ part of *cggR* (Ludwig *et al*., 2001).

To assay a possible effect of SR1 on GapA or CggR translation initiation, two translational *gapA-lacZ* fusions pCGR2 and pCGR4 were constructed and integrated into the *amyE*-locus of the *B. subtilis* chromosome of the DB104 wild-type and *Δsr1*-strains. Whereas pCGR2 contains the entire *cggR*-ORF under its own promoter and *cre*-site and, additionally, 50 codons of *gapA* with its own SD sequence, pCGR4 comprises only the 5′ part of *gapA* starting from the processing site including the SD and the 50 5′ *gapA* codons, but under control of the heterologous promoter pIII (Brantl *et al.*, 1992). Integrants were grown in TY till OD_{560} = 5.0 (maximum expression of SR1), and β-galactosidase activities determined. As

shown in Table 3, in the case of both pCGR2 and pCGR4, the presence or absence of SR1 did not affect the amount of β-galactosidase. Therefore, we can conclude that SR1 does not promote translation of the processed monocistronic *gapA* transcript. Since in the case of pCGR4, the use of the heterologous promoter might have altered the ratio between SR1 and *gapA*-mRNA in favour of *gapA* thus preventing the visualization of an effect of SR1, we resorted to the integration of a translational *gapA-lacZ*-fusions at its natural site. For this purpose, plasmid pLKG1 was constructed that allows the integration of a *gapA-lacZ* fusion by single crossing-over at its native location. The measurement of β-galactosidase activities from the corresponding integrants at OD_{560} = 5.0 confirmed the results obtained with the previously assayed integrants in the *amyE* locus: no significant differences in the β−galactosidase activities between wild-type and *sr1*-knockout strain were found. These data indicate that SR1 does not affect translation from the *gapA* SD sequence.

To further corroborate this result, we performed a toeprinting analysis with *in vitro* synthesized *gapA* mRNA starting from the processing site and an unprocessed *cggR-gapA* mRNA fragment which contains additionally 98 nt upstream of the processing site. As control, *ahrC* mRNA, the translation initiation of which is inhibited by SR1 as described previously (Heidrich *et al*., 2007), was used in toeprinting with the same SR1 preparation. As shown in Fig. 5, in the presence of initiator $tRNA^{fMet}$, 30S ribosomal subunits bind to the *ahrC* and to the *gapA* translation initiation region and block reverse transcription of a labelled primer, annealed downstream, at the characteristic positions +15 and +16, respectively (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 $tRNA^{fMet}$. However, addition of a 50 or a 100-fold excess of SR1 to *gapA* RNA did not alter the ratio between terminated and read-through RT product, i.e. did not promote or inhibit 30S binding to *gapA* significantly. Instead, a slight decrease of the relative toeprint to 80 % was observed. In the case of the longer mRNA that mimicks the unprocessed transcript, a slight increase of the relative toeprint to 160 % was observed. By contrast, binding of SR1 to *ahrC* mRNA clearly interfered with ternary complex formation, resulting in a significantly weaker toeprint signal. Compared to the effect of SR1 on 30S binding on *ahrC*-mRNA, which was already reduced to 10 % in the presence of 50-fold excess of SR1 and almost zero at 100-fold excess, the observed changes in the case of the *gapA* and *cggR* mRNAs were negligible. These data argue against an effect of SR1 on translation of *gapA* mRNA.

In summary, both translational *lacZ* fusions and toeprinting demonstrate that the mechanism used by SR1 is not the promotion of GapA translation.

SR1 prevents degradation of* gapA-*operon mRNA

The higher amount of *gap*-operon-mRNA in the presence of SR1 might be due to stabilization of this mRNA by the sRNA. Therefore, first the stability of *gapA*-operon mRNA in the presence and absence of SR1 was investigated. DB104 and DB104(*Δsr1::cat*) were cultivated till OD_{560} = 5.0, rifampicin was added to a final concentration of 200 μg/ml, and time samples were taken. The half-life of *gapA*-operon mRNA in the presence of SR1 was determined by Northern blotting to be •11 min (Fig. 6A). Since in the absence of

SR1 the *gapA*-operon *mRNA* was almost not detectable in Northern blots, we resorted to quantitative RT-PCR as described in *Experimental Procedures*. As shown in Fig. 6B, the signals of *gapA*-operon mRNA in the absence of SR1 decreased much faster than in the presence of SR1. A half-life determination with this method yielded about 8 min in the presence of SR1 and about 55 sec in the absence of SR1. This indicates that *gapA*-operon mRNA is more rapidly degraded in the *sr1* knockout strain, and SR1 somehow stabilizes the *gapA*-operon-mRNA.

Some SR1 mutants in a predicted complementary region with gapA are not able to complement the effect of the* sr1*-knockout

Previously, we have shown that SR1 is a *bona fide* antisense RNA that acts by basepairing with its primary target *ahrC*-mRNA. SR1 inhibits *ahrC* translation initiation by introducing structural changes downstream of the *ahrC* SD, but does not affect the amount of *ahrC* mRNA (Heidrich *et al*., 2006 and 2007). To analyze if a basepairing interaction between SR1 and *gapA* in the predicted region is needed for the stabilizing effect of SR1, a series of pWSR1 derivatives allowing the inducible expression of mutated SR1 species were constructed. The mutations were generated within the predicted complementary region between SR1 and *gapA* (Figure 4, overview on mutants see Fig. 7A). All mutants were tested in Northern blots for their ability to complement the *gapA* defect in an *sr1* knockout strain. Mutant M1 contains an 11 bp exchange in the loop region of SR1 affecting the 3' half of the region complementary with *gapA* (Fig. 7B). Northern blots showed that this mutant SR1 is no longer able to compensate the loss of chromosomal wild-type SR1 in stabilizing the *gapA* operon transcripts (Fig. 7B). Based on this result, we aimed to narrow down the region decisive for the contact between SR1 and *gapA* by constructing a series of mutant species (Fig. 7A). Mutants M2, M3 and M6 were, surprisingly, still able to complement the effect of the *sr1* knockout strain, whereas mutants M9 and M13 proved to be not functional in complementation (Fig. 7B). By contrast, two mutants within the 3' part of SR1 (M7 and M8) carrying point mutations in the sequence previously found to be involved in the SR1/*ahrC* interaction, behaved like wild-type pWSR1 (not shown). Two controls, M5 and M12, were constructed. M5 lacks the region between stem-loop SL1 and the terminator stem-loop that is involved in the previously discovered interaction between SR1 and *ahrC* RNA (Heidrich *et al.*, 2006). In M12 the start codon AUG of the small SR1 ORF (*sr1p*) was replaced by the stop codon UAG. Unexpectedly, both control plasmids were unable to complement the *sr1* defect on *gapA* (Fig 7B). This result indicated that the SR1 encoded 39 aa peptide SR1P (Fig. 4B) is involved in the effect of SR1 on *gapA*. Formerly, the translation of this peptide was not detectable in single-copy conditions from the chromosome (Licht *et al*., 2005), whereas here, multiy-copy plasmids were used. The result with M12 would, at the same time, explain the nonfunctionality of mutants M1, M9 and M13, since in these cases the introduced bp exchanges altered two to four codons within the N-terminus of SR1P (Fig. 8B).

A small SR1 encoded peptide, SR1P, is expressed from a multicopy plasmid and is conserved among Bacillus species

To analyse the expression of *sr1p* from the multicopy plasmid pWH353, two plasmids were constructed: pWSR1/M14 contained the *sr1p* gene with a C-terminal extension encoding a 3x FLAG tag, and pWSR1/M15 contained the *sr1p* gene with an N-terminal extension encoding a 3x FLAG tag (Fig 7C). Both plasmids were transferred to strain DB104 (*Δsr1::cat*) and found to be functional in complementation of the *sr1* knockout. Thus, neither the C- nor the N-terminal tag impaired the function of SR1. To visualize the FLAG-tagged SR1P on a protein gel, DB104 (*Δsr1::cat*, pWSR1/M14 or M15) were grown till OD_{560} = 5.0, SR1 expression induced with anhydro-tetracyline, protein crude extracts prepared by sonication and separated on a 17.5 % SDS polyacrylamide gel. Western blotting was performed using anti-FLAG antibodies as described in *Experimental Procedures*. As shown in Fig. 8A, a protein was observed from the N-terminally FLAG-tagged construct pWSR1/M15. This indicates that the weak SD sequence 5′ of the *sr1p* ORF is functional in *B. subtilis*. A sequence alignment of SR1P from different firmicutes shows a high conservation in the N-terminal 9 amino acids with cysteines at positions 6 and 9, in the central part (FEDEK motif, aa 16 to 20) and in three cysteine residues at positions 28, 32 and 34 within the C-terminus (Fig. 1S). SR1P does not contain basic stretches or any known putative RNA binding motifs, but has, by contrast, a calculated pI value of 3.9. SR1P homologues were not found outside the genus *Bacillus* or *Geobacillus* in any other Gram-positive or Gram-negative bacteria.

SR1 acts on* gapA *as a peptide encoding mRNA and not as a* bona fide *sRNA

To find out whether SR1 acts on *gapA* soleley as a peptide encoding mRNA or as both a peptide encoding and a *bona fide* antisense RNA, we used the following approach: The *sr1p* sequence was altered at the wobble positions of the 5′ 28 codons yielding plasmid pWSR1/M19. By this mutagenesis, the putative complementarity of the 5′ and central part of SR1 to any region in *gapA* mRNA was altered, whereas the transcription and translation initiation of *sr1p* RNA as well as the composition of SR1P remained unaffected. Furthermore, plasmids pWSR1/M20 containing the heterologous BsrF transcription terminator (Preis *et al.*, 2009) instead of the SR1 terminator and pWSR1/M21, carrying the BsrF terminator and replacements at the C-terminal 9 wobble positions of *sr1p* were constructed (Fig. 9A). A heterologous terminator was used since in the case of SR1/*ahrC* the 5′ part of the SR1 terminator was found to be involved in the initial basepairing between both RNAs. Additionally, plasmid pWSR1/M22 containing 23 nt exchanges within the 33 5′ nt of SR1 upstream of the SR1P SD sequence that, however, preserved the secondary structure, was generated (a complete deletion of the sequence upstream of the SR1P SD was not possible, since the resulting SR1 RNA was unstable and detectable only in extremely low amounts). Using Northern blotting as above, we observed that all of these mutants were still able to complement the effect of the *sr1* knockout on the *gapA* operon (Fig. 9B).

Since we could not exclude that SR1 acts additionally as a basepairing sRNA within the same region that encodes the peptide, loop mutant pWSR1M13 was chosen for the construction of a compensatory mutation in *gapA* within the chromosome as described in *Experimental Procedures.* This mutant was chosen since it was located in the only predicted region of complementarity between *gapA* and SR1, and it was the only mutant that could, without altering the GapA protein sequence significantly,

be compensated by mutating *gapA*. In the resulting strain GAPM with the mutated *gapA* gene, the *sr1* gene was replaced by an MLS^R gene. This strain was transformed with plasmid pWSR1/M13 and complementation was assayed by Northern blotting. As shown in Fig. 9C, pWSR1/M13 could not complement the effect of the *sr1* knockout on the compensatory mutation in the *gapA* gene in the *B. subtilis* chromosome. Taken together all these data demonstrate that SR1 acts solely as a peptide encoding mRNA and not as a basepairing sRNA on *gapA*.

***A peptide lacking the C-terminal 9 amino acids can complement the SR1 defect on* gapA**

The alignment of the SR1 encoded peptides of different *Bacillus* species (Fig. 1S) revealed that only the N-terminal 30 amino acids and Cys32 and Cys34 are highly conserved. To analyse the requirements of the C-terminal amino acids for SR1P function, mutants M29 and M30 containing a stop codon instead of codon 31 or codon 35 of SR1P, respectively, were constructed and analysed in Northern blotting. As shown in Fig. 9B, at least for normal regulation of *gapA* mRNA levels, both mutants were still functional. By contrast, the deletion of two more C-terminal amino acids, among them Cys29, rendered SR1P nonfunctional (mutant M27 carrying a stop codon instead of codon 29). These data show that the C-terminal 9 amino acids, among them Cys32 and Cys34, are not required for the function of SR1P.

Analysis of RNase deficient mutants indicates that neither of the known RNases is involved in degradation of* gapA *mRNA

To analyse whether the stabilizing effect of SR1P on *gapA* RNA was due to an alteration of the activity of an RNAse involved in *gapA* degradation, DB104 knockout strains for the following RNases were constructed: RNase III, RNases J1, J2, PNPase, YhaM, RpH. However, except for J2, no significant alteration in the amount of *gapA* operon mRNA was found in these knockout strains (Fig. 2S). Surprisingly, in the J2 knockout strain, a strongly reduced amount of *gapA* mRNA was observed, for which we – so far – do not have an explanation. Double knockout strains lacking *sr1* and the corresponding RNase gene behaved like the *sr1* knockout strain. These data indicate that no known RNase is involved in the degradation of *gapA* mRNA.

SR1P interacts with GapA

To purify a potential interaction partner of SR1P, two plasmids encoding *sr1p* derivatives (pWSR1/M25 and pWSR1/M31) were constructed. Whereas pWSR1/M25 encodes SR1P C-terminally fused to a Strep tag, pWSR1/M31 encodes SR1P C-terminally fused to a His_6 tag. As a negative control, pWSR1/M26 was constructed derived from pWSR1/M13 (Fig. 7) which encodes SR1P with two amino acid exchanges (C6A, D8H, see Fig. 8) also C-terminally fused to a Strep tag. (For schematic representations of the plasmids see Fig. 10A). As a second negative control served pOU75H encoding the C-terminally His_6-tagged heterologous protein CcpN (Licht *et al*., 2005). Northern blot analyses ensured that pWSR1/M25 and pWSR1/M31 were functional in complementing the *sr1* knockout, whereas pWSR1/M26 was not. All three plasmids were transferred to DB104(*Δsr1::cat*). From the three mutant strains as well as from DB104

(*Δsr1::cat*, pWSR1) encoding the non-tagged SR1P, protein crude extracts were obtained and processed through streptactin (pWSR1/M25, pWSR1/M26 and pWSR1) and Ni-NTA agarose (pWSR1/M31 and pWSR1) columns as described in *Experimental procedures*. All elution profiles are shown in Fig. 3S. Aliquots of the individual elutions fractions E3 were separated on a 17.5 % Tris-glycine-gel and stained with Coomassie blue (Fig. 10B and D). In the cases of the tagged wild-type SR1P (pWSR1/M25 and pWSR1/M31) a prominent band of approximately 45 kD was visible. This band was absent in pWSR1/M26 (nonfunctional tagged SR1P), pWSR1 (non-tagged SR1P) and pOU75H (tagged CcpN). In M25 and M26, the tagged SR1P was visible, too. Sequencing of the 7 N-terminal amino acids of the 45 kD bands obtained with M25 and M31 identified them as GapA. This result was confirmed by Western blotting with antibodies against GapA (Fig. 10C). Apparently, we co-eluted SR1P and GapA from the columns, suggesting an interaction between both proteins. Since the co-elution experiment with pWSR1/M26 encoding a C-terminally Strep-tagged mutant peptide that was not functional in complementation of *Δsr1* did not yield the GapA band, we conclude that GapA can co-elute only with SR1P that is functional *in vivo*. The negative result with pOUH75 indicates that the interaction between GapA and SR1P is specific and not due to the His_6-tag (Fig. 10B and C and Fig. 3S B).

DISCUSSION

A few trans-encoded sRNAs target exactly one mRNA. In the other cases, two scenarios are conceivable: several sRNAs might regulate the same mRNA target or one sRNA might regulate more than one target mRNA. Examples are known for both scenarios: e.g. *rpoS* is regulated by at least four small RNAs: OxyS, DsrA, RprA and 6S RNA (rev. in Brantl, 2009) and *glmS* mRNA in *E. coli* is regulated by GlmY and GlmZ (Reichenbach *et al*., 2008; Urban and Vogel, 2008). Likewise, for a number of trans-encoded basepairing sRNAs more than one target has been identified. For instance, *E. coli* DsrA acts negatively on *hns* and positively on *rpoS* and is predicted to have further targets (Lease *et al*., 2004) and *E. coli* RyhB involved in iron metabolism has at least 18 targets (rev. in Massé *et al.,* 2007). Recently, for CyaR, beside the already known *ompX* (Papenfort *et al*., 2008), three novel targets, *nadE, luxS* and *ygaE* were identified (De Lay and Gottesman, 2009). To date, in Gram-positive bacteria, RNAIII from *S. aureus* and the recently discovered FsrA from *B. subtilis*, are the only sRNAs for which several targets have been found (Boisset *et al.*, 2007, Gaballa *et al.,* 2008). Furthermore, one sRNA can use different mechanisms on different targets. For example, GcvB was shown to inhibit translation of several target mRNAs encoding ABC transporters, however, its binding on the *gltI* target upstream of the region that can be protected by the 30S ribosomal subunit suggests that in this case, an alternative, still unknown mechanism, might operate (Sharma *et al*., 2007).

Interestingly, in three cases, small ORFs present on sRNAs are essential for their regulatory function. The 514 nt staphylococcal RNAIII acts both as an mRNA encoding δ hemolysin (Morfeldt *et al*., 1995) and as an antisense RNA regulating *hla, spa, sa1000, sa2353* and *rot* (rev. in Repoila and Darfeuille, 2009). The 459 nt streptococcal Pel RNA, a regulator of the virulence factors, encodes streptolysin S (Mangold *et al.,* 2004). *E. coli* sRNA SgrS was found to encode the 43 aa polypeptide SgrT, which inhibits glucose transporter activity by an unknown mechanism and acts in the same pathway as SgrS that inhibits translation of the *ptsG* mRNA (Wadler and Vanderpool, 2007). Recently, the novel Hfq binding sRNA PhrS from *Pseudomonas aeruginosa* was discovered that also contains a translated 37 aa ORF, but the function of the encoded peptide ist still elusive (Sonnleitner *et al*., 2008).

Here, we report on the second target for the *B. subtilis* sRNA SR1, the *gapA* operon. Whereas SR1 acts on its first identified target, *ahrC* mRNA, as a *bona fide* antisense RNA by basepairing with *ahrC* mRNA thereby inhibiting translation initiation (Heidrich *et al*., 2006 and 2007), the small polypeptide SR1P encoded by *sr1* was found to be responsible for the effect on the *gapA* operon. Formerly, using an *sr1p-lacZ* translational fusion integrated as single copy into the chromosome, we could not detect any translation of this SR1 ORF in *B. subtilis* (Licht *et al*., 2005). However, to investigate the effect on *gapA* operon-mRNA, we used the multi-copy plasmid pWSR1 (about 50 copies/chromosome) to provide SR1 *in trans*. Since our first hypothesis was that SR1 might interact with *gapA* mRNA in a complementary region overlapping the SD sequence, a series of pWSR1 mutants altered in this region was assayed (Fig. 7) and, indeed, some of them could not complement the *Δsr1* effect on *gapA*. However, two control mutants showed that the *sr1*

ORF termed *sr1p* is required for complementation. The use of a FLAG-tagged SR1P allowed to detect an expression of *sr1p* from a multicopy plasmid by Western blotting. An alignment of SR1P from *Bacillus subtilis* with ORFs from other firmicutes revealed a high degree of sequence conservation (Fig. 1S).

We demonstrate that SR1 does neither affect transcription nor translation of the *gapA* operon nor its processing, but, instead, inhibits degradation of *gapA* operon mRNAs (Fig. 6). Furthermore, we show that SR1P interacts with GapA, the main translation product of the *gapA* operon. The calculated acidic pI (3.9) of SR1P and the lack of any RNA binding motifs make an additional RNA binding capacity of this small polypeptide rather unlikely.

How does the interaction between SR1P and GapA lead to the stabilization of the *gapA* operon mRNAs? Three hypotheses are conceivable: 1) During translation, SR1P might bind GapA and, thereby, stall the ribosomes on the *gapA* operon mRNAs. The mRNA protected by the ribosomes will not be accessible to RNases, and thus, cannot be degraded. The stalled polysomes will not allow access of tmRNA. 2) SR1P may guide *gapA* operon mRNA *via* binding to nascent GapA to a kind of cytoplasmic foci in the prokaryotic cell, where – similar to the eukaryotic P bodies (e.g. Liu *et al.*, 2005) – mRNAs interacting with sRNAs are transiently assembled and prevented from being translated. One could speculate that the interaction of SR1P with nascent GapA converts *gapA*-operon mRNA with bound ribosomes into a kind of tightly packed structure that does neither allow RNA degradation nor continuation of translation. 3) GapA was not found in the *B. subtilis* degradosome (Commichau *et al*., 2009). However, it has been shown that GAPDHs from all domains of life, among them that of *Bacillus stearothermophilus*, are able to cleave RNA *in vitro* (Evguenieva-Hackenberg *et al*., 2002). Therefore, we hypothesize that SR1P could mask the RNase activity site on GapA thus inhibiting degradation of *gapA* operon mRNAs. Additionally, ribosome stalling would impede translation. In the absence of SR1, GapA could moonlight as an RNase and cleave *gapA*-operon mRNA and, most probably, some other, still unknown mRNAs.

What is the physiological relevance of the stabilizing effect of SR1 on the *gapA*-operon mRNA? All enzymes encoded by the operon are required during glycolysis, when SR1 is repressed by CcpN and CcpA (Licht *et al*., 2005). We suggest the following explanation: When *Bacillus* has exhausted all glucose and other sugars that are fed into glycolysis, e.g. in stationary phase, SR1 transcription is increased about 10-fold to about 250 molecules/cell (Heidrich *et al*., 2007). The resulting SR1P molecules stabilize the few *gapA*-operon-mRNA molecules which are still present within the cell and allow only the synthesis of small amounts of GapA. When glucose is fed into the medium, SR1 transcription is repressed by CcpN/CcpA, since SR1 is no longer needed at high concentrations. At the same time, due to rapid degradation of SR1 (half-life 3-4 min), SR1P-mediated ribosome stalling is abrogated allowing immediate translation of a large amount of GapA and the other glycolytic enzymes. Furthermore, CggR-mediated repression of the *gapA* operon transcription is lifted. Most probably, SR1 provides, additionally to CggR, a backup level for control ensuring an immediate response to the appearance of glucose in the environment of *B. subtilis*.

SR1 – so far the first dual-function sRNA discovered in *B. subtilis* – represents an unprecedented case as it uses almost the same RNA regions to act as an antisense RNA on one target (*ahrC*) and as a

peptide encoding mRNA on another target (*gapA* operon). However, the region required for the initial contact between SR1 and *ahrC*-RNA (region G, Heidrich *et al.*, 2007) is located outside of the SR1P encoding region, allowing evolution to play around without affecting the peptide encoding region.

What is the advantage of dual-function sRNAs? The use of the same promoter and the same transcript for different functions would save coding capacity. Furthermore, since transcription is energy-consuming, the use of the same transcript as a regulatory RNA on the one hand and peptide encoding mRNA on the other hand might save energy. Additionally, in cases where peptide and sRNA act in the same pathway (e.g. virulence), the relative amounts of sRNA and peptide are kept constant when both regulators are under control of the same promoter responding to an environmental signal. To date, only a few dual-function sRNAs are known, but it can be expected that their number will increase with the discovery of novel sRNAs from other genomes.

EXPERIMENTAL PROCEDURES

Enzymes and chemicals

Chemicals used were of the highest purity available. Taq DNA polymerase was purchased from Roche or SphaeroQ, Netherlands, respectively, and Thermoscript reverse transcriptase and M-MuLV reverse transcriptase from Invitrogene and Fermentas, respectively. Firepol polymerase was purchased from Solis Biodyne, Estonia.

Strains, media and growth conditions

E. coli strains DH5α and DH10B were used for cloning. *B. subtilis* strains DB104 (Kawamura and Doi, 1984), GP550 and GP231 and *E. coli* strains were grown in complex TY medium (Licht *et al.*, 2005). *B. subtilis* RNase knockout strains SSB334 (ΔJ1, ΔJ2), SSB340 (ΔJ2), BG 503 (*Δrph, ΔyhaM, Δrnr*), BG119 (*ΔrnP*) and BG218 (defect in rnase III) were grown in TY.

Construction of plasmids for the in vivo *reporter gene test system and measurements of β-galactosidase activities*

For the construction of pACC1 containing a *cggr-lacZ* transcriptional fusion, a PCR on chromosomal DNA with primers SB1217 and SB1218 was performed, the resulting fragment digested with BamHI and EcoRI and inserted into the pAC6 vector. For the construction of the *gapA* translational fusion under control of the natural *gapA*-operon promoter upstream of *cggR*, chromosomal DNA from *B. subtilis* DB104 was used as template in a PCR reaction with upstream primer SB1084 and downstream primer SB1086 (Primers are listed in Table 1S). The fragment was digested with BamHI and EcoRI and inserted into the BamHI/EcoRI vector of pGGA1 yielding plasmid pCGR2 (all plasmids are listed in Table 1). The same approach was used for the construction of the *gapA* translational fusion under control of the heterologous promoter pIII, however, using the alternative upstream primer SB1092 comprising pIII immediately upstream of the processing site. The resulting plasmid was designated pCGR4. Both pCGR2 and pCGR4 contain the *amyE* back and front regions and can be integrated into the *amyE* locus by double crossing-over. Plasmid pLKG1 designed for the integration into the native locus by single crossing-over, was constructed as follows: First, vector pBLK1 was constructed by insertion of the NotI(filled in)/BamHI fragment of pGGA1 with the BgaB and kanamycin resistance genes into the EcoRI(filled in)/BamHI vector of pBR322. Subsequently, the BamHI/EcoRI fragment of pCGR2 was inserted into EcoRI/BamHI digested vector pBLK1 yielding pLKG1. Plasmids pCGR2 and pCGR4 were integrated into the *amyE* locus by double crossing over, and pLKG1 into its native locus and measurements of the β-galactosidase activities were performed as described previously (Brantl, 1994).

Construction of an integration vector for replacement of the sr1 *gene by the MLS^R gene from plasmid pE194*

Plasmid pINT1E for the replacement of the chromosomal *sr1* gene by the MLS^R gene was constructed as follows: The EcoRI/SalI fragment of plasmid pTaq7 comprising the MLS^R gene of staphylococcal plasmid

pE194 was isolated, and, together with the SalI/PstI fragment of plasmid pCBACK containing the 800 bp region downstream from the *sr1* terminator, inserted into the EcoRI/PstI digested pUC19 vector resulting in plasmid pEBACK1. The EcoRI/PstI fragment of pEBACK1 was jointly cloned with the EcoRI/BamHI fragment of plasmid pFRONT1 encompassing the 800 bp upstream of the *sr1* promoter into the pUC19 BamHI/PstI vector yielding plasmid pINT1E.

