Heat-shock-induced refolding entails rapid degradation of bsrG toxin mRNA by RNases Y and J1

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Gene regulation accomplished by alternative folding of an mRNA is a widely used mechanism. Classical examples are the various transcriptional attenuation mechanisms that employ, for example, leader peptide translation, or binding of a modified protein, an uncharged tRNA or an antisense RNA to the 5′ untranslated region of an mRNA. With the discovery of transcriptional and translational riboswitches, it became clear that small metabolites or even metal ions can also alter RNA secondary structures and, hence, gene expression. In addition, biophysical factors like temperature can affect RNA folding, as exemplified by RNA thermometers. We have investigated in detail the type I toxin–antitoxin system bsrG/SR4 from Bacillus subtilis. The antitoxin SR4 is a cis-encoded regulatory RNA that neutralizes BsrG toxin action. SR4 prevents toxin expression by promoting degradation of the toxin mRNA and inhibiting its translation. In addition, upon temperature shock the amount of toxin mRNA decreases significantly. Here, we demonstrate that heat shock induces a refolding in the central region of the toxin mRNA that makes it more accessible to degradation by RNases Y and J1. Furthermore, we show that BsrG might play a role at the onset of stationary phase, when the antitoxin SR4 can no longer prevent toxin synthesis.

INTRODUCTION

RNA molecules fold into complex three-dimensional structures that are crucial for the biological function of non-coding RNA (e.g. tRNA and rRNA) and mRNAs affecting their transcription, maturation, translation and degradation. RNA-mediated gene regulation is of central importance for bacterial cells, since it is faster and more energy-efficient than the synthesis and action of transcription factors. The first example for alternative folding of an mRNA was discovered in 1981 in the Escherichia coli tryptophan operon and termed transcriptional attenuation (reviewed by Yanofsky, 2007). In the following years, attenuation mechanisms using binding of a modified protein, an uncharged tRNA or a cis-encoded antisense RNA were found (reviewed by Brantl, 2004). Furthermore, structured RNA elements like riboswitches or RNA thermometers and sRNA-mediated RNA–RNA interactions are key players in gene expression control by alternatively folded RNA (summarized by Breuer, 2012; Brantl & Brückner, 2014; Righetti & Narberhaus, 2014). Thereby, conformational alterations induced in mRNA by a molecule or a stress factor that acts in cis or in trans affect gene expression.

Riboswitches are cis-active elements in the 5′ untranslated regions (UTRs) of mRNAs that selectively bind small molecular mass ligands and control transcription or translation of the downstream ORFs by a conformational switch (Dambach & Winkler, 2009; Breuer, 2012). Identified riboswitch ligands include vitamins, amino acids, purines (reviewed by Roth & Breuer, 2009) or even metal ions like Mg2+ (e.g. see Coppins et al. 2007). In addition, one pH-dependent riboswitch has been reported (Nechooshtