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Dual-function sRNA encoded peptide SR1P modulates moonlighting activity of *B. subtilis* GapA

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ABSTRACT

SR1 is a dual-function sRNA from *B. subtilis* that acts as a base-pairing regulatory RNA and as a peptide-encoding mRNA. Both functions of SR1 are highly conserved. Previously, we uncovered that the SR1 encoded peptide SR1P binds the glycolytic enzyme GapA resulting in stabilization of *gapA* mRNA. Here, we demonstrate that GapA interacts with RNases Y and J1, and this interaction was RNA-independent. About 1% of GapA molecules purified from *B. subtilis* carry RNase J1 and about 2% RNase Y. In contrast to the GapA/RNase Y interaction, the GapA/RNaseJ1 interaction was stronger in the presence of SR1P. GapA/SR1P-J1/Y displayed *in vitro* RNase activity on known RNase J1 substrates. Moreover, the RNase J1 substrate SR5 has altered half-lives in a Δ*gapA* strain and a Δ*sr1* strain, suggesting *in vivo* functions of the GapA/SR1P/J1 interaction. Our results demonstrate that the metabolic enzyme GapA moonlights in recruiting RNases while GapA bound SR1P promotes binding of RNase J1 and enhances its activity.

Abbreviations: aa, amino acid; BSA, bovine serum albumin; DRaCALA, Differential Radial Capillary Action of Ligand Assay

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Introduction

Small regulatory RNAs (sRNAs) are the most important post-transcriptional regulators in all 3 kingdoms of life (rev. in[1-3]). During the past years, computer-based searches combined with RNAseq identified on average 100–200 sRNAs/genome in a multitude of Gram-negative and Gram-positive species. These sRNAs can be divided into 2 major groups, base-pairing and protein-binding sRNAs. Regulatory mechanisms employed by all base-pairing sRNAs comprise inhibition or activation of translation and promotion of RNA decay or stability. Additionally, inhibition of primer maturation, transcriptional attenuation and transcriptional interference has been reported for some cis-encoded sRNAs (rev. in[4]). Dual-function sRNAs are a subgroup of trans-encoded sRNAs. On the one hand they act as base-pairing sRNAs, but on the other hand as peptide encoding mRNAs. The first example was *S. aureus* RNAIII encoding δ-haemolysin (26 aa) and using a base-pairing mechanism to activate translation of *hla* mRNA[5] or repress translation of *rot, spa, coa* and *lytM* (e.g.[6,7]; rev. in[8]). Subsequently, the streptolysin SLS-ORF of *Streptococcus* Pel RNA[9] and the 43-codon-SgrT ORF on *E. coli* SgrS[10] were identified. SgrS and SgrT downregulate PtsG glucose transporter activity and have a physiologically redundant, but mechanistically distinct function in inhibition.[10] Recently, *S. aureus* sRNA Psm-mec was discovered that encodes the 22 aa secreted cytolytic toxin PSMα and inhibits translation of *agrA* mRNA by base-pairing with the coding sequence.[11] By contrast, no functions have been uncovered to date for the *hyp7* ORF on *Clostridium perfringens* VR,[12] the 32-codon RivX-ORF,[13] the RSs0019-ORF of *Rhodobacter sphaeroides*[14] and the 37-codon PhrS-ORF of *Pseudomonas aeruginosa*.[15]

The 205 nt long sRNA SR1 from the *B. subtilis* genome was found by a combination of computer predictions and Northern blotting.[16] Using 7 complementary regions, SR1 base-pairs with its primary target, *ahrC* mRNA, the transcriptional activator of the *rocABC* and *rocDEF* arginine catabolic operons.[17] Base-pairing induces structural changes downstream of the *ahrC* ribosome-binding site that inhibit translation initiation.[18] SR1 is expressed under gluconeogenic and repressed under glycolytic conditions by CcpN binding to 2 operator sites upstream of and overlapping p_{sr1}[19] and, to a minor extent, CcpA binding to a *cre*-site at −139.[16] CcpN requires both ATP and a slightly acidic pH [20] to prevent promoter escape *via* direct contacts with the α-subunit of the *B. subtilis* RNA polymerase.[21] In 2010 we demonstrated that the small ORF on SR1 is translated into a 39 aa peptide SR1P (synonym YkzW) that binds GapA (glyceraldehyde-3-P-dehydrogenase A) resulting in stabilization of *gapA* mRNA.[22] SR1 is the first dual-function sRNA in *B. subtilis*. A computer-based search identified 23 SR1 homologues in *Bacillus, Geobacillus, Anoxybacillus* and *Brevibacillus* species.[23] All homologues share a high structural similarity with *B. subtilis* SR1, and the encoded SR1P peptides are

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highly similar. In the *Bacillus cereus* group, the *sr1p* region is present in triplicate or duplicate resulting in longer SR1 species. Both SR1 functions – the base-pairing and the mRNA function – have been conserved over ≈one billion years of evolution.[23]

Bacterial RNA degradation occurs in a multiprotein complex, the so-called degradosome, which displays a high degree of evolutionary divergence. Whereas the *E. coli* degradosome is assembled around the C-terminus of the main endoribonuclease RNase E and contains 3′-5′ exoribonuclease PnpA, a helicase and the glycolytic enzyme enolase,[24] the *B. subtilis* degradosome was proposed to comprise the major endoribonuclease RNase Y, 3′-5′ exoribonuclease PnpA, RNases J1, J2, helicase CshA and the glycolytic enzymes enolase (Eno) and phosphofructokinase (PfkA).[25] RNases J1 and J2 were first described to be endonucleases[26] and J1 was thought to be the main endonuclease in *B. subtilis*, but later, J1 and J2 were found to have in addition so far unprecedented 5′-3′ exoribonuclease activities (rev. in 27; 28). Instead, RNase Y was discovered in 2009,[25] later investigated in more detail[29] and confirmed to be the main endoribonuclease in *B. subtilis* that fulfils the same role as RNase E in *E. coli*.

Here, we report on the biological function of the GapA/SR1P interaction. We demonstrate that the glycolytic enzyme GapA moonlights in RNA degradation by recruiting RNases. Far Western blots show that GapA can bind the RNases J1 and Y and SR1P promotes the GapA-J1 interaction. Furthermore, it enhances the RNase activity of GapA-bound RNase J1. A certain percentage of GapA and GapA/SR1P isolated from *B. subtilis* contain RNase J1 and RNase Y. GapA/SR1P isolated from a wild-type, but not from a Δ*rnjA* strain, is able to degrade a known RNase J1 substrate *in vitro*. This finding could be substantiated by an increased half-life of the J1 substrate in a Δ*gapA* strain. The *gapA* RNA itself that is stabilized in the presence of SR1P,[22] is not only – as shown previously – substrate of RNase Y, which makes a stabilizing cut upstream of the *gapA* ORF shown before,[25,30] but also for RNase J2 and, to a much lesser extent, RNase J1. We present a working model on the function of GapA/SR1P in RNA degradation in *B. subtilis* and speculate how these interactions lead to the observed stabilization of *gapA* mRNA. Taken together our data suggest a broader function for GapA/SR1P in RNA turnover.

Results

B. subtilis *GapA cannot bind* gapA *operon mRNA*

Previously, we discovered that SR1P interacts with GapA and that this interaction results in stabilization of the *gapA* mRNA.[22] Based on these results, the following scenarios are conceivable: In the absence of SR1P, GapA could i) act as RNase and degrade its own RNA or ii) recruit an RNase that degrades *gapA* mRNA. Thereby, SR1P might induce a conformational change in GapA that either inhibits intrinsic RNase activity or prevents binding of an RNase. Alternatively, if stabilization of *gapA* RNA in the presence of SR1P is due to stabilizing cuts by one or several RNases, SR1P might induce a conformational change in GapA that either promotes an internal RNase activity of GapA or facilitates binding of an RNase responsible for such a cut(s).