Construction of plasmids for tet-inducible overexpression of mutated SR1 species

To express mutant SR1 species under a tetracycline inducible promoter, two parallel PCR reactions were performed with primer SB1171 and mutant primer 1 on plasmid pUCSR1 as template and mutant primer 2 and SB1176 (or, for shorter versions, SB317), respectively, on chromosomal DNA as template, the fragments isolated from agarose gels and subjected to a third PCR reaction with the outer primers SB1171 and SB1176 (or SB317). The obtained fragments were digested with HindIII and inserted into the HindIII vector of pWSR1. Inserts were confirmed by sequencing. The constructed mutant plasmids are listed in Table 1 and the corresponding mutant primer pairs used are listed in Table 1S. Plasmids pWSR1/M25 and pWSR1/M26 were constructed by a two step PCR using pWSR1 and pWSR1/M13, respectively, as templates, and first primer pair SB348/SB1375 and, after purification of the resulting fragment, primer pair SB348/SB1402 for the second PCR. Plasmid pWSR1/M31 was constructed by an alternative two-step PCR with pWSR1 as template, outer primer pair SB348/SB317 and mutagenic primers SB1445/SB1446.

Construction of a compensatory mutation in the gapA *gene in the* B. subtilis *chromosome*

Plasmid pGAPM1 carrying a compensatory *gapA* mutation to the mutation in pWSR1/M13 was constructed as follows: A two step PCR with primer pairs SB1241/1244 and SB1242/1243 was performed on chromosomal DNA as template. The second PCR was performed with primer pair SB1243/SB1244, the resulting fragment digested with PstI and EcoRI and inserted into the pUC19 vector. After sequencing, the PstI/NcoI fragment of pUC19gapM was inserted into the PstI/NcoI-cleaved plasmid pGP231 generating plasmid pGAPM1. Strain GP550 carrying a mutation in *cggR* was cotransformed with *ScaI*-linearized pGAPM1 and chromosomal DNA from strain GP311 carrying a kanamycin resistant cassette for replacement of the *cggR-lacZ* fusion in the GP550 *amyE* locus, and kanamycin resistant, chloramphenicol sensitive colonies were selected. In the resulting mutant strain *B. subtilis* GAPM, the kanamycin cassette in the *amyE* locus was replaced by ScaI-cleaved pAC6 vector by selecting for chloramphenicol resistant, kanamycin sensitive transformants.

Preparation of total RNA, RNA gel electrophoresis and Northern blotting

B. subtilis strains were cultivated till OD_{560} = 5.0 and frozen in liquid nitrogen in 2 ml vials. The pellets from 8 ml culture were dissolved in 500 µl Tri Reagent (Ambion) in 2 ml vials and treated with 200 µl glass beads three times for 30 min each in a Biospec bead-beater interrupted by cooling on ice. Subsequently, 200 µl distilled water were added and the disrupted cells were centrifuged for 2 min at 13.000 rpm. The supernatant was subjected to three phenol-chloroform and one chloroform extractions,

followed by ethanol precipitation. Pellets were washed with 80% ethanol. The pellets from 40 ml culture were finally dissolved in 400 µl sterile bidistilled water, and the RNA concentration was determined by UV spectrometry at 260 nm. In some cases, total RNA was prepared by the hot-phenol method as described (Heidrich *et al*., 2006). RNA gel electrophoresis on 1.5 % agarose gels and Northern blotting were carried out as described previously (Heidrich *et al.*, 2006).

Calculation of the relative amounts of gapA *operon mRNA in Northern blots*

Northern blots (as shown in Fig. 7B) were evaluated with a Fuji PhosphorImager and quantified using the program TINA-PC BAS 2.09. All single bands in one lane visualized with a probe against *gapA* were quantified and their intensities summarized, and, to avoid loading errors, corrected by the signals obtained with the probe against 23S rRNA. Then, the corrected amounts for the sum of all *gapA* species for a specific mutant pWSR1/MX (X = number of the mutant) were divided by the amount of *gapA* calculated for the wild-type pWSR1 run on the same gel. In the case of the empty pWH353 vector, this resulted in values between 0.05 and 0.2 (regarded to be the relative amounts of *gapA* compared to the wild-type). If the resulting number for a mutant was •0.2 (relative amount of *gapA*), this mutant was considered to be unable to complement the SR1 knockout-effect on the *gapA* operon. If the resulting number for a mutant was between 0.6 and 1.0 or >1.0, this mutant was considered to be still functional.

Quantitative real time PCR (qRT-PCR)

For quantitative real time PCR, the Applied Biosystems StepOne real-time PCR system, the GeneAmp fast PCR Master Mix and the fluorescence dye EvaGreen were used. As reference served primers located in the BsrF-RNA, as target two primers in the mRNA of the *gapA* gene were used (a list of all primers can be found in Table 1S). The PCR conditions on the cDNA were optimized in the Applied Biosystems fast cycler "Verity". Ratios were calculated by the $\Delta\Delta C_T$ method (Pfaffl, 2002).

Purification of Strep-tagged and His_6-tagged proteins

B. subtilis strain DB104*(Δsr1::cat)* containing either of the plasmids pWSR1, pWSR1/M25, pWSR1/M26 or pWSR1/M31 was grown in 1 l TY medium till onset of stationary phase, induced with 0.4 µg/ml anhydro-tetracycline for 20 min and harvested by centrifugation at 4 °C and 8000 rpm. Pellets were frozen over night. Subsequently, they were disrupted in 10 ml total volume of equilibration buffer (150 mM NaCl, 100 mM Tris-HCl pH 8.0, 1 mM EDTA) for 1.5 min at 2000 rpm in the Mikro-Dismembrator (Sartorius). Resulting crude extracts were centrifuged twice at 13.000 rpm and 4 °C and supernatants applied to Streptactin- (pWSR1, M25, M26) or Ni-NTA-columns (pWSR1, M31). Washing, elution and regeneration of the 1.0 ml columns were performed according to the manufacturer IBA (streptactin column) or as previously described (Steinmetzer and Brantl, 1997). Six 500 µl elution fractions were collected for each column, and 22.5 µl of each fraction tested on SDS-Tris-glycine PAA gels.

Western blotting

Protein extracts were prepared by sonication from the corresponding *B. subtilis* strains as described previously (Licht *et al.*, 2005) and adjusted to equal concentrations using the Bradford assay. Equal amounts of protein extracts were separated on 15.5 % or 17.5 % (in the case of the peptide SR1P) SDS/polyacrylamide (PAA) gels and transferred onto PVDF membranes at 12 V for 90 min by semidry blotting in transfer buffer containing 5.8 g Tris-HCl, 2.9 glycine, 0.37 g SDS and 200 ml methanol per liter. Membranes were blocked for 1 hr with PBST containing 0.5 % gelatine and subsequently incubated with polyclonal antisera against GapA (1: 30.000) or CggR (1: 1.000) for 1 h. Subsequently, filters were washed four times for 10 min each in PBST, followed by 1 h incubation with the second antibody (anti-rabbit-horseradish peroxidase, 1: 2000) and washed again 6 times for 10 min in PBST. Afterwards, membranes were incubated with 10 ml substrate solution containing 50 mM Tris pH 7.5, 0.9 g/ml diaminobenzidine and 5 µl H_2O_2 until bands were visible. To stop the reaction, membranes were washed with distilled water. Blots were digitized with a Scan-Prisa 640U (Acer) scanner and analyzed with TINA-PC BAS 2.09 software. 10x PBS buffer is composed of 80 g NaCl, 5.04 g KOH, 15.6 g NaH_2PO_4 x 1 H_2O in 1 l distilled water. 1x PBST contains additionally 0.5 ml Tween 20 per liter. For the detection of FLAG-tagged SR1P, both a commercial anti-FLAG antiserum from SIGMA and a secondary goat anti mouse IgG antibody conjugated to alkaline phosphatase from Santa Cruz Biotechnology were used, and detection was performed as described by the supplier.

Toeprinting analysis

The toeprinting assays were carried out using 30S ribosomal subunits, *gapA* mRNA and $tRNA^{fMet}$ as described previously (Heidrich *et al.*, 2007). The 5′-[^{32}P]-labelled *gapA*-specific oligonucleotide SB1107 (5′ CCG CTA CTA CCT CAA CTT) complementary to *gapA* mRNA was used as a primer for cDNA synthesis in the toeprinting reactions. An aliquot of 0.04 pmol of *gapA* mRNA annealed to primer SB1107 was incubated at 37°C without or with 0.4 pmol of 30S subunits and 8 pmol of uncharged $tRNA^{fMet}$ (Sigma) before supplementing with 1µl M-MuLV-RT (80 units). In the control toeprint with *ahrC*-mRNA, primer SB1068 (5′ TAC CGT GGC CTG CGT TAC) was used. cDNA synthesis was performed at 37°C. Reactions were stopped after 10 minutes 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, the corresponding mRNA and SR1 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 (TINA-BAS 2.09).

Transcriptome analysis

Strains DB104 and DB104(*Δsr1*) from two independent cultivations as well as the anhydro-tetracycline induced strains DB104(*Δsr1*) and DB104(*Δsr1::cat*, pWSR1) from four independent cultivations were used for the microarray analysis. Cell harvesting and preparation of total RNA were performed as described above. RNA samples were DNase-treated with the RNase-free DNase kit (Qiagen) according to the manufacturer's instructions and purified using RNeasy mini columns (Qiagen). RNA quality was checked

by Agilent's 2100 Bioanalyzer with the RNA 6000 Nano LabChip Kit. Generation of the Cy3/Cy5-labeled cDNAs and hybridization to *B. subtilis* whole-genome DNA microarrays (Eurogentec) were performed as described previously (Jürgen *et al*., 2005). The slides were scanned with a ScanArray Express scanner (PerkinElmer Life and Analytical Sciences). Quantitation of the signal and background intensities of the individual spots was carried out with the ScanArray Express image analysis software. Data were analyzed using the GeneSpring software (Agilent Technologies). Raw signal intensities were first transformed by intensity dependent LOWESS normalization. The mRNA abundance was considered to be different between DB104 and DB104(*Δsr1*) strains and between the anhydro-tetracycline induced strains DB104(*Δsr1*) and DB104(*Δsr1*, pWSR1) strains, if the ratio of the normalized Cy5 and Cy3 signal intensities was at least 2-fold in each of the two biological replicates.

REFERENCES

Afonyushkin, T., Vecerek, B., Moll, I., Bläsi, U., and Kaberdin, V.R. (2005) Both RNase E and RNAse III control the stability of *sodB* mRNA upon translational inhibition by the small regulatory RNA RyhB. *Nucleic Acids Res* **33**: 1678-1689.

Barrick, J.E., Sudarsan, N., Weinberg, Z., Ruzzo, W.L., Breaker, R.R. (2005) 6S RNA is a widespread regulator of eubacterial RNA polymerase that resembles an open promoter. *RNA* **11**: 744-784.

Boisset, S., Geissmann, T., Huntzinger, E., Fechter, P., Bendridi, N., Possedko, M., Chevalier, C., *et al.* (2007) *Staphylococcus aureus* RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. *Genes Dev* **21**: 1353-1366.

Bouvier, M., Sharma, C.M., Mika, F., Nierhaus, K.H., and Vogel, J. (2008) Small RNA binding to 5' mRNA coding region inhibits translational initiation. *Mol Cell* **32**: 827-837.

Brantl, S. (1994) The *copR* gene product of plasmid pIP501 acts as a transcriptional repressor at the essential *repR* promotor. *Mol Microbiol* **14**: 473-483.

Brantl, S. (2009) Bacterial chromosome-encoded small regulatory RNAs. *Future Microbiol* **4**: 85-103.

Brantl, S., Nuez, B., and Behnke, D. (1992) *In vitro* and *in vivo* analysis of transcription within the replication region of plasmid pIP501. *Mol Gen Genet* **234**: 105-112.

Christiansen, J.K., Nielsen, J.S., Ebersbach, T., Valentin-Hansen, P., Søgaard-Andersen, L., and Kallipolitis, B.H. (2006) Identification of small Hfq-binding RNAs in *Listeria monocytogenes*. *RNA* **12**: 1-14.

Commichau, F.M., Rothe, F.M., Herzberg, C., Wagner, E., Hellwig, D., Lehnik-Habrink, M., Hammer, E., *et al.* (2009) Novel activities of glycolytic enzymes in *Bacillus subtilis*: Interactions with essential proteins involved in mRNA processing. *Mol Cell Proteomics* **8**: 1350-1360.

De Lay, N., and Gottesman, S. (2009) The Crp-activated small noncoding regulatory RNA CyaR (RyeE) links nutritional status to group behavior. *J Bacteriol* **191**: 461-476.

Doan, T., and Aymerich; S. (2003) Regulation of the central glycolytic genes in *Bacillus subtilis*: binding of the repressor CggR to its single DNA target sequence is modulated by fructose-1,6-bisphosphate. *Mol Microbiol* **47**: 1709-1721.

Eiamphungporn, W., and Helmann, J. (2009) Extracytoplasmic function σ factors regulate expression of the *Bacillus subtilis yabE* gene via a cis-acting antisense RNA. *J. Bacteriol.* **191**: 1101-1105.

Even, S., Pellegrini, O., Zig, L., Labas, V., Vinh, J., Brechemmier-Baey, D., and Putzer, H. (2005) Ribonucleases J1 and J2: two novel endoribonucleases in *B. subtilis* with functional homology to *E. coli* RNase E. *Nucleic Acids Res* **33**: 22141-2152.

Evguenieva-Hackenberg, E., Schiltz, E., and Klug, G. (2002) Dehydrogenases from all three domains of life cleave RNA. *J Biol Chem* **277**: 46145-46150.

Faires, N., Tobisch, S., Bachem, S., Martin-Verstraete, I., Hecker, M., and Stülke, J. (1999) The catabolite control protein CcpA controls ammonium assimilation in *Bacillus subtilis. J Mol Microbiol Biotechnol* **1**: 141-148.