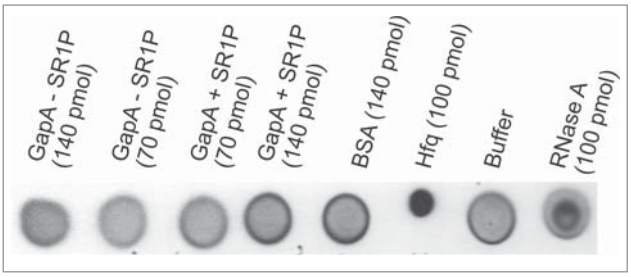

Figure 1. GapA does not bind its own mRNA. DRaCALA with 60 fmol *in vitro* transcribed, [α-^{32}P]UTP-labeled *gapA* operon mRNA was mixed with indicated amounts of GapA purified from a *B. subtilis* wild-type or Δ*sr1* strain as described in *Materials and Methods*.

To investigate whether or not GapA can bind its own RNA, we employed DRaCALA (Differential Radial Capillary Action of Ligand Assay, ref. 31) with internally labeled 2.5 kb *cggR-gapA-pgk*' mRNA and GapA or GapA/SR1P purified from *B. subtilis* as 'ligands' along with control proteins of the same concentration. BSA and buffer served as negative, Hfq and RNase A as positive controls. Fig. 1 reveals that Hfq known to have a strong RNA binding capacity but no RNase activity bound the substrate RNA tightly, indicated by a dark small dot on the filter. RNase A that simultaneously binds and cleaves RNA yielded a larger, less dark spot. By contrast, GapA and GapA/SR1P from *B. subtilis* behaved like the negative controls, i.e. were not able to retain the labeled RNA. Therefore, we conclude that GapA does neither bind its own RNA nor act as RNase on *gapA* operon RNA. Instead, it might be involved in a more complex protein-protein interaction that affects the stability of *gapA* RNA, and SR1P might interfere with this interaction.

Localization of regions within the gapA *operon mRNA required for stabilization of* gapA *RNA*

To narrow down the regions of the *gapA* operon mRNA required for stabilization by GapA/SR1P, we constructed a series of plasmids comprising wild-type and truncated regions of the *gapA* operon (Fig. 2). These plasmids were integrated into the *amyE* locus of a Δ*gapA*/Δ*sr1* knockout strain and either an empty vector or multicopy-plasmid pWSR1 providing SR1 in trans [22] were transferred into these strains. Cells were grown in complex TY medium until onset of stationary phase (OD_{560} = 4.5), *sr1* transcription induced by anhydro-tetracycline, total RNA isolated and subjected to Northern blotting (Fig. S1, summarized in Fig. 2). Surprisingly, neither the *gapA* ORF itself (pMGG1) nor the *gapA* ORF with the 5′ part of the downstream *pgk* region (pMGG13) or the bicistronic *cggR-gapA* region lacking the downstream *pgk* part (pMCG1) needed SR1P for stabilization of *gapA* mRNA, as they were already stable in its absence. Moreover, *gapA* with the downstream *pgk* region comprising in addition progressively longer 3′ portions of *cggR* (pCG4 to pCG7), but lacking the 5′ UTR and RBS of *cggR*, were also stable in the absence of SR1P. We only observed a stabilizing effect of SR1P with full-length *cggR-gapA-pgk*' (pMCG2), a mutant lacking the *gapA* sequence, but containing entire *cggR* with the 5′ UTR of *gapA* as well as the *pgk* downstream region (pMCG8), and a mutant containing solely p_{cggR}

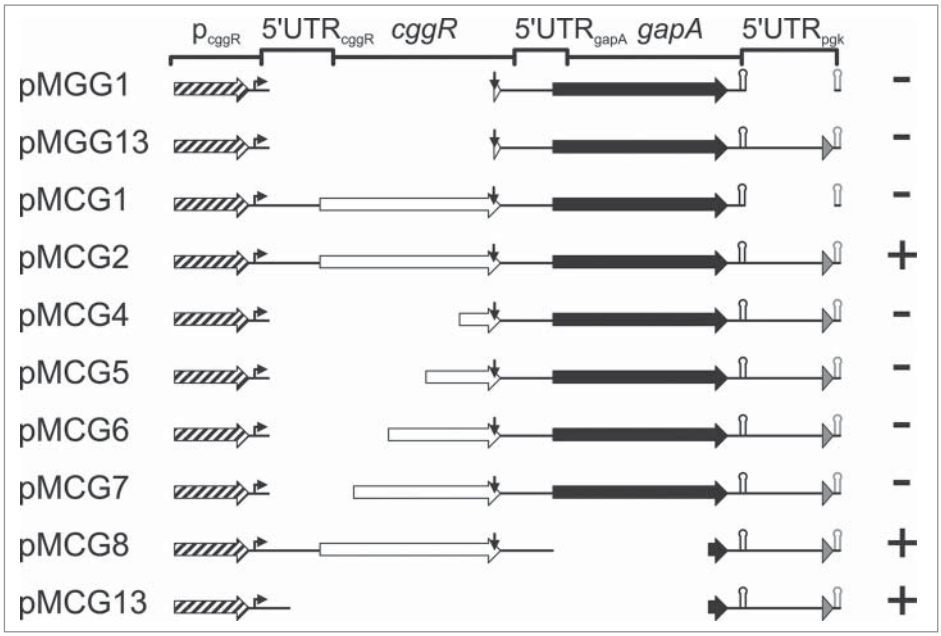

Figure 2. Localization of *gapA* operon RNA regions dependent on stabilization by GapA/SR1P. Schematic representation of the analyzed *gapA* operon mutants. Mutants were integrated into the *amyE* locus of *B. subtilis* MG1P (Δ*sr1*::*phleo*; Δ*gapA*::*ery*). For pMCG8 and pMCG13 that do not encode a functional *gapA* gene, strain MG2P (Δ*sr1*::*phleo*; Δ*gapA*::*ery*, ΔthrC::*gapA*) was used. Strains were transformed with either inducible *sr1* overexpression plasmid pWSR1 or empty vector pWH353. Cells were grown as described in *Materials and Methods* and SR1P dependent stabilization of mutated *gapA* operon RNA analyzed by Northern blotting (Fig. S1). Genes: boxed arrows (*cggR*: white; *gapA*: black; *pgk'*: gray); p_{cggR} with 200 nt upstream region: hatched arrow; transcription start site: bent arrow; RNaseY processing site: vertical arrow; alternative *gapA* terminator: black hairpin; artificial *bsrF* terminator: gray hairpin. +, SR1P required for stabilization; -, RNAs stable in the absence of SR1P (concluded from 3 independent experiments).

with the 5′ UTR of *cggR* and the downstream part of *gapA* with *pgk'* (pMCG13). Apparently, neither the *gapA* nor the *cggR* coding sequence were needed for SR1P-dependent stabilization, but regions upstream and downstream of them. These results suggest destabilizing cuts within these 2 regions in the absence of SR1P or stabilizing cuts in the presence of SR1P.

B. subtilis *GapA interacts with RNase J1 and RNase Y, and SR1P promotes the GapA/J1 interaction*

In our previous co-elution assays, Strep-tagged or His-tagged SR1P was bound to an affinity column and crude protein extracts from *B. subtilis* Δ*sr1* strains were applied.[22,23] In the elution fractions, we did not observe any band in addition to GapA. However, since we used cytosolic extracts, a hypothetical third interaction partner localized in the membrane might have escaped our attention. Therefore, we repeated the co-elution with crude extracts containing cytosolic and membrane proteins, and used a larger scale to prepare highly concentrated extracts. These were applied onto a streptactin column with already bound GapA/SR1P. After elution with desthiobiotin we observed in the protein gel besides GapA additional weak bands corresponding to proteins of ≈55–60 kDa (Fig. S2). Unfortunately, it was not possible to identify these proteins unequivocally by mass spectrometry, as each of the bands contained at least 10–20 proteins, and all scored below the threshold of the validation program. However, among these proteins were also RNases J1 and Y. This prompted us to employ Far Western blotting to study a potential direct interaction between GapA and these RNases. First, we used $GapA_{Strep}$ and GapA as targets and RNase $J1_{His}$ and RNase Y_{His} as bait (Fig. 3A). Detection was performed with anti-His-tag antibodies, which proved to be specific (lanes 6–9). RNases J1 and Y bound specifically both GapA preparations (lanes 10, 11, 14, 15), indicating that the Strep-tag does not interfere with binding. In the second Far Western blot (Fig. 3B), we used RNases $J1_{His}$ and Y_{His} as targets and $GapA_{Strep}$ with or without SR1P as bait, and detection was with anti-Strep-tag antibodies, which were also specific (lanes 6–9). As expected, $GapA_{Strep}$ and $GapA_{Strep}$/SR1P bound to RNases J1 and Y.