Gaballa, A., Antelmann, H., Aguilar, C., Khakh, S.-K., Song, K.-B., Smaldone, T., and Helmann, J.D. (2008) The *Bacillus subtilis* iron-sparing response is mediated by a Fur-regulated small RNA and three small, basic proteins. *Proc Natl Acad Sci USA* **105**: 11927-11932.

Geissmann, T., Chevalier, C., Cros, M.-J., Boisset, S., Fechter, P., Noirot, C., Schrenzel, J., François, P., Vandenesch, F., Gaspin, C., and Romby, P. (2009) A search for small noncoding RNAs in *Staphylococcus aureus* reveals a conserved sequence motif for regulation. *Nucleic Acids Res* **37**: 7239-7257.

Halfmann, A., Kovács, M., Hakenbeck, R., and Brückner, R. (2007) Identification of the genes directly controlled by the response regulator CiaR in *Streptococcus pneumoniae*: five out of 15 promoters drive expression of small non-coding RNAs. *Mol Microbiol* **66**: 110-126.

Heidrich, N., Chinali, A., Gerth, U., and Brantl, S. (2006) The small untranslated RNA SR1 from the *B. subtilis* genome is involved in the regulation of arginine catabolism. *Mol Microbiol* **62**: 520-536.

Heidrich, N., Moll, I., and Brantl, S. (2007) *In vitro* analysis of the interaction between the small RNA SR1 and its primary target *ahrC* mRNA. *Nucleic Acids Res* **35**: 331-346.

Hershberg, R., Altuvia, S., and Margalit, H. (2003) A survey of small RNA-encoding genes in *Escherichia coli*. *Nucleic Acids Res* **31**: 1813-1820.

Huntzinger, E., Boisset, S., Saveanu, C., Benito, Y., Geissmann, T., Namane, A., Lina, G., *et al.* (2005) *Staphylococcus aureaus* RNAIII and the endoribonuclease III coordinately regulate *spa* gene expression. *EMBO J* **24**: 824-835.

Jürgen, B., Tobisch, S., Wumpelmann, M., Gordes, D., Koch, A., Thurow, K., Albrecht, D., *et al.* (2005) Global expression profiling of *Bacillus subtilis* cells during industrial-close fed-batch fermentations with different nitrogen sources. *Biotechnol Bioeng* **92**: 277-298.

Kawamura, F., and Doi, R.H. (1984) Construction of a *Bacillus subtilis* double mutant deficient in extracellular alkaline and neutral proteases. *J Bacteriol* **160**: 442-444.

Kobayashi, K., Ehrlich, S.D., Albertini, A., Amati, G., Andersen, K.K., Arnaud, M., Asai, K., *et al.* (2003) Essential *Bacillus subtilis* genes. *Proc. Natl. Acad. Sci USA* **100**: 4678-4683.

Lease, R.A., Smith, D., McDonough, K., and Belfort, M. (2004) The small noncoding DsrA RNA is an acid resistance regulator in *Escherichia coli*. *J Bacteriol* **186**: 6179-6185.

Lee, M., Zhang, S., Saha, S., Santa Anna, S., Jiang, C., and Perkins J. (2001) RNA expression analysis using an antisense *Bacillus subtilis* genome array. *J Bacteriol* **183**: 7371-7380.

Licht, A., Preis, S. and Brantl, S. (2005) Implication of CcpN in the regulation of a novel untranslated RNA (SR1) in *B. subtilis*. *Mol Microbiol* **58**: 189-206.

Liu, J., Valencia-Sanchez, M. A., Hannon, G. J., and Parker, R. (2005) MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat Cell Biol* **7**: 719-723.

Ludwig, H., Homuth, G., Schmallisch, M., Dyka, F.M., Hecker, M., and Stülke, J. (2001) Transcription of glycolytic genes and opeons in *Bacillus subtilis*: evidence for the presence of multiple levels of control of the *gapA* operon. *Mol Microbiol* **41**: 409-422.

Majdalani, N., Hernandez, D., and Gottesman S. (2002) Regulation and mode of action of the second small RNA activator of RpoS translation, RprA. *Mol Microbiol* **46**: 813-826.

Mandin, P., Repoila, F., Vergassola, M., Geissmann, T., and Cossart, P. (2007) Identification of new nonconding RNAs in *Listeria monocytogenes* and prediction of mRNA targets. *Nucleic Acids Res* **35**: 962-974.

Mangold, M., Siller, M., Roppenser, B., Vlaminck, B.J., Penfound T.A., Klein, R., Novak, R., *et al.* (2004) Synthesis of group A streptococcal virulence factors is controlled by a regulatory RNA molecule. *Mol Microbiol* **53**: 1515-1527.

Massé; E., Salvail, H., Desnoyers, G., and Arguin, M. (2007) Small RNAs controlling iron metabolism. *Curr Opin Microbiol* **10**: 140-145.

Meinken, C., Blencke, H.-M., Ludwig, H., and Stülke, J. (2003) Expression of the glycolytic *gapA* operon in *Bacillus subtilis*: differential synthesis of proteins encoded by the operon. *Microbiol* **149**: 751-761.

Morfeldt, E., Taylor, D., von Gabain, A., and Arvidson, S. (1995) Activation of alpha-toxin translation in *Staphylococcus aureus* by the trans-encoded antisense RNA, RNAIII. *EMBO J* **14**: 4569-4577.

Morita, T., Maki, K., and Aiba, H. (2005). RNase E-based ribonucleoprotein complexes: mechanical basis of mRNA destabilization mediated by bacterial non-coding RNAs. *Genes Dev* **19**: 2176-2186.

Nielsen, J.S., Olsen, A.S., Bonde, M., Valentin-Hansen, P., and Kallipolitis, B.H. (2008) Identification of a sigma B-dependent small noncoding RNA in *Listeria monocytogenes*. *J Bacteriol* **190**: 6264-6270.

Nielsen, J.S., Kristensen Lei, L., Ebersbach, T., Olsen, A. S., Klitgaard, J. K., Valentin-Hansen, P., and Kallipolitis, B. H. (2010) Defining a role for Hfq in Gram-positive bacteria: evidence for Hfq-dependent antisense regulation in *Listeria monocytogenes*. *Nucleic Acids Res* **38**: 907-919.

Opdyke, J.A., Kang, J.G., and Storz, G. (2004) GadY, a small-RNA regulator of acid response genes in *Escherichia coli*. *J Bacteriol* **186**: 6698-6705.

Papenfort, K., Pfeiffer, V., Sonawane, A., Hinton, J.C.D., and Vogel, J. (2008) Systematic deletion of *Salmonella* small RNA genes identifies CyaR, a conserved CRP-dependent riboregulator of OmpX synthesis. *Mol Microbiol* **68**: 890-906.

Pfaffl, M.W. (2002) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. **29**: 2002-2007.

Pichon, C., and Felden, B. (2005) Small RNA genes expressed from *Staphyloccoccus aureus* genomic and pathogenicity islands with specific expression among pathogenic strains. *Proc Natl Acad Sci USA* **102**: 14249-14254.

Preis, H., Eckart, R.A., Gudipati, R.K., Heidrich, N., and Brantl, S. (2009) CodY activates the transcription of a small RNA in *Bacillus subtilis*. *J Bacteriol* **191**: 5446-5457.

Reichenbach, B., Maes, A., Kalamorz, F., Hajnsdorf, E., and Görke, B. (2008) The small RNA GlmY acts upstream of the sRNA GlmZ in the activation of *glmS* expression and is subject to regulation by polyadenylation in *Escherichia coli*. *Nucleic Acids Res* **36**: 2570-2780.

Repoila, F., and Darfeuille, F. (2009) Small regulatory non-coding RNAs in bacteria: physiology and mechanistic aspects. *Biol Cell* **101**: 117-131.

Saito, S., Kakeshita, H., and Nakamura, K. (2009) Novel small RNA-encoding genes in the intergenic regions of *Bacillus subtilis*. *Gene* **428**: 2-8.

Sharma, C.M., Darfeuille, F., Plantinga, T.H., and Vogel, J. (2007) A small RNA regulates multiple ABC transporter mRNAs by targeting C/A-rich elements inside and upstream of ribosome binding sites. *Genes Dev* **21**: 2804-2817.

Silvaggi, J.M., Perkins, J.B., and Losick, R. (2005) Small untranslated RNA antitoxin in *Bacillus subtilis*. *J Bacteriol* **187**: 6641-6650.

Silvaggi, J.M., Perkins, J.B., and Losick, R. (2006) Genes for small, noncoding RNAs under sporulation control in *Bacillus subtilis*. *J Bacteriol* **188**: 532-541.

Sonnleitner, E., Sorger-Domenigg, T., Madej, M.J., Findeiss, S., Hackermüller, J., Hüttenhofer, A., Stadler, P.F., Bläsi, U. and Moll, I. (2008) Detection of small RNAs in *Pseudomonas aeruginosa* by RNomics and structure-based bioinformatic tools. *Microbiol.* **154**: 3175-3187.

Steinmetzer, K., and Brantl, S. (1997) Plasmid pIP501 encoded transcriptional repressor CopR binds asymmetrically at two consecutive major grooves of the DNA. *J Mol Biol* 269: 684-693.

Storz, G., Altuvia, S., and Wassarman, K.M. (2005) An abundance of RNA regulators. *Annu Rev Biochem* **74**: 199-217.

Stülke, J., Martin-Verstraete, I., Zagorec, M., Rose, M., Klier, A., and Rapoport, G. (1997) Induction of the *Bacillus subtilis ptsGHI* operon by glucose is controlled by a novel antiterminator, GlcT. *Mol Microbiol* **25**: 65-78.

Toledo-Arana, A., Repoila, F. and Cossart, P. (2007) Small noncoding RNAs controlling pathogenesis. *Curr Opin Microbiol* **10**: 182-188.

Urban, J.H., and Vogel, J. (2008) Two seemingly homologous noncoding RNAs act hierarchically to activate *glmS* mRNA translation. *PloS Biol* 6: e64.

Valentin-Hansen, P., Eriksen, M., and Udesen, C. (2004) The bacterial Sm-like protein Hfq: a key player in RNA transactions. *Mol Microbiol* **51**: 1525-1533.

Vogel, J., and Papenfort, K. (2006) Small noncoding RNAs and the bacterial outer membrane. *Curr Opin Microbiol* **9**: 605-611.

Wadler, C.S., and Vanderpool, C.K. (2007) A dual function for a bacterial small RNA: SgrS performs base pairing dependent regulation and encodes a functional polypeptide. *Proc Natl Acad Sci USA* **104**: 20454-2459.

Waters, L.S., and Storz, G. (2009) Regulatory RNAs in bacteria. *Cell* **136**: 615-628.

Zhang, A., Wassarman, K.M., Ortega, J., Steven, A.C., and Storz, G. (2002) The Sm-like Hfq protein increases OxyS RNA interaction with target mRNAs. *Mol Cell* **9**: 11-22.

ACKNOWLEDGEMENTS

The authors thank Jörg Stülke, Göttingen, for the polyclonal antisera against GapA and CggR, for strains GP231 and GP550 and for valuable and critical comments. We are grateful to Berhard Schlott, FLI Jena, for the N-terminal sequencing of GapA and to Diana Imhof, FSU Jena, for synthesizing SR1P. We thank Isabella Moll, Vienna, for the 30S extracts. We acknowledge Harald Putzer, Paris, for strains SSB344 and SSB340 and D. Bechhofer, New York, for strains BG503, BG119 and BG218. We thank Jette Anklam, Greifswald, and and Yvonne Leuschner, HKI, Jena, for excellent technical assistance. This work was supported by grant Br1552/6-3 from the Deutsche Forschungsgemeinschaft to S. B. and by grant 03ZIK011 from the Bundesministerium für Bildung und Forschung to U. M. Nadja Heidrich was partially financed by grant BR1552/7-1 of the Research Focus Progamme SPP1258/1 from the Deutsche Forschungsgemeinschaft to S. B.

Figure Legends

Figure 1. Schematic representation of the transcripts originating from the *gapA* operon

The open reading frames of the *gapA* operon are shown as boxes. Short arrows indicate the two promoters, and circles the two terminators. P denotes the processing site 60 codons upstream of the *cggR* stop codon. Transcripts are shown as long arrows with black circles at their ends for pI transcripts (upstream of *cggR*) and a grey circle for the pII transcript. Transcript ends that result from processing are indicated by a dashed black line. The six transcripts reported previously are numbered with Roman numerals. The representation is based on the results of Ludwig *et al.*, 2001 and Fig. 2 in Meinken *et al.*, 2003.

Figure 2. Effects of SR1, Hfq and CcpN on the amounts of the *gapA* and *cggR* transcripts

Cells were grown at 37 °C in TY, samples taken at the indicated OD_{560} and used for the preparation of total RNA. The RNA was treated with glyoxal, separated on 1.5 % agarose gels, blotted onto nylon membranes and hybridized with [α-^{32}P]-dATP-labelled DNA probes specific for *gapA* and *cggR*, respectively. Filters were reprobed with a probe against SR1 prepared as described previously (Heidrich *et al.*, 2006). To allow for the correction of loading errors, filters were reprobed with a [γ^{32}-P]-ATP-labelled oligonucleotide specific for 23S rRNA as described (Heidrich *et al.*, 2006). Autoradiographs of the Northern blots are shown. The size of the detected transcripts is indicated, U, unprocessed bicistronic transcript, P, processed transcript.

A) comparison of wild-type, *sr1* knockout and *hfq* knockout strain at two different optical densities.

B) Inducible overexpression of *sr1* from a multicopy plasmid complements the defect of the *sr1* knockout strain. pWH353, empty vector; pWSR1, *sr1* overexpression vector. Strains containing pWH353 or pWSR1 were induced with anhydro-tetracycline for 15 min.