GapA isolated from B. subtilis *wild-type strains contains RNases J1 and Y*

To corroborate the results from the Far Western blotting with an independent method, we used co-elution assays followed by Western blotting with antibodies against RNase J1 and RNase Y (Fig. 4). To investigate if RNA might be needed to bridge the interaction between GapA and RNase J1 or Y, we treated an aliquot of the protein crude extract with RNase A prior to loading onto the streptactin column. Fig. 4A shows the Western blot with RNase J antibodies and Fig. 4B that with RNase Y antibodies. In both cases, GapA without SR1P and GapA/SR1P copurified the RNase, and this was independent of the presence of RNA (Figs. 4A and B, lanes 1 vs. 2 and 3 vs. 4). Whereas co-elution of RNase Y with GapA was SR1P-independent, GapA/SR1P compared to GapA alone bound approximately 3-fold higher amounts of RNase J1. To substantiate that RNA was neither needed to visualize the GapA/RNase J1 nor the GapA/RNase Y interaction in Far Western blots, we used the same RNase A-treated aliquot to repeat the Far Western blot (Fig. S3). No RNase J was detectable when GapA was prepared from the Δ*rnjA* strain indicating that GapA co-purifies exclusively RNase J1 and not RNase J2 (Fig. 4A). Based on band

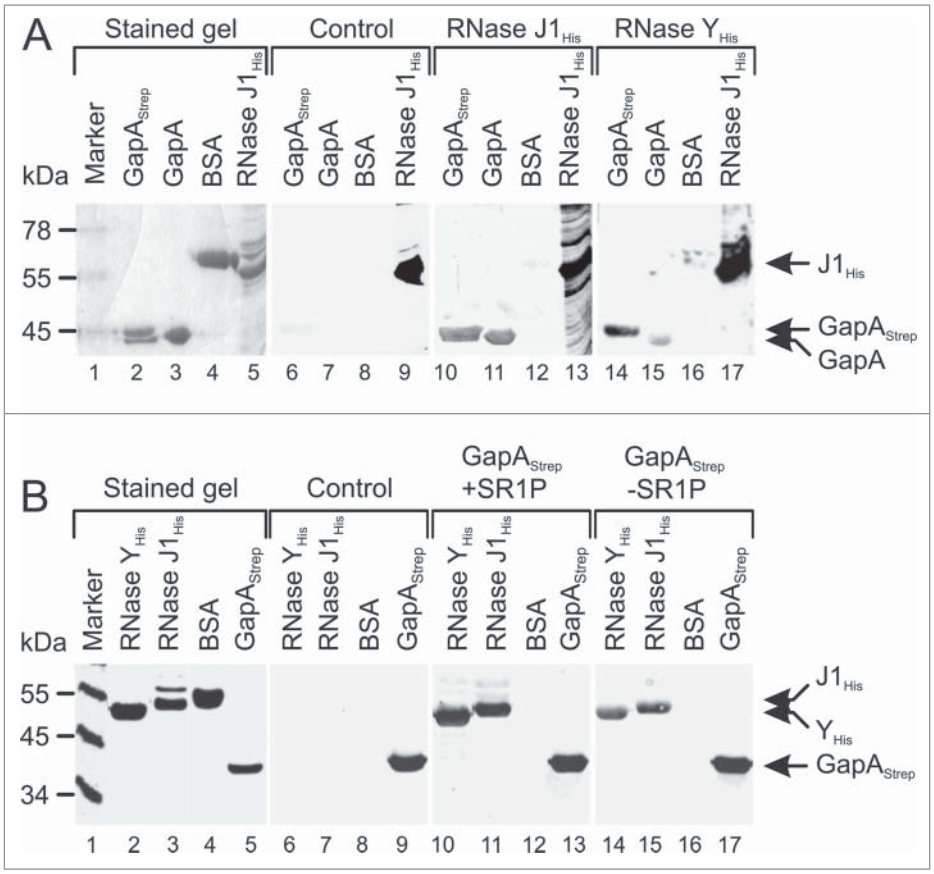

Figure 3. $GapA_{Strep}$ and GapA interact with RNases J1 and RNase Y. Far-Western blotting. A representative blot of 3 independently performed experiments is shown. Proteins were separated on 10% SDS-PAA gels and either stained with Coomassie (lanes 1–5) or blotted on PVDF membrane (lanes 6–17) as described in *Materials and Methods*. His-tagged RNases J1 and Y were purified from *E. coli*, $GapA_{Strep}$ was purified from *B. subtilis* DB104 (Δ*amyE*::*gapA*$_{Strep}$) and untagged GapA was co-purified with Strep-tagged SR1P from *B. subtilis* Δ*sr1*::*cat* (pWSR1/M25). (A) After blocking all blots were incubated with PBST gelatine (control, lanes 6–9) or PBST-gelatine containing either 170 μg RNase $J1_{His}$ (lanes 10–13) or 170 μg RNase Y_{His} (lanes 14–17 RNase binding was detected with mouse anti-His-tag antibodies. Both RNases were able to bind $GapA_{Strep}$ and GapA. (B) Far Western Blot as in A) except that blots were incubated with 100 μg $GapA_{Strep}$ purified from either *B. subtilis* DB104 (Δ*amyE*::*gapA*$_{Strep}$) (lanes 10–13) or *B. subtilis* DB104 (Δ*sr1*::*phleo*, Δ*amyE*::*gapA*$_{Strep}$) (lanes 14–17). $GapA_{Strep}$ binding was detected with mouse anti-Strep-tag antibodies.

intensities in Fig. 4A, we calculated a stoichiometric GapA/SR1P-RNase J1 ratio of 80:1, i.e. 1.25% of GapA molecules from the wild-type strain carried RNase J1. In the case of GapA lacking SR1P, the ratio was about 200:1 corresponding to 0.5%. About 2% of both the GapA and the GapA/SR1P preparations contained RNase Y as calculated from Fig. 4B. However, due to the purification procedure that did not involve cross-linking, we cannot exclude that *in vivo* a considerably larger fraction of GapA might be bound to RNase J1 or Y.

We conclude that GapA and GapA/SR1P interact directly with RNase J1 and RNase Y, and this interaction is RNA-independent. Whereas SR1P promotes the GapA/RNase J1 interaction, the GapA/RNase Y interaction is not influenced by SR1P.

GapA/SR1P preparations from B. subtilis *can cleave a known RNase J1 substrate* in vitro

As our GapA and GapA/SR1P preparations from *B. subtilis* contain sub-stoichiometric concentrations of RNase J1, these preparations should exhibit RNase J1 activity. In Figs. 2 and S1 we have shown that for the stabilizing effect of SR1 a *gapA* mRNA of at least 2.5 kb is required. Degradation of an RNA of this length cannot be analyzed in a PAA gel, and agarose gels cannot be dried to visualize sharp bands. Therefore, we used to test our assumption as first substrate the small (162 nt) internally labeled sRNA SR5, the antitoxin of a type I toxin-antitoxin system investigated in our group. SR5 has a 2-fold longer half-life in a Δ*rnjA* strain, whereas its half-life was not affected by RNase Y.[32] In an *in vitro* degradation assay (Fig. 5A), purified RNase J1 digested SR5 similarly to RNase A used in parallel. By contrast, the same amount of BSA or RNase Y added in the same buffer had no cleavage activity. Therefore, SR5 seemed to be a suitable substrate for the analysis of RNase J1-dependent degradation. To analyze if GapA-J1/Y (GapA with co-purified RNases J1 and Y) or GapA/SR1P-J1/Y can degrade SR5, we tested GapA purified from either *B. subtilis* wild-type or a Δ*sr1* strain. Whereas SR5 was completely degraded after addition of 10 pmol GapA/SR1P-J1/Y, the same amount of SR1P-free GapA-J1/Y was not able to cleave SR5 (Fig. 5B). This indicates that only the GapA/SR1P-J1/Y complex displays RNase activity. Moreover, the 0.125 pmol J1 (calculated from Fig. 4A) that are present in 10 pmol GapA/SR1P-J1/Y could degrade the substrate (Fig. 5B), whereas 0.1 pmol of purified RNase J1 could not (Fig. 5B). Therefore, we conclude that not only binding, but also the activity of RNase J1 is enhanced by the presence of SR1P in the purified GapA-J1/Y complex. In contrast to GapA/SR1P-J1/Y purified from the wild-type, GapA/SR1P-Y purified from an isogenic Δ*rnjA* strain (lane 6) was not able to degrade SR5 (Fig. 5B) confirming that the RNase activity of the GapA/SR1P preparation observed in