Figure 3. Effects of SR1 and Hfq on the intracellular amounts of GapA and CggR

Cells were grown in TY medium to OD_{560} = 5.0 (maximum expression of SR1), protein lysates were obtained by sonication and subsequent centrifugation, and separated on 12.5 % SDS polyacrylamide gels. Western blotting and quantification of the gels were performed as described in *Experimental Procedures*. GapA and CggR were detected by rabbit polyclonal antibodies. As an internal control, a polyclonal rabbit antiserum against CcpN was used. The numbers below the gels indicate the relative amounts of GapA and CggR calculated by using the internal control CcpN, a protein that is constitutively expressed independent of growth phase or the presence of Hfq or SR1.

Figure 4. Complementarity between *gapA* operon and SR1

A) Schematic representation of the *cggR-gapA* region predicted to be complementary to SR1

B) Secondary structure of SR1 with highlighted region predicted to be complementary to *gapA* (left) and with small ORF SR1P (right).

Figure 5. Toeprinting analysis

Ternary complex formation upon addition of different amounts of SR1 to $gapA_{160}$ mRNA, $cggR\text{-}gapA_{258}$ mRNA, and, as control, $ahrC_{196}$-mRNA (for details, see *Experimental Procedures* and *Results*).

In *gapA* and *cggR-gapA*, the AUG codons are labelled by black dots. The toeprint signal relative to A of the start codon is marked. Addition of 30S ribosomal subunits and initiator tRNA (lanes 2 and 3) as well as increasing concentrations (50-fold and 100-fold excess) of the regulatory RNAs (lanes 4 and 5) are indicated above the gels. In all cases, the RNA sequencing reactions (C U G A) were carried out with the same end-labelled oligonucleotide as in the toeprint analysis assays.

For all three toeprints, the same SR1 preparation was used.

A) Toeprinting analysis with *ahrC* mRNA, $gapA_{160}$-mRNA and $cggR\text{-}gapA_{258}$ mRNA

Autoradiographs of ternary complex formation in the absence or presence of SR1 are shown.

B) A schematic representation indicates the RNA species used as templates. The processing site and the *gapA* SD sequence are shown as black and grey rectangles, respectively.

C) Calculation of the relative toeprints

Figure 6. Stability of *gapA* mRNA in the presence and absence of SR1

DB104 and DB104(*Δsr1::cat*) were grown in TY till OD_{560} = 5.0, treated with 200 µg/ml rifampicin, time samples taken, and total RNA prepared.

A Northern blot with time samples of DB104. The autoradiograph of the gel was quantified with TINA-BAS 2.09 and the half-life of *gapA* mRNA determined to be 11 min. No determination could be performed with the *Δsr1* strain since *gapA* is almost not visible (see Fig. 2).

B The RNA of wild-type and *sr1* knockout strain was further purified with DNase and RNeasy columns and subjected to qRT-PCR. The diagram shows the amounts of *gapA* mRNA from DB104 and DB104(*Δsr1::cat*) at different time points. The data were averaged from three independent determinations.

Figure 7. Effect of SR1 mutants on *gapA* mRNA

A) Schematic overview on SR1 mutants M1 to M13

In the wild-type sequence, the region predicted to be complementary to *gapA* is shaded with grey. In the mutants, the nucleotides altered are encircled. Below the mutant designations, the relative amounts of *gapA*-operon mRNA calculated from the gels in 7 B) according to *Experimental procedures* are indicated.

+ and – indicate the ability of the corresponding mutants to complement the *Δsr1* effect on *gapA*.

B) Ability of the SR1 mutants to complement the effects of the *sr1* knockout strain on the *gapA* operon

B. subtilis DB104(*Δsr1::cat*) was transformed with the pWSR1 derivatives expressing mutant SR1 species, grown at 37° C in TY medium till OD_{560} = 5.0 and used for the preparation of total RNA. The RNA was treated with glyoxal, separated on 1.5 % agarose gels, blotted onto nylon membranes and hybridized with [α-^{32}P]-dATP-labelled DNA probe specific for *gapA*. Filters were reprobed against SR1 and against 23S rRNA as loading control as in Fig. 2. Autoradiographs of the Northern blots are shown.

C) Analysis of FLAG-tagged SR1P

Top: Schematic representation of mutants M14 and M15 that carry the entire wild-type SR1 sequence, however, a C-terminal or N-terminal 3x FLAG-tag (shaded with dark-grey) added to the SR1P-ORF. Bottom: Autoradiograph of Northern blot used to assay the ability of M14 and M15 to complement the *Δsr1* effect.

Figure 8. Western blotting detects FLAG-tagged SR1P from multicopy plasmid

A) *B. subtilis* strains DB104(*Δsr1::cat*) with the empty vector pWH353 or with the overexpression plasmids pWSR1/M14 (SR1P-C-FLAG) or M15 (N-FLAG-SR1P) were grown in TY till OD_{560} = 5.0, induced for 15 min with anhydro-tetracycline, 10 ml cells were pelleted, resolved in 0.5 ml buffer and subjected to sonication. 5 and 20 µl of each crude extract were separated on a 17.5 % SDS-Tris-glycine-PAA gel and Western blotted with anti-FLAG antibodies as described in *Experimental Procedures*.

B) Comparison of the amino acid sequences of mutated SR1P species with the wild-type sequence. Altered residues are shaded in grey. + indicates functionality *in vivo*.

Figure 9. The SR1-encoded peptide SR1P is responsible for the effect on the *gapA* operon

A) Top: Overview on the nucleotide-replacements introduced to alter the wobble positions of *sr1p* and of the 5' end of SR1.

Bottom: Schematic overview on mutants pWSR1/M19, pWSR1/M21, pWSR1/M22, pWSR1/M27, pWSR1/M29 and pWSR1/M30.

B) Ability of mutants pWSR1/M19, M20, M21, M22, M27, M29 and M30 to complement the *sr1* defect on *gapA*

Autoradiographs of Northern blots are shown

C) Top: schematic drawing of basepairing between *gapAM1* and SR1/M13, middle: N-termini of mutated and wild-type *gapA*, bottom: Analysis of the compensatory mutation *gapAM1* in the genome in the absence and presence of plasmid pWSR1/M13 carrying the complementary mutation.

Autoradiographs of Northern blots are shown.

Figure 10. SR1P interacts with GapA

A) Schematic representation of the plasmids used for the identification of an interaction partner of SR1P.

B) DB104(*Δsr1::cat)* containing one of the plasmids pWSR1, pWSR1/M25, pWSR1/M26 or pWSR1/31 was grown in TY till stationary phase, induced with anhydro-tetracyline, harvested, disrupted, and protein extracts passed either through streptactin- or Ni-NTA-columns, depending on the added tag as described in *Experimental procedures*. The non-tagged peptide expressed from pWSR1 was also passed in parallel through both columns and used as negative control. As a second negative control, DB104(*ΔccpN::cat*, pOU75H) expressing the C-terminally His_6-tagged heterologous protein CcpN, was used. The elution profiles with crude extract, flow-through and all washing and elution fractions can be seen in Fig. 3S.

An aliquot of each elution fraction E3 was separated on a 17.5 % SDS-Tris-glycine PAA gel along with a size marker (M) and stained with Coomassie blue. The 45 kD bands in the fractions of pWSR1/M25 and pWSR1/M31 were blotted onto PVDF membrane and the sequence of the 7 N-terminal amino acids determined to be AVKVGIN (corresponding to GapA).

C) Western blot of the gel shown in B) with a polyclonal antiserum against GapA.

D) Coomassie blue stained 20 % SDS-Tris-Tricine PAA gel with a chemically synthesized SR1P and the elution fractions of pWSR1 (non-tagged peptide) and pWSR1/M25 from the streptactin column.

Table 1: Plasmids used in this study

Plasmid	Description	Reference
pINT1	integration vector for replacement of *sr1* gene by CAT	Licht *et al.*, 2005
pFRONT	pUC19 with 800 bp upstream of *sr1* promoter	Licht *et al.*, 2005
pCBACK	pUC19 with cat gene and 800 bp downstream of *sr1*	Licht *et al.*, 2005
pUCSR1	pUC19 with promoterless *sr1* gene	Licht *et al.*, 2005
pWSR1	*B. subtilis* vector for inducibile induction of SR1 transcription with tetracycline, Km^R	Licht *et al.*, 2005
pGP231	vector with *gapA* gene as Pst/NcoI fragment	Stülke
pAC6	vector for integration of transcriptional *lacZ* fusions	Stülke *et al.*, 1997
pGGA1	integration vector for *amyE* gene with *ahrC* insert, heat-stable β-galactosidase from *B. stearothermophilus* without SD for translational fusions, Km^R, Ap^R	Heidrich *et al.*, 2006
pTaq7	pUC19 with MLS^R gene of pE194	Brantl, unpublished
pACC1	pAC6 with *cggR-lacZ* transcriptional fusion	this study
pEBACK1	as pCBACK, but with MLS^R gene of pE194	this study
pINT1E	as pINT1, but with MLS^R gene	this study
pWSR1/M1	pWSR1 with 11 bp exchange in loop 1 of SR1	this study
pWSR1/M2	pWSR1 with 5 bp exchange in loop 1 of SR1	this study
pWSR1/M3	pWSR1 with 4 bp exchange in loop 1 of SR1	this study
pWSR1/M5	pWSR1 with internal deletion in SR1 (nt 113-172)	this study
pWSR1/M6	pWSR1 with 2 bp exchange in loop 1 of SR1	this study
pWSR1/M7	pWSR1 with 3 bp exchanges at codons 28/29	this study
pWSR1/M8	pWSR1 with 2 bp exchange at codon 37	this study
pWSR1/M9	pWSR1 with 6 bp exchange in loop 1 of SR1	this study
pWSR1/M12	pWSR1 with UAG instead AUG at *sr1p*	this study
pWSR1/M13	pWSR1 with 4 bp exchange in loop 1 of SR1	this study
pWSR1/M14	pWSR1 with C-terminal FLAG-Tag at *sr1p*	this study
pWSR1/M15	pWSR1 with N-terminal FLAG-Tag at *sr1p*	this study
pWSR1/M19*	pWSR1 with 28 single bp exchanges in *sr1p*	this study
pWSR1/M20*	pWSR1 with heterologous terminator from BsrF	this study
pWSR1/M21*	pWSR1 with 10 single bp exchanges in *sr1p* and BsrF transcription terminator	this study
pWSR1/M22*	pWSR1 with nt exchanges in 5′ untranslated region of *sr1*	this study
pWSR1/M25*	pWSR1 with C-terminal Strep-Tag	this study
pWSR1/M26	pSWR1/M13 with C-terminalem Strept-Tag	this study
pWSR1/M27	pWSR1 with stop codon after 28^{th} codon of SR1P	this study

pWSR1/M29	pWSR1 with stop codon after 30th codon of SR1P	this study
pWSR1/M30	pWSR1 with stop codon after 34th codon of SR1P	this study
pWSR1/M31*	pWSR1 with C-terminal His_6-Tag	this study
pBLK1	pBR322 with gaB and Km^R gene from pGF-BgaB	this study
pCGR2	pGGA1 with *gapA-gaB* translational fusion containing the *cggR* gene and its native promoter	this study
pCGR4	pGGA1 with *gapA-gaB* translational fusion containing the *gapA* under promoter pIII	this study
pLKG1	pBLK1 with BamHI/EcoRI fragment of pCGR2	this study
pUCGAPM1	pUC19 with mutated *gapA* gene	this study
pGAPM1	pGP231 with mutated *gapA* gene of pUCGAPM	this study

*Only the SR1 RNA, but not the peptide SR1P is mutated.

Table 2: Results of the transcriptome analyses

Gene	Ratio *Δsr1*/wild-type	Ratio overexpression SR1/*Δsr1*
gapA	0.04	33.80
cggR	0.01	46.15
pgm	0.22	3.59
tpiA	0.23	2.76
eno	0.24	3.37
pgk	0.30	2.98

The values for the overexpression strain represent averages of four independent experiments performed with four independently grown cultures, the values for the knockout/wild-type strain have been determined twice from two independently grown cultures.

Table 3: β-galactosidase activities

Strain	5′ *cggR/gapA* sequence	β-galactosidase activity (Miller units)
DB104::pACC1	p_{CggR}, transcriptional fusion	1349±29
DB104::pACC1 (*Δsr1::phleo*)	p_{CggR}, transcriptional fusion	1301±17
DB104::pAC6	no	1.8±0.4
DB104::pAC6 (*Δsr1::phleo*)	no	2.2±0.5
DB104::pCGR2	p_{CggR}, *cggR*, 50, SD + codons *gapA*	105±11

DB104::pCGR2 (*Δsr1::cat*)	p_{CggR}, *cggR*, 50, SD + codons *gapA*		92±8
DB104:: pCGR4	pIII, SD + 50 codons *gapA*		50±3.5
DB104::pCGR4 (*Δsr1::cat*)	pIII, SD + 50 codons *gapA*		57±6
DB104::pGF-BgaB	no		2.5±0.4
DB104::pGF-BgaB (*Δsr1::cat*)	no		2.1±0.6
DB104::pLKG1	p_{CggR}, *cggR*, SD + 50 codons *gapA*	76±5	
DB104::pLKG1 (*Δsr1::cat*)	p_{CggR}, *cggR*, SD + 50 codons *gapA*	72±7	
DB104::pBLK1	no		1.9±0.5
DB104::pBLK1 (*Δsr1::cat*)	no		2.3±0.2

All values represent averages of at least three independent determinations. Plasmids pAC6, pGF-BgaB and pBLK1 are the empty vectors. All translational fusions contain *cggR/gapA* sequences fused in frame to the promoterless, SD less *gaB* gene encoding the heat-stable β-galactosidase of *B. stearothermophilus.* Whereas plasmids pCGR2 and pCGR4 were inserted into the *amyE* locus of the *B. subtilis* chromosome by double crossing over, plasmid pLKG1 was inserted into its natural location by single crossing over. β-galactosidase activities were measured at 28° C for pACC1 and pAC6 and at 55° C for the other integrants.

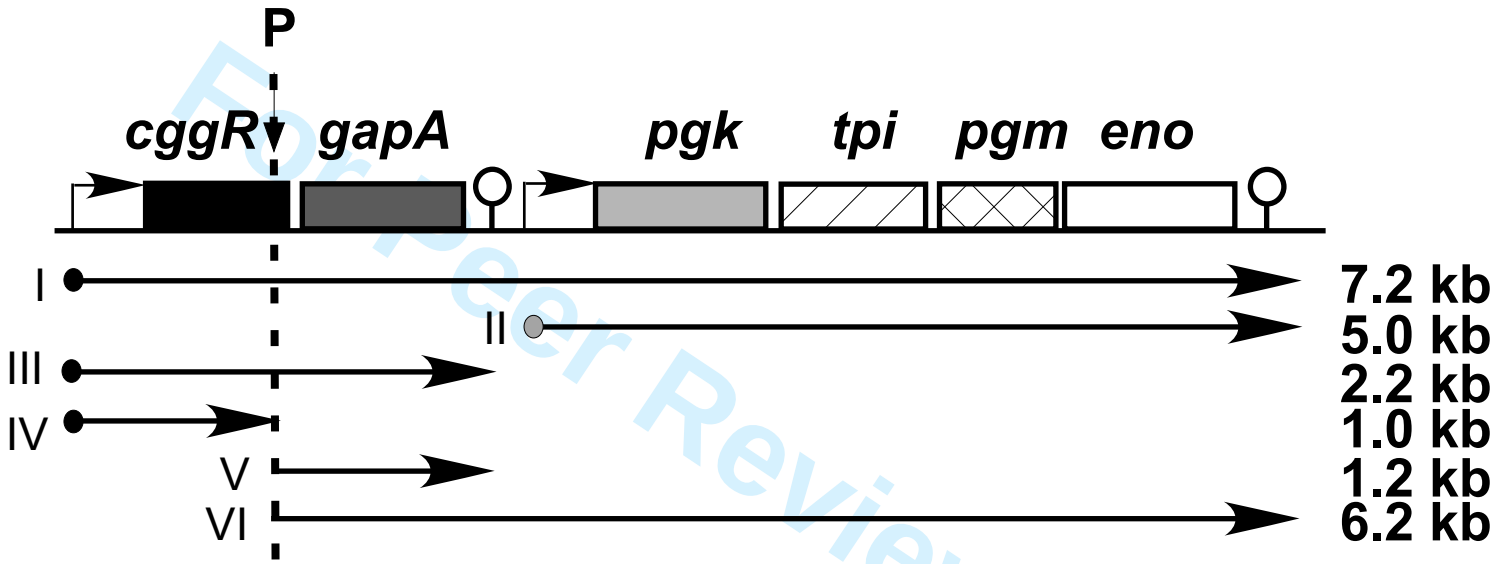


Fig. 1 Gimpel *et al.*

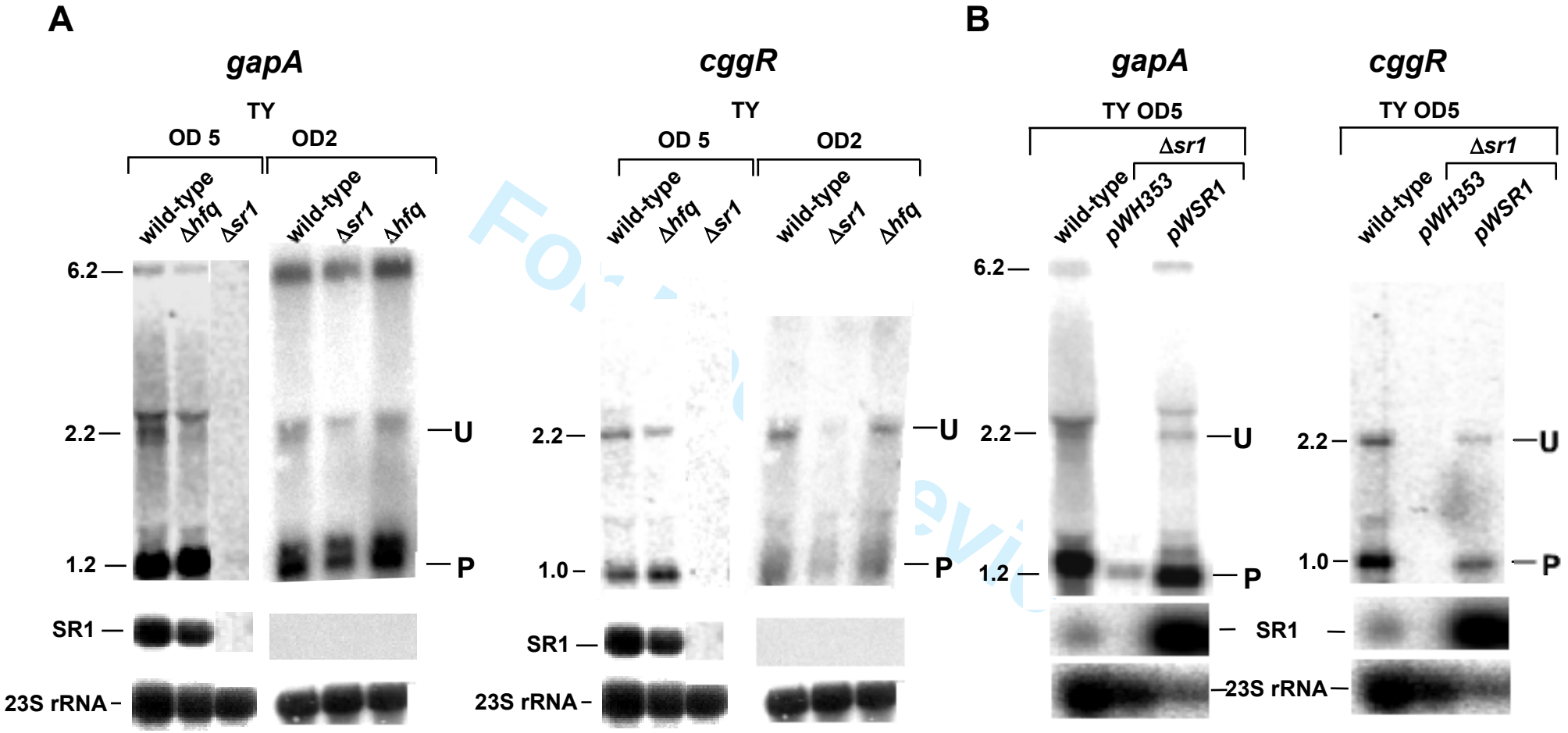


Fig. 2 Gimpel *et al.*

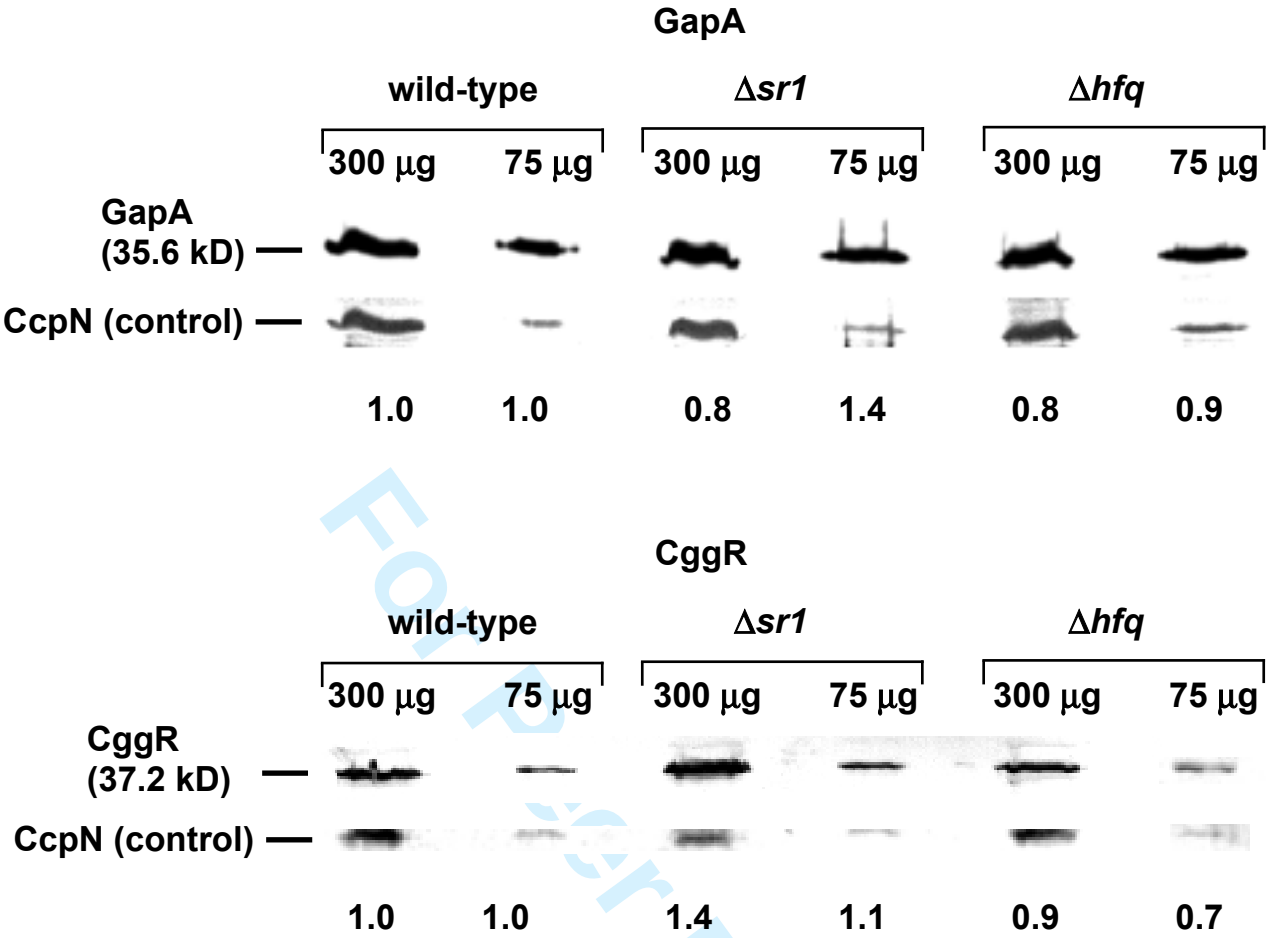
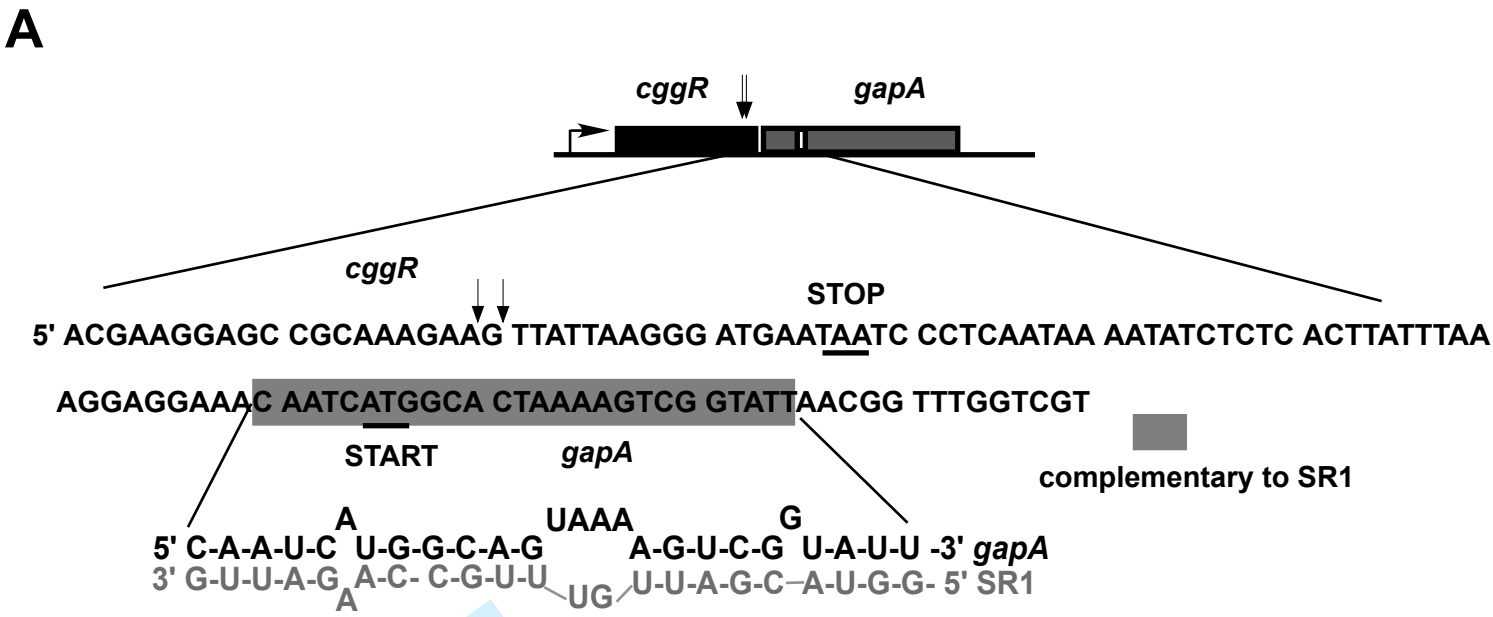