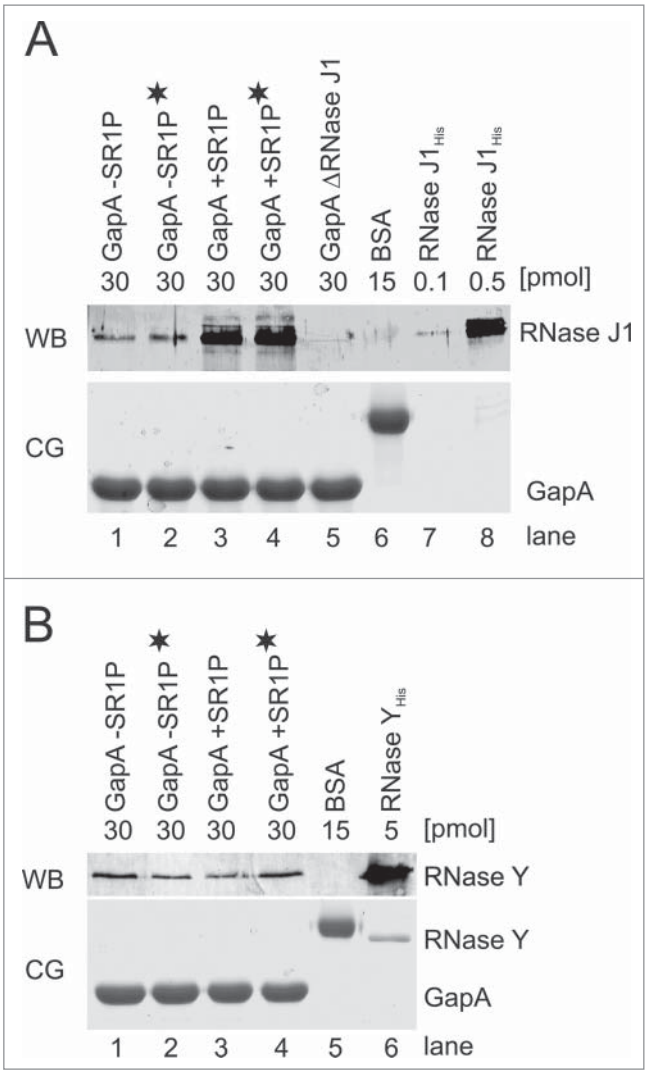

Figure 4. RNases J1 and Y co-purify with GapA. Co-elution assays [22] followed by Western blotting as described in *Materials and Methods*. A) Top: Western blot (WB) for detection of co-eluted RNase J1 within GapA preparations. $GapA_{Strep}$ was either purified from *B. subtilis* DB104 (Δ*amyE*::*gapA*$_{Strep}$), DB104 (Δ*sr1*::*phleo*; Δ*amyE*::*gapA*$_{Strep}$) or DB104 (Δ*rnjA*::*spec*; Δ*amyE*::*gapA*$_{Strep}$). To exclude that RNA bridges a possible interaction, an aliquot of the protein crude extracts was incubated with RNase A prior to protein purification (indicated by black asterisk). BSA and RNase $J1_{His}$ purified from *E. coli* serve as controls. RNase J specific antibodies were used. Bottom: Coomassie stained gel (CG) of the Western blot (loading control). The amount of protein loaded onto the gels is indicated. (B) Top: Western Blot (WB) as in (A) for detection of co-eluted RNase Y within GapA preparations. RNase Y specific antibodies were used. Bottom: CG of Western blot as loading control. The amount of protein loaded onto the gels is indicated.

lane 5 was indeed due to co-purified RNase J1. Fig. 5C presents a time-course for the degradation of SR5 with 10 pmol RNase J1 purified from *E. coli* (left) and 10 pmol GapA/SR1P-J1/Y purified from *B. subtilis* (right). In both cases, a similar degradation pattern was observed.

To demonstrate that GapA/SR1P-J1/Y has a specific RNase J1 activity, we employed a degradation assay with a known RNase J1 target, *B. subtilis* threonyl tRNA.[33] As shown in Figs. 5D and S4, a similar degradation pattern of threonyl tRNA was observed for RNase J1 and GapA/SR1P-J1/Y from *B. subtilis*, but not for GapA-J1/Y alone. This confirms that degradation of SR5 is not due to an unspecific co-purified RNase activity, but to the co-purified RNase J1. In addition, to analyze if RNase J1 acts as endo- or as 5′-3′ exoribonuclease, we performed a degradation assay with 5′ labeled SR5 and purified RNase J1 (Fig. S5). The degradation pattern shows both small degradation products that run with the migration front and internal cleavage products. Therefore, we conclude that RNase J1 does not only act as an endonuclease, but most probably also as 5′-3′ exoribonuclease on SR5.

Based on these results we conclude that only GapA purified from a *B. subtilis* strain expressing both SR1P and RNase J1 has RNase activity on a known J1 substrate, and this activity is due to the presence of sub-stoichiometric amounts of RNase J1 bound to and co-purified with GapA/SR1P.

The ability of the GapA/SR1P-J1 complex to degrade RNA is growth-phase dependent

Formerly, we demonstrated that SR1 is ≈20-fold repressed by CcpN under glycolytic conditions (e.g., complex TY medium, log phase), but expressed under gluconeogenic conditions (e.g. TY medium, stationary phase).[16] When we assayed GapA/SR1P-J1/Y preparations from log phase cultures (Fig. 5E, lanes 6 and 7), we observed no RNase activity on our substrate RNA SR5, whereas those from stationary phase cultures used before (Fig. 5E, lanes 3 and 4) displayed RNase activity. These data confirm that SR1P that is much more abundant in cells from stationary phase TY cultures, does indeed modulate the GapA-RNase J1-complex.

Neither His-tagged SR1P ($SR1P_{His6}$) purified separately by affinity chromatography (Fig. 5E, lane 8) nor $SR1P_{His6}$ added afterwards to a GapA preparation from the Δ*sr1* strain from log or stationary phase (Fig. 5E, lanes 2 and 5) were active in degrading SR5 ruling out an intrinsic RNase activity of SR1P or the GapA/SR1P complex. Apparently, subsequent binding of SR1P did not increase the amount of RNase J1 already bound to GapA before. Thus, we conclude that SR1P triggers the RNase activity only when it is co-expressed with GapA.

Whereas RNase J1 binds SR5, GapA does not, confirming that it is not an RNA binding protein

In the DRaCALA (Fig. 1) we had only tested if GapA can bind its own (*gapA* operon) mRNA, which could be excluded. However, as SR5 proved to be a suitable substrate for degradation by RNase J1 and by GapA/SR1P containing sub-stoichiometric J1 amounts, we used labeled SR5 to perform an electrophoretic mobility shift assay (EMSA) with increasing concentrations of RNase J1 and in parallel with the same concentrations of GapA/SR1P purified from *B. subtilis*. As shown in Fig. S6, already 6 pmol of J1 completely bound SR5 whereas 54 pmol of GapA containing SR1P did not suffice to produce a complex with even a small percentage of labeled SR5. The amount of RNase J1 present in the 54 pmol of the GapA/SR1P preparation would not result in a visible shift as it is still below the lowest amount of $RNase\ J1_{His}$ that was used in parallel. This result confirms that GapA is not an RNA binding protein, as it can neither bind its own RNA nor a small RNA that is degraded by GapA/SR1P-J1/Y.

The half-life of the RNase J1 substrate SR5 is altered in a gapA knockout strain

SR5 is 2-fold more stable in the absence of RNase J1.[32] Figs. 3 and 4 demonstrate that GapA can interact with RNase J1.