Fig. 3 Gimpel *et al.*



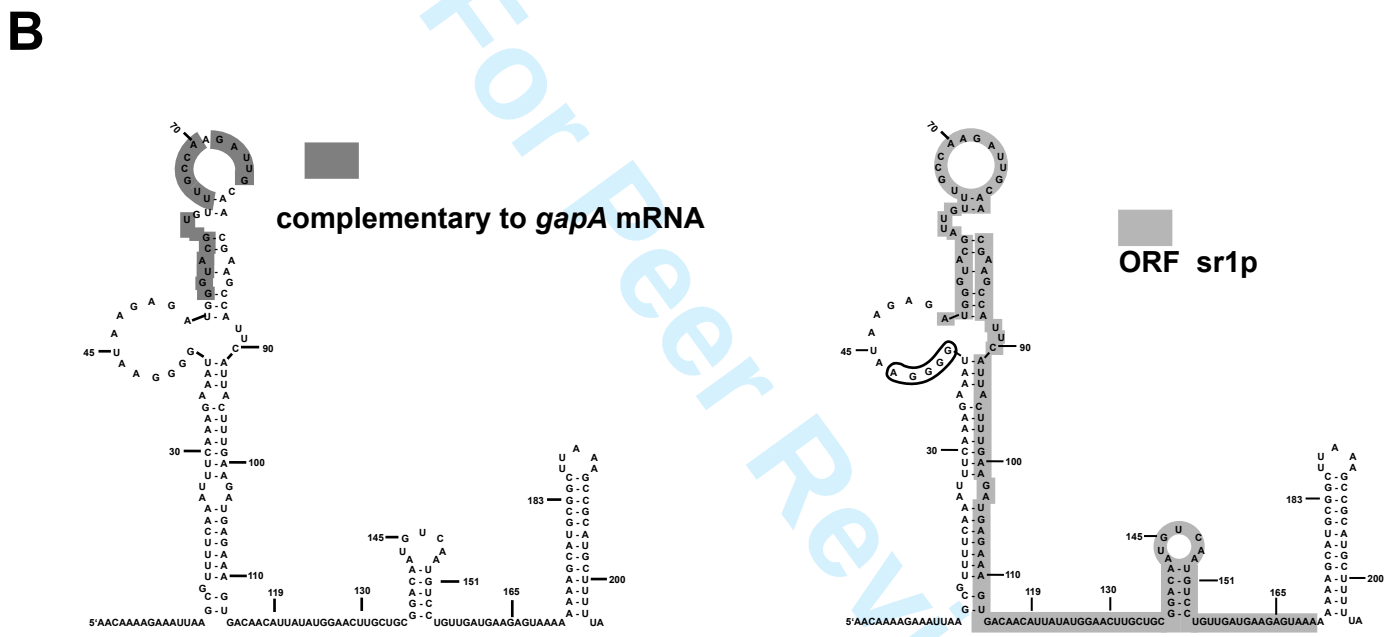


Fig. 4 *Gimpel et al.*

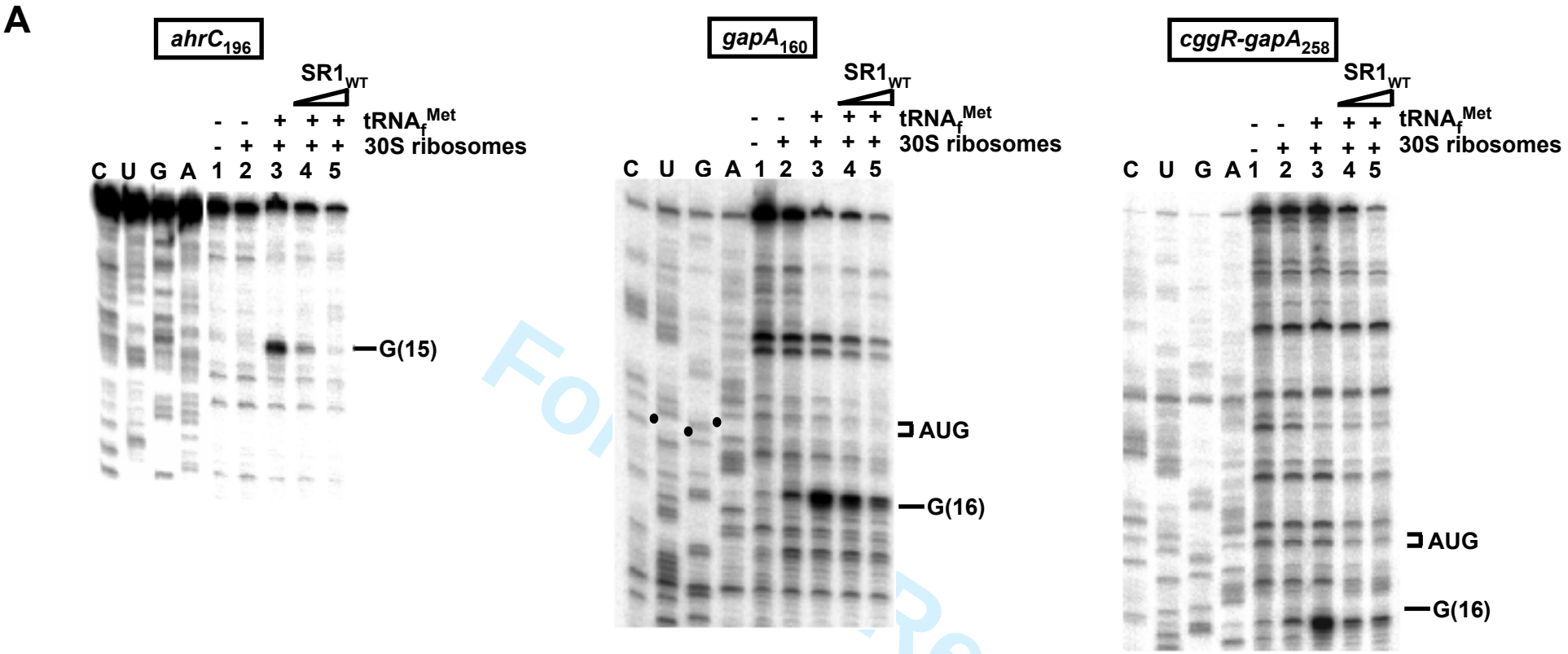


Fig. 5 Gimpel *et al.*

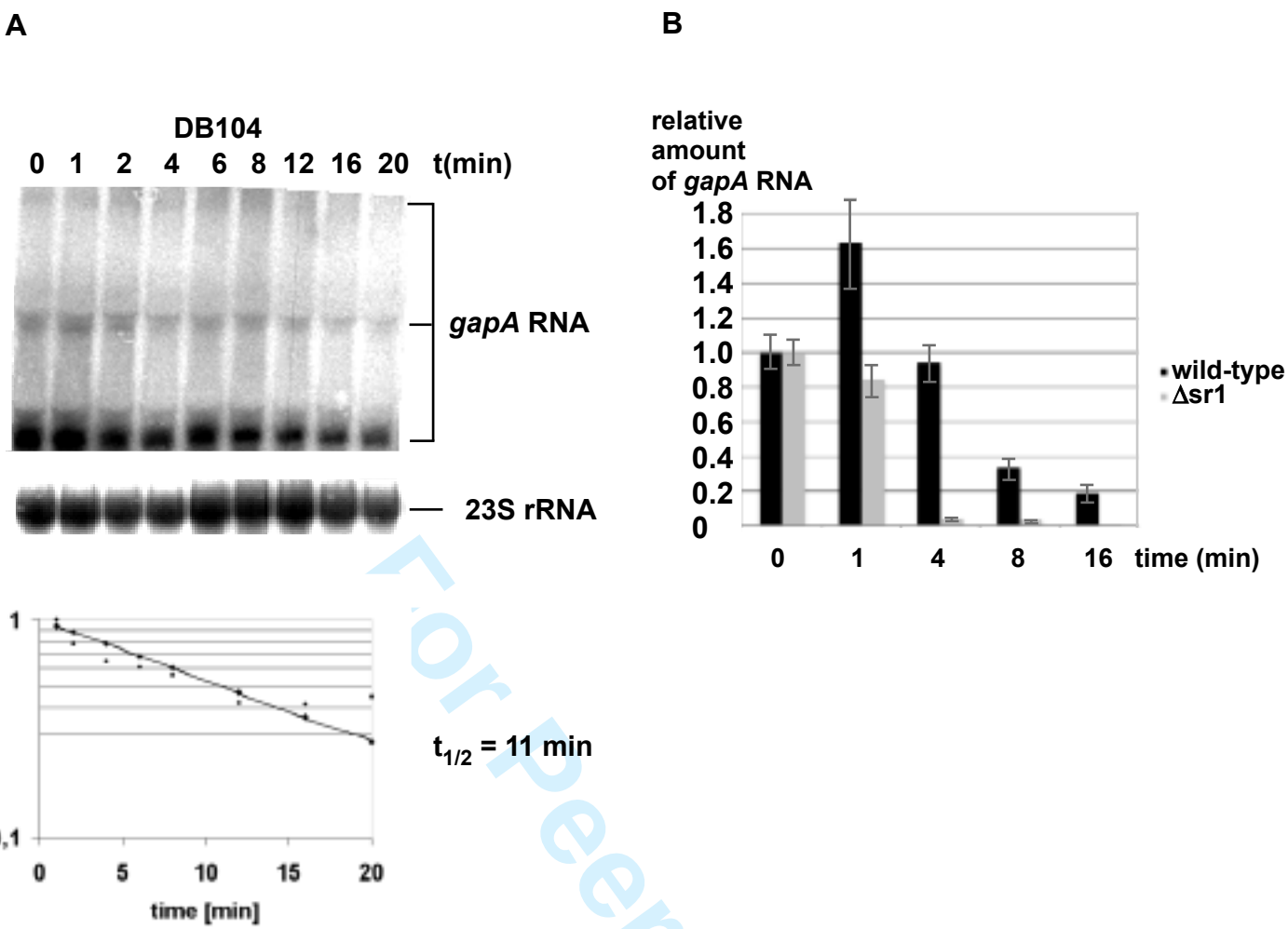
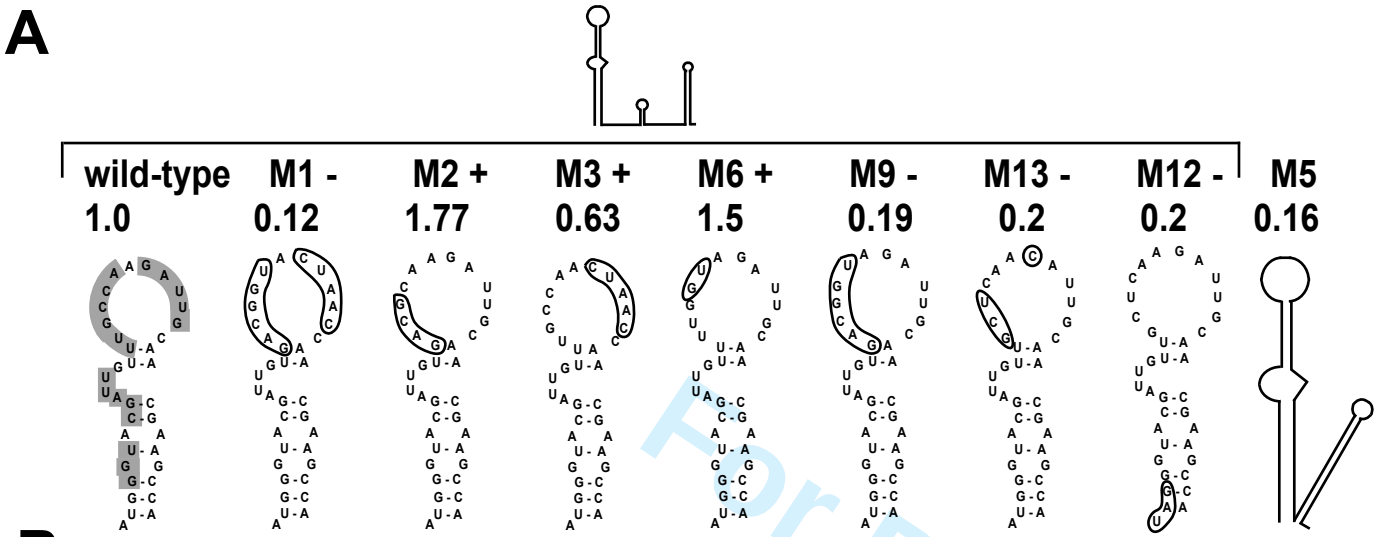
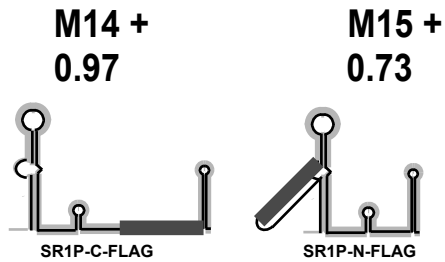
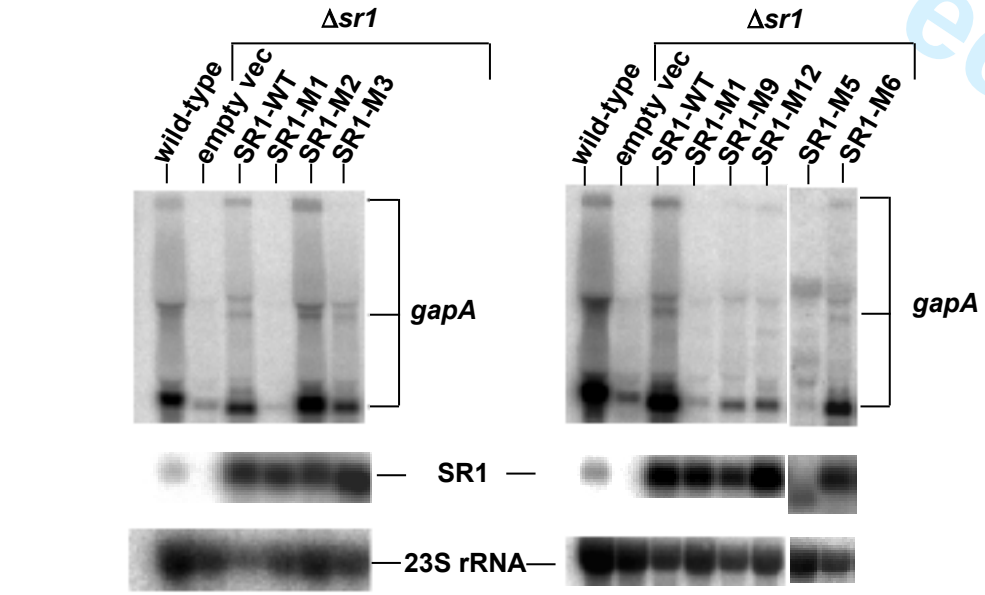