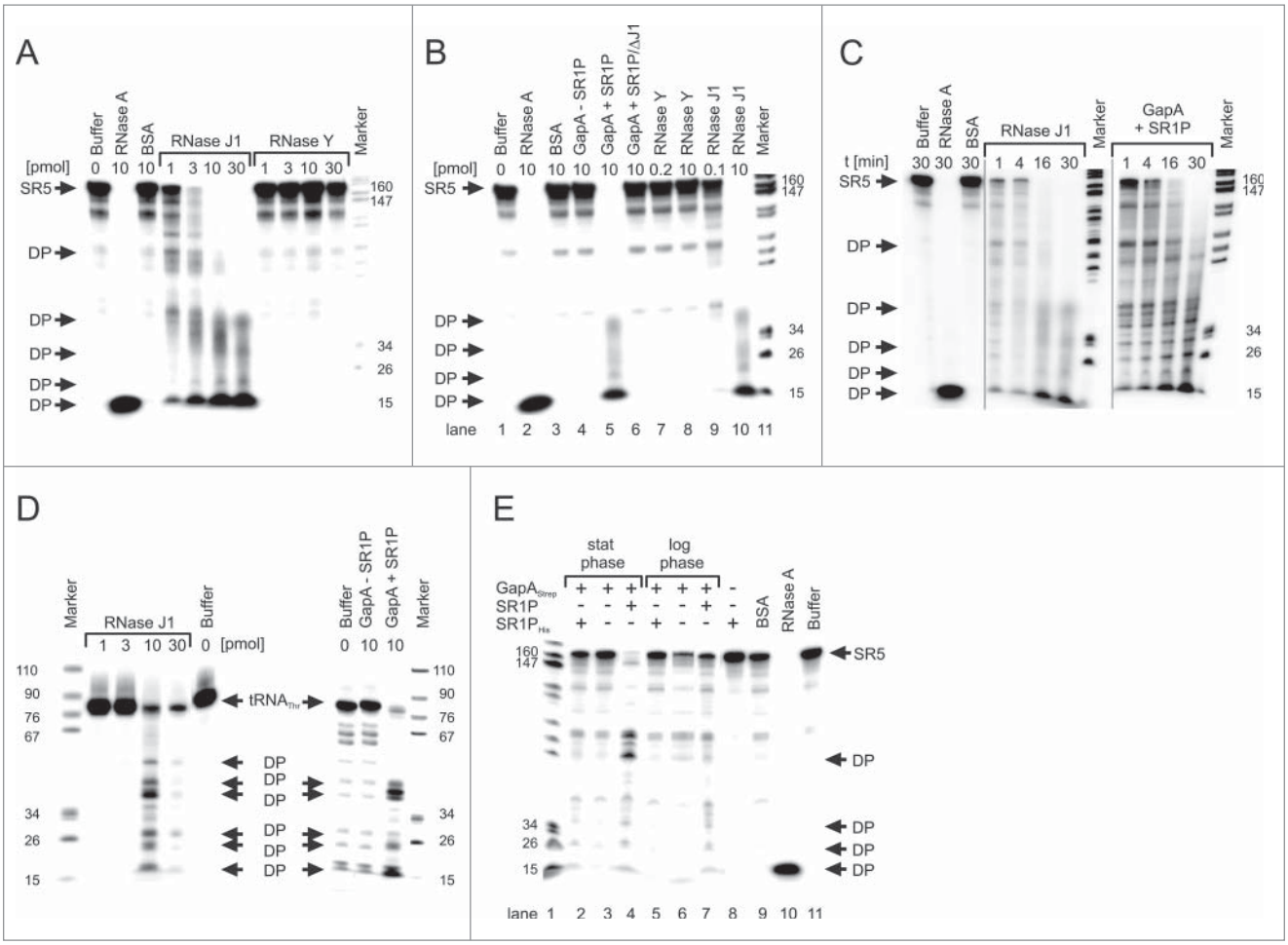

Figure 5. GapA/SR1P is involved in RNA degradation. RNA degradation assays with internally [α-^{32}P]-UTP-labeled sRNA SR5. BSA and protein-free buffer were used as negative controls and 10 pmol RNase A as positive control. [γ-^{32}P]-ATP-labeled pBR322xMspI served as size marker. Incubation was for 30 min at 37°C, followed by denaturation and separation on 8% denaturing polyacrylamide (PAA) gels, if not stated otherwise. (A) SR5 was incubated with RNase Y and RNase J1. The amount of protein used, full-length SR5 and degradation products (DP) are indicated. (B) Degradation assay as in A) with $GapA_{Strep}$/SR1P purified from *B. subtilis* DB104 (Δ*amyE::gapA*$_{Strep}$), $GapA_{Strep}$ purified from *B. subtilis* DB104 (Δ*sr1::phleo*, Δ*amyE::gapA*$_{Strep}$) and $GapA_{Strep}$/SR1P purified from the Δ*rnjA* strain. (C) Time-course RNA degradation assay. SR5 was incubated with 10 pmol $GapA_{Strep}$ purified from *Bacillus subtilis* DB104 (Δ*amyE::gapA*$_{Strep}$) or 10 pmol RNase $J1_{His}$ purified from *E. coli*. Incubation times and degradation products (DP) are indicated. (D) Degradation of the known RNase J1 target threonyl tRNA with purified RNases J1 and Y and $GapA_{Strep}$ +/−-SR1P. A schematic representation of the RNase J1 cleavage pattern of threonyl tRNA based on [33] is shown in Fig. S3. (E) Assay as in A) with $GapA_{Strep}$ purified from *B. subtilis* cells harvested from log- or stationary phase TY cultures. 10pmol of $GapA_{Strep}$ with or without co-purified SR1P were used. To exclude that the presence of SR1P increases an intrinsic RNase activity of GapA rather than that of co-purified RNase J1, $SR1P_{His}$ was purified separately and added to the degradation assays. Neither $SR1P_{His}$ alone (lane 8) nor addition of $SR1P_{His}$ to SR1P-free $GapA_{Strep}$ (lanes 2 and 5) increased the RNase activity. SR1P, peptide co-purified with $GapA_{Strep}$; $SR1P_{His;}$ 50 pmol peptide purified separately and added later to purified $GapA_{Strep}$.

Moreover, GapA/SR1P preparations from *B. subtilis* wild-type strains contain sub-stoichiometric amounts of RNase J1 and are active in degrading SR5 *in vitro* (Fig. 5). This prompted us to assay if GapA affects the stability of SR5 *in vivo*. To this end, we determined the half-life of SR5 in a Δ*gapA* strain and a Δ*sr1* strain compared to the isogenic wild-type strain. As shown in Fig. 6, the half-life of SR5 was 4.6 min in the wild-type strain 168 and with 11.5 min more than 2-fold higher in the Δ*gapA* strain, supporting that GapA is indeed involved in the degradation of SR5 by RNase J1. The determined half-life in the absence of GapA (11.5 min) is in perfect agreement with the 2-fold higher half-life in the absence of RNase J1 (12.2 min, ref. 32). In the Δ*sr1* strain, the half-life of SR5 was with 7.2 min 1.5-fold higher compared to the wild-type strain, confirming a smaller, but also detectable contribution of SR1P to the degradation of SR5 *in vivo*. Nevertheless the observed increased half-life of SR5 in the Δ*sr1* strain is in line with the effect observed in the Δ*gapA* and the Δ*rnjA* strains and corroborates the participation of both GapA and SR1P in SR5 degradation.

Taken together, this data indicates a role of GapA and SR1P in the modulation of RNA degradation *in vivo*.

B. subtilis gapA *mRNA is not only a substrate for cleavage by RNase Y, but also by RNases J2 and J1*

Formerly, it had been shown by Stülke and colleagues that the main endoribonuclease RNase Y makes a stabilizing cut upstream of the *gapA* ORF within the 3′ end of the *cggR* ORF yielding a 1.2 kb *gapA* RNA species that is more stable than the bicistronic 2.2 kb *cggR-gapA* RNA.[25,30] On the other hand, we had demonstrated that the monocistronic 1.2 kb *gapA* mRNA is more stable in the presence of SR1P.[22] As shown above, GapA/SR1P interacts with RNases J1 and Y. This prompted us to investigate if the stability of *gapA* RNA is also dependent on RNase J1 or its *in vivo* interaction partner RNase J2. To this

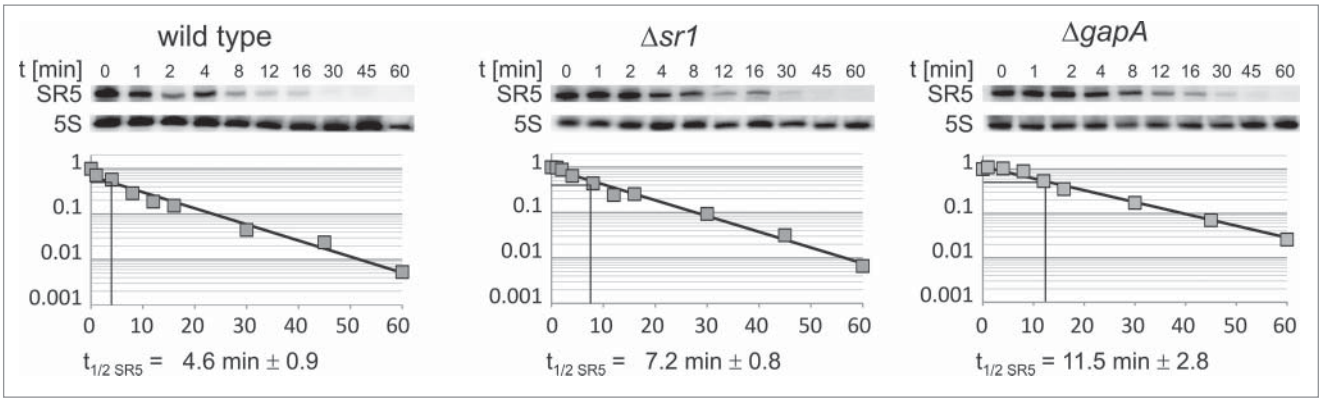

Figure 6. Determination of SR5 half-lives. *B. subtilis* strains 168, 168 (Δ*sr1*::*cat*) and 168 (Δ*gapA*::*ery*) were grown in complex TY medium until onset of stationary growth phase, samples taken at the indicated times after rifampicin addition, total RNA prepared and separated on 6% denaturing PAA gels as described.[16] [α-^{32}P]UTP-labeled riboprobes were used. Reprobing was performed with [γ-^{32}P]ATP-labeled SB767 specific for 5S rRNA. Autoradiograms of Northern blots are shown. Half-lives presented under the gels are averaged from 3 independent determinations.

end, we determined the half-life of the 1.2 kb *gapA* RNA in isogenic wild-type, Δ*rnjA* and Δ*rnjB* strains. Whereas the half-life of *gapA* mRNA in the wild-type 168 strain was 4.5 min, it was with 10.9 min 2- to 3-fold longer in the Δ*rnjB* strain lacking RNase J2 and with ≈6 min 1.5-fold increased in the Δ*rnjA* strain lacking RNase J1 (Fig. 7). Our data (Figs. 2 and S1) suggest that regions upstream and downstream of the *cggR-gapA* coding sequence are substrate for destabilizing cuts in the absence of SR1P. The results in Fig. 7 demonstrate that RNase J2 is involved in degradation of *gapA* RNA. RNases J1 and J2 form a complex that is likely the predominant form *in vivo*.[34] RNase J2 is a very weak 5′-3′ exoribonuclease, whereas J1 and the J1/J2 complex have robust 5′-3′ exoribonuclease activity *in vitro*. However, J2 might, in the complex with J1, be also an endoribonuclease.[34] Most likely, RNase J2 is, in complex with RNase J1, responsible for degradation of the *gapA* operon RNA.

Discussion

To date, the biological function of the encoded peptides has been unravelled for only a handful of dual-function sRNAs (see also Introduction). In all these cases, the peptide function is connected to the base-pairing function of the RNAs: Both in *S. aureus* RNAIII and Psm-mec RNA as well as in *Streptococcus pyogenes* Pel RNA, the encoded peptides – δ-haemolysin, PSMα and streptolysin SLS – and the base-pairing functions of the sRNAs play a role in virulence.[8,11,9] Likewise, the base-pairing function of *E. coli* SgrS is important for glucose and mannose metabolism, and the SgrS-encoded peptide SgrT down-regulates PtsG glucose transporter activity.[10,35,36]

By contrast, we report that the 2 functions of *B. subtilis* SR1 are relevant in different pathways: Whereas SR1 acts as a base-pairing sRNA in arginine catabolism,[17,18] SR1P plays a broader role, as it is involved in modulating RNA degradation: SR1P binds GapA,[22] promotes GapA binding of RNase J1 and this binding enhances the activity of GapA-bound RNase J1 on its corresponding substrates. On the one hand, we demonstrated that GapA can bind RNase J1 *in vitro*, and this binding is promoted by SR1P. On the other hand, we showed that the GapA/SR1P complex isolated from the *B. subtilis* wild-type, but not from the RNase J1 knockout strain, contains sub-stoichiometric amounts of RNase J1, which enable it to degrade 2 known *in vivo* substrates of RNase J1, the sRNA SR5, and threonyl tRNA, *in vitro*. Furthermore, the presence or absence of GapA and SR1P does also affect SR5 degradation *in vivo* supporting that the GapA/SR1P-RNase J1 interaction has a biological function. The smaller effect of the *sr1* deletion compared to the *gapA* deletion might be attributed to the fact that GapA is the

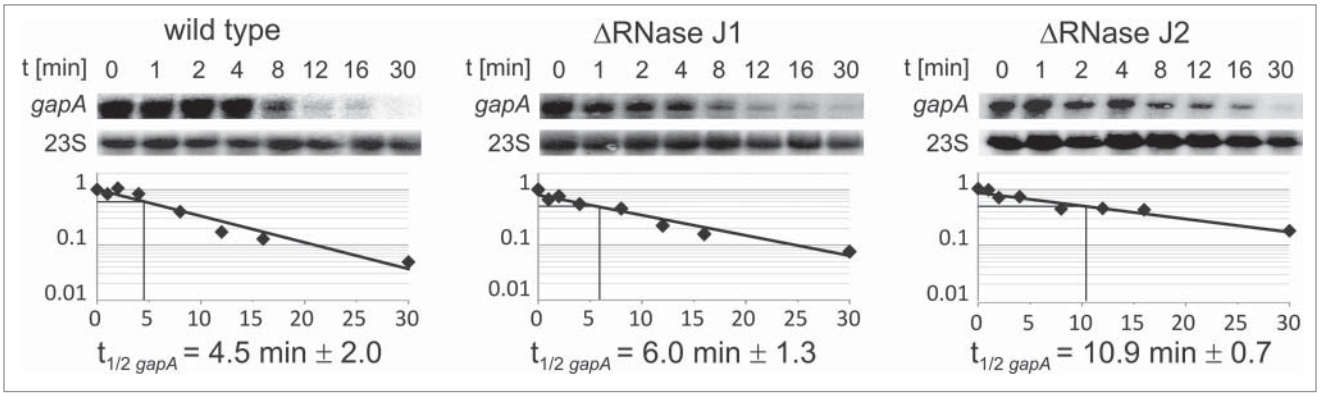

Figure 7. Determination of *gapA* mRNA half-life. *B. subtilis* strains 168, 168 (Δ*rnjA*::*spec*); 168 (Δ*rnjB*::*ery*) were grown in complex TY medium until onset of stationary growth phase, samples taken at the indicated times after rifampicin addition, total RNA prepared and separated on 1.5% agarose gels, probed and reprobed as described.[22] Autoradiograms of representative Northern blots are shown. Half-lives are averaged from at least 2 independent determinations.

moonlighting protein whereas SR1P is only the mediator of this moonlighting activity.

As GapA binding to RNases J1 and Y was independent of the presence of RNA, we can exclude a bridging function of RNA in these interactions.