Fig. 6 Gimpel *et al.*







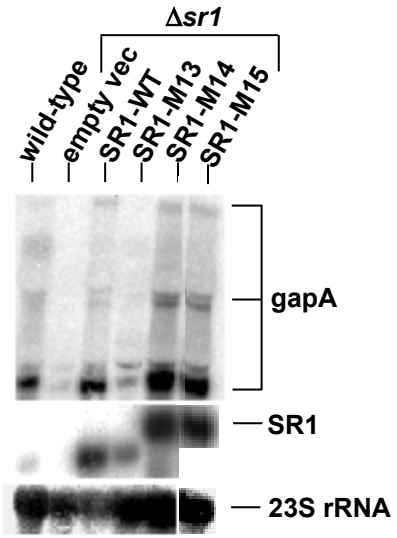
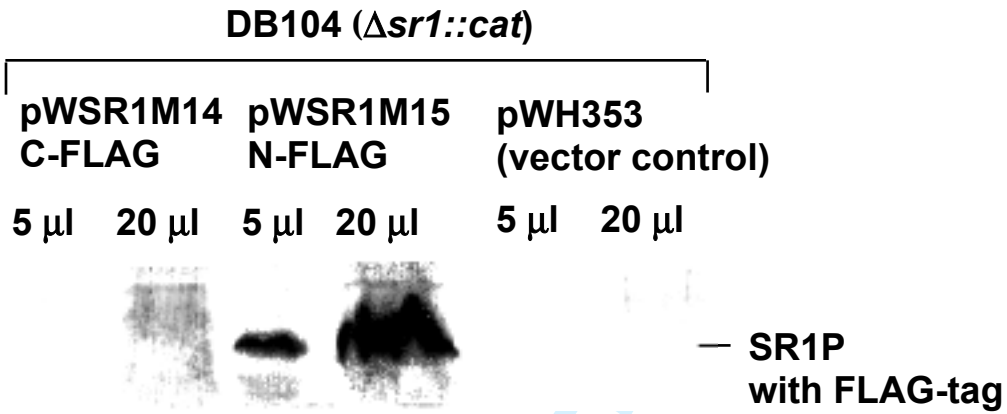


Fig. 7 Gimpel *et al.*

A



B

Alterations in SR1P in different mutants

aa positions	1								10	11	
wild-type	M	G	T	I	V	C	Q	D	C	N	+
M1	M	G	T	I	V	T	V	L	T	N	-
M2	M	G	T	I	V	T	Q	D	C	N	+
M3	M	G	T	I	V	C	Q	L	T	N	+
M6	M	G	T	I	V	C	V	D	C	N	+
M9	M	G	T	I	V	T	V	D	C	N	-
M13	M	G	T	I	V	A	Q	H	C	N	-

aa positions	21								30	31							39		
wild-type	V	T	T	L	Y	G	T	C	C	G	Q	C	G	C	P	V	D	E	E	+
M7	V	T	T	L	Y	G	T	S	S	G	Q	C	G	C	P	V	D	E	E	+
M8	V	T	T	L	Y	G	T	C	C	G	Q	C	G	C	P	V	S	E	E	+

Fig. 8 Gimpel *et al.*

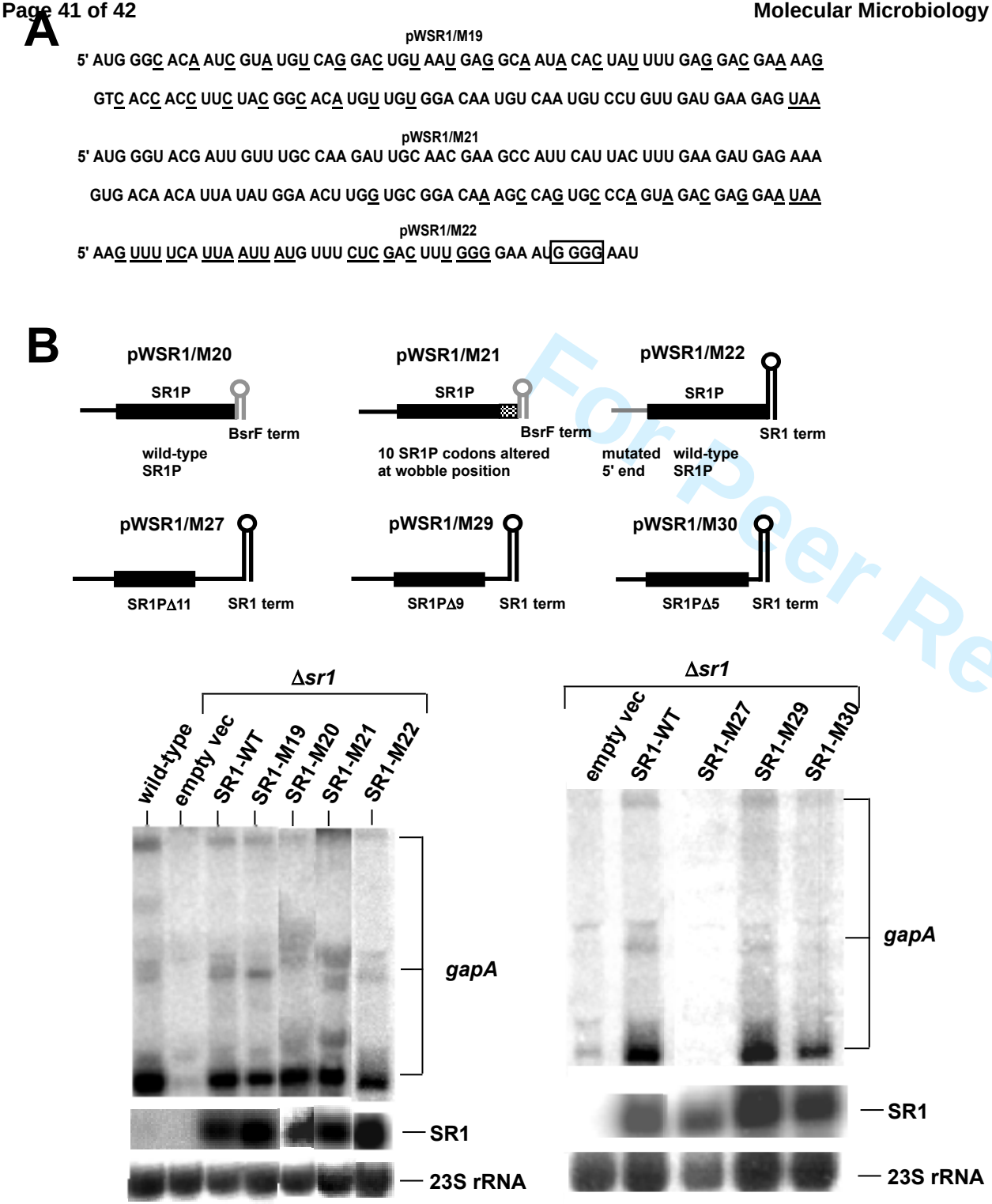
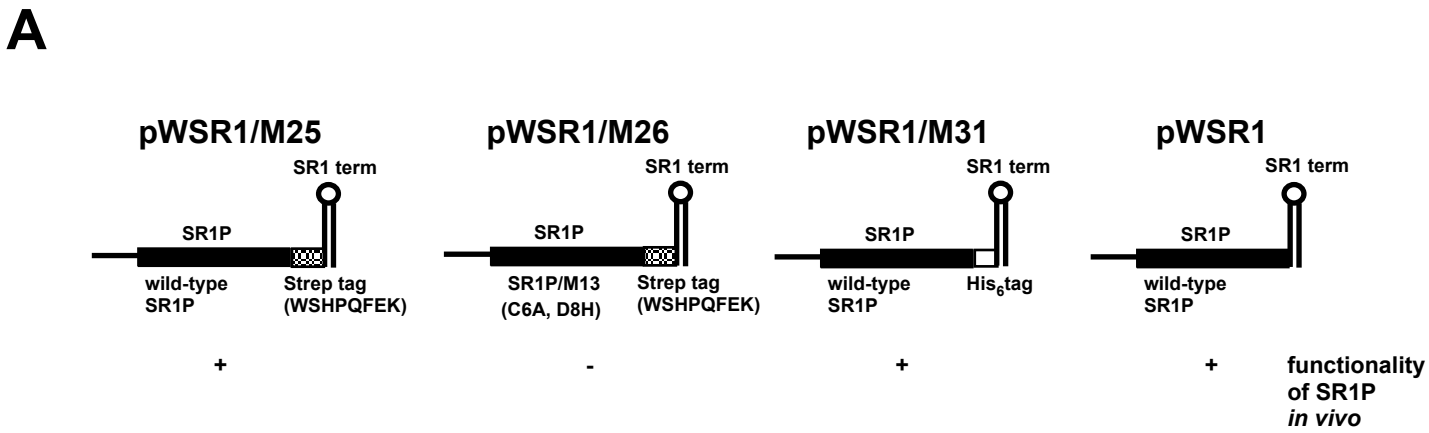
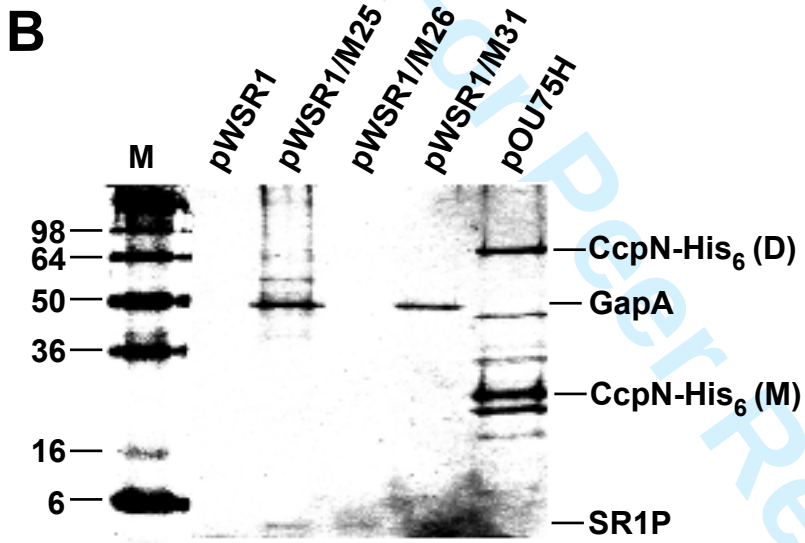
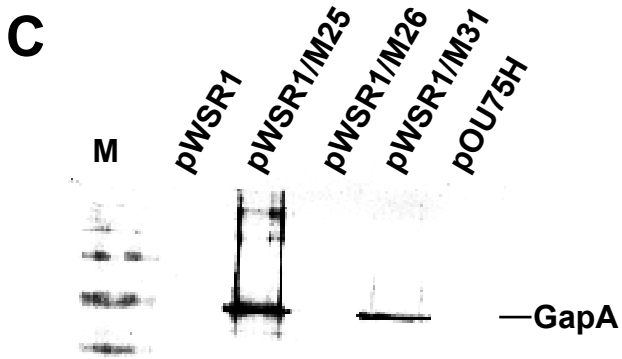


Fig. 9 Gimpel *et al.*







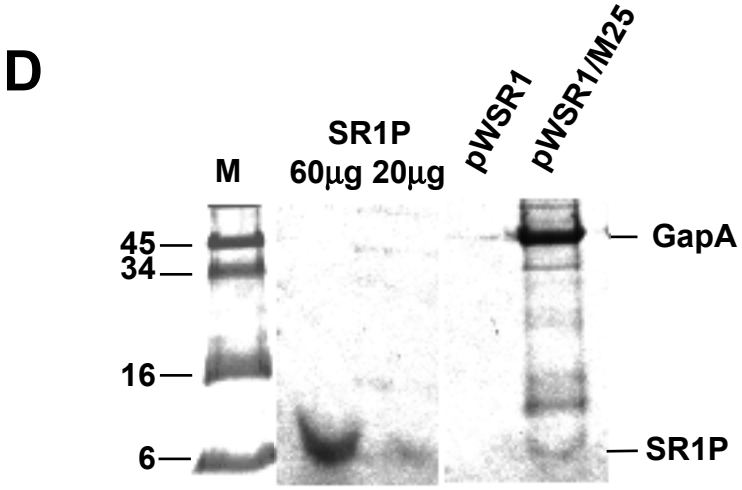


Fig. 10 Gimpel *et al.*