Our findings indicate that GapA might affect the proposed *B. subtilis* degradosome, which was reported to comprise the main endoribonuclease RNase Y, the main 3′-5′-exoribonuclease PnpA, RNases J1 and J2, helicase CshA, and the glycolytic enzymes Eno and PfkA.[25] However, in contrast to the *E. coli* degradosome, the *B. subtilis* degradosome cannot be isolated in the absence of cross-linking reagents indicating that it has a more dynamic structure.[37] Using a bacterial 2-hybrid system, it was shown that RNase J1 interacts with RNase Y, PnpA and PfkA. RNase Y interacts in addition with PnpA, CshA, Eno and PfkA, whereas PnpA interacts with RNase J1, RNase Y and PfkA.[25] However, the authors did not report interactions of GapA with itself (GapA is a tetramer) or with one of the proposed degradosome components. On the one hand, this might be due to masked GapA interactions sites in the constructed chimera. On the other hand, it might also be attributed to the use of a 2-hybrid system from *E. coli* that does not encode SR1P in its genome.[23] Later, some of the initially found interactions were confirmed biochemically with purified proteins, among them Eno-PfkA, Eno-RNase Y, PNPase-RNase J1 and PNPase-RNase Y, whereas the RNase Y-RNase J1 interaction could not be corroborated.[38] Based on our data we speculate about a possible involvement of GapA/SR1P in the dynamic *B. subtilis* degradosome: i) GapA/SR1P could provide additional interactions with RNase J1 and RNase Y, ii) GapA/SR1P could replace PfkA or enolase as scaffolding component, or iii) GapA/SR1P could displace RNase J2 from its interaction with RNase J1 (Fig. 8).

How can we explain our previous finding of SR1P-dependent stabilization of *gapA* operon mRNA [22]? Our current data suggest that, on the one hand, regions upstream and downstream of *cggR-gapA* are necessary for degradation in the absence of SR1P (Fig. 2), most likely because proper folding of the full-length mRNA is important for recognition by RNases. On the other hand, the stable 1.2 kb *gapA* RNA is generated by two independent processing events: one stabilizing endonucleolytic cut by RNase Y [25] and prevention of a destabilizing endonucleolytic cleavage by RNase J2. We speculate that the stabilizing effect of SR1P might be due to one of the above mentioned alterations in the *B. subtilis* degradosome. As RNase J2 seems to be important for *gapA* mRNA degradation (Fig. 7) an SR1P-mediated displacement of RNase J2 from its interaction with RNase J1 could be a possible scenario for the observed stabilization of *gapA* mRNA (Fig. S7).

In 2012 Newman *et al.* observed that citrate modulates *B. subtilis* enolase activity. They argued that glycolytic enzymes like Eno and PfkA may act as sensors of nutritional stress and may coordinate this with the RNA degrading machinery to decrease the rate of global mRNA turnover under energy-limiting conditions.[38] Interestingly, already in 2011 it was reported that citrate alters the activity of *E. coli* PNPase by direct binding to the enzyme [39] suggesting that metabolism and RNA degradation may be linked in all organisms. In our case, CcpN-mediated ≈20-fold repression of *sr1* transcription during glycolysis prevents the synthesis of high amounts of SR1P under these conditions whereas under gluconeogenic conditions, the amount of SR1 increases to at least 200–250 molcules/cell.[16,18] It is difficult to estimate the amount of SR1P expressed from these SR1 molecules, as an additional regulation of *sr1p* translation cannot be entirely excluded. Nevertheless, the different RNase activities of logarithmic and stationary phase GapA/SR1P-J1/Y preparations (Fig. 5E) support that SR1P might, by promoting the GapA-RNase J1 interaction, also act as a sensor to modulate RNA degradation in response to the nutritional state of the cell. In addition, *B. subtilis* has two Gap enzymes, GapA active in glycolysis and GapB active in gluconeogenesis.[40] Thus, GapA is not required in metabolism under gluconeogenic conditions when high amounts of SR1P are available. Consequently, under these conditions, it can moonlight in RNA degradation by recruiting RNase Y or RNase J1. SR1P improves GapA-RNase J1 binding. In addition, it promotes in GapA-J1 complexes the degradation of substrates of this RNase, as shown for SR5.

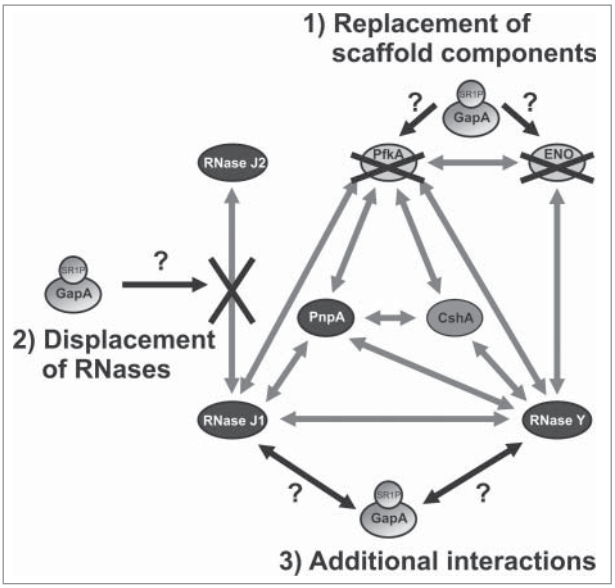

Figure 8. Working model on the potential function(s) of GapA/SR1P in the *B. subtilis* degradosome. Illustration of the possible effects of GapA/SR1P on the integrity of the *B. subtilis* degradosome. The interaction of GapA/SR1P might lead to 1) the replacement of the metabolic enzymes PfkA or enolase; 2) the displacement of RNase J2 from its interaction with RNase J1, or 3) additional interactions with RNase J1 and Y. In all cases the presence of GapA/SR1P within the degradosome might result in modulation of the RNase activity or specificity of the RNA degradation machinery. Gray arrows: interactions between the components of the RNA degradosome as observed by Commichau et al., 2009 [25] and Lehnik-Habrink et al., 2010,[48] black arrows and black crosses: possible effects of GapA/SR1P; gray ovals: proteins.

As we did not detect – in addition to GapA – RNase J1 or RNase Y in our routine co-elution experiments with tagged SR1P (e.g., [22]), we suggest that the interactions between GapA/SR1P or GapA with RNase J1 or RNase Y are weak, so that only a few molecules of GapA retain the bound RNases. Weak interactions would also facilitate a rapid exchange of transient degradosome components. Furthermore, in the case of J1, different populations might be present in *B. subtilis*, some associated with the proposed degradosome, others free in the cytosol. Both assumptions would also explain why Newman *et al.* [38] could not confirm biochemically an interaction of RNase J1 with RNase Y, which provides – bound to the membrane – the scaffold for the degradosome. The existence of a larger amount of J1 outside of the degradosome would also suggest that not all J1 targets might

depend on GapA or GapA/SR1P. However, this does not seem to apply to the J1 target SR5, for which the 2-fold increased half-life in the Δ*gapA* strain is in perfect agreement with the 2-fold increased half-life in the Δ*rnjA* strain compared to the isogenic wild-type strain.[32]

In 2002, Hackenberg *et al.* reported that dehydrogenases from rabbit muscle, 2 bacterial species (*E. coli* and *B. stearothermophilus*) and the hyperthermophilic archeon *Sulfolobus solfataricus* cleave RNA.[41] In 2012, we discovered in Bacillales, but neither in other Gram-positive nor in Gram-negative bacteria, 23 homologues of SR1, whose base-pairing and peptide-encoding functions are highly conserved over one billion years of evolution.[23] Therefore, we assume that SR1P might play a comparable role in the other Bacillales. Furthermore, as all GAPDHs are highly similar, it cannot be excluded that other bacterial GAPDHs might also recruit and modulate the activity of RNases, perhaps in some cases depending on small, so far unknown, peptides, and that the role of GapA in RNA degradation is not confined to Bacillales like *B. subtilis*. In the light of our present data it is tempting to speculate that the RNase activities observed for the bacterial GAPDH's by Hackenberg *et al.* might be also modulated by so far unknown co-purified peptides or, alternatively, the result of substoichiometric amounts of a co-purified RNase rather than GAPDH itself.

Interestingly, in *E. coli*, an alteration of RNase E activity by binding of small proteins has been discovered some years ago: The 17.4 kDa protein RraA [42] or the 15.6 kDa RraB [43] bind weakly – with a K_D value in micromolar range – to and inhibit RNase E. RraA masks the RNA binding domains in the C-terminus of RNase E and additionally binds to the helicase RhlB [44] leading to global changes in RNA turnover. It was argued that remodelling of the degradosome by such proteins allows for differential regulation of RNA cleavages in *E. coli*. RraA is widely distributed among Gram-negative bacteria and also plants,[42] whereas RraB is confined to γ-proteobacteria. Gao *et al.* proposed that RraA and RraB might also exert indirect effects on the amount of free, not degradosome bound PNPase or RhlB.[43] These data suggest that modulation of RNase activity involving weak interactions with peptides or small proteins might be a more common principle in the regulation of RNA turnover.

Future research will focus on the effect of the GapA-RNase Y interaction on specific targets, the detailed analysis of a genome-wide impact of GapA on RNA degradation, the molecular mechanism by which SR1P binding to GapA activates bound RNase J1 and the identification of functional homologs of SR1P in other Gram-positive bacteria.

Materials and methods

Strains, growth conditions, enzymes

E. coli strain TG1 was used for cloning and *B. subtilis* strain DB104 (Δ*amyE*::pMGG19) or DB104 (Δ*sr1::cat*; Δ*amyE*::pMGG19) for GapA purification with or without SR1P. *E. coli* strains CCE093 and SSC420 were used for purification of RNase $J1_{His}$ or RNase Y_{His} (all strains are listed in Table S1). TY medium [16] and TFB medium (12 g bacto tryptone, 24 g yeast extract, 4 ml glycerol, 2.31 g KH_2PO_4, 12.54 g K_2HPO_4 x 3 H_2O/l) served as complex medium for *B. subtilis* and *E. coli*, respectively. Taq-polymerases were purchased from Roche or Solis Biodyne (Estonia) and T7 RNA polymerase from New England Biolabs.

Protein purification and co-elution assay

Strep-tagged GapA carrying or lacking SR1P was purified from *B. subtilis* strain DB104 (Δ*amyE*::$gapA_{Strep}$) or DB104 (Δ*sr1::cat;* Δ*amyE*::$gapA_{Strep}$) as described [22] except that 1.5% Triton-X100 was added to the lysis buffer. For purification of His-tagged RNases J1 and Y, 1 l TFB medium was inoculated with an overnight culture to OD_{560} of 0.1 and grown for 1.5 hr. RNase expression was induced with 2 mM IPTG for 3 hours, cells harvested by centrifugation and pellets frozen overnight. Protein extracts were prepared by sonication and supernatants applied to 1 ml Ni-NTA-columns. Washing and elution were performed as described.[45] Six 500 μl elution fractions were collected for each column, stored at 4°C and, prior to use, concentrated using Amicon(R) Ultra 10k centrifugal filters. Proteins were centrifuged at 4°C with 16.000 g until no further concentration was possible, washed twice with 100 μl storage buffer (300 mM NaCl, 50 mM Na phosphate pH7.5, 25% glycerol) and again centrifuged as before. Proteins were collected by reverse spin with 50 μl storage buffer for 2 min at 1000 g. For purification of $SR1P_{His}$, 2 l TY medium were inoculated with an overnight culture of *B. subtilis* strain MG1P (pWSR1/M31) to an OD_{560} of 0.2, grown for 7 hr, and *sr1* expression induced for 20 min by addition of 0.5 μg/ml anhydro-tetracycline. Cells were harvested by centrifugation, protein crude extracts prepared as described,[22] and applied to 1 ml Ni-NTA columns as above. Prior to use, $SR1P_{His}$ was separated from elution fractions by Amicon(R) Ultra 10k centrifugal filters, and the filtrate concentrated with Amicon(R) Ultra 3k centrifugal filters. Concentration, washing with storage buffer and protein collection was performed as above. The co-elution assay was performed as described.[22]

Western blotting and Far Western blotting

Western blotting was performed as described,[22] but a polyclonal antiserum raised against RNase J2 that recognizes both RNase J1 and RNase J2 (H. Putzer, personal communication) (1:5000) was used. For Far-Western blotting, proteins were separated on SDS/PAA gels and transferred onto PVDF membranes that were blocked for 2 hr. Blots were incubated overnight with 10 ml PBST-gelatine or PBST-gelatine supplemented with either 100 μg $GapA_{Strep}$ purified from *B. subtilis* or 170 μg His-tagged RNase purified from *E. coli*. Binding of $GapA_{Strep}$ and His-tagged RNase was detected by incubation with mouse-anti-Strep-tag antibody (1:1000; IBA Göttingen) or mouse-anti-His-tag antibody (1:2000; IBA Göttingen), respectively, and subsequently horseradish peroxidase coupled anti-mouse antibody (1:2000; IBA Göttingen).

DRaCALA (Differential Radial Capillary Action of Ligand Assay)

In vitro transcribed [α-^{32}P]-UTP labeled RNA was diluted to a final concentration of 24 nM. 2.5 μl RNA were mixed with 2.5 μl purified protein and incubated for 10 min at 25°C. Subsequently, the reaction mixture was spotted onto nitrocellulose

membrane NC45 (pore size 0.45 μm, Serva Heidelberg). Spots were allowed to dry for 30 min at room temperature and signals detected after overnight exposure to a phosphorImager plate using Aida Image Analyzer v.4.50.

RNA degradation assay

One μl *in vitro* transcribed, [α-^{32}P]-UTP labeled RNA (10.000 cpm) was incubated with 2 μl 5x reaction buffer (100 mM Tris-HCl pH8.0; 40 mM $MgCl_2$, 500 mM NH_4Cl; 0,5 mM DTT) and 7 μl diluted protein for 30 min at 37°C. The reaction was stopped by addition of 10 μl formamide loading dye [16] and 5 min at 95°C. Samples were separated on 6% to 12.5% denaturing PAA gels. Dried gels were analyzed by phosphorImaging using Aida Image Analyzer v.4.50.

RNA preparation, Northern blotting, in vitro transcription and EMSA

Strains were cultivated until onset of stationary phase, 0.5 ml samples flash-frozen in liquid nitrogen and stored at -20°C. Preparation of total RNA, separation on 6% denaturing PAA gels, Northern blotting and determination of RNA half-lives were carried out as described previously[16,17] except that Aida Image Analyzer v.4.50 was used. [α-^{32}P]-UTP labeled riboprobes (SR5) or [α-^{32}P]-dATP labeled DNA fragments (*gapA*, SR1) were used for detection and reprobing performed as described.[16,22] *In vitro* transcription for the generation of [α-^{32}P]-UTP labeled RNAs was carried out as described.[46] EMSA was carried out as described.[18]

Plasmid constructions

For construction of plasmid pMGG13, a PCR was performed on chromosomal DNA from *B. subtilis* DB104 with primer pair SB1818/SB1993, the resulting fragment digested with BsrGI and BamHI and inserted into vector pMGG1[47] (primers are listed in Table S2, plasmids in Table S3). For expression of the bicistronic *cggR-gapA* mRNA, a fragment was generated by PCR with primer pair SB1217/SB1083 as above, digested with EcoRI/BsrGI and inserted into pMGG1 yielding pMCG1. Plasmid pMCG2 was constructed by ligation of the 1.2 kb BsrGI/BamHI fragment from pMGG13 into pMCG1. Plasmids that allow expression of 5′ truncated *cggR-gapA* mRNA were constructed as follows: The 1.7 kb EcoRI/BamHI p_{cggR}-*gapA* fragment from pMGG13 was ligated into pMG7 yielding plasmid pMGG14 that was later used as a host for the '*cggR-gapA* fragments. Next, '*cggR-gapA* fragments were generated by PCR on pMCG2 as template using primer SB1083 and either primer SB2142, SB2249, SB2250 or SB2251. The obtained fragments were inserted into the pMGG14 PstI vector yielding plasmids pMCG4-7. Plasmid pMCG8 that lacks most of the *gapA* ORF was constructed using 2 independent PCRs on pMCG2 as template and primer pairs SB1217/SB2291 and SB2292/SB1993, respectively, the resulting fragments digested with EcoRI/Acc65I or Acc65I/BamHI and jointly inserted into pUC19. The resulting 1.8 kb EcoRI/BamHI fragment was cloned into pMG6 yielding pMCG8. Further truncation of the *cggR* portion from the 3′-end was achieved by integration of a fragment generated by PCR with primer pair SB1217/SB2387 using pMCG2 as template. The resulting fragment was digested with EcoRI and Acc65I and ligated into pMCG8 yielding pMCG13. Plasmid pMGG19 for the expression of $gapA_{Strep}$ from the *amyE* locus was generated by insertion of a fragment obtained by 2 subsequent PCRs, the first with primer pair SB1818/SB2437, and the second with primer pair SB1818/SB2438, into the HindIII/BamHI vector of pMG7.[47] All inserts were confirmed by sequencing.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

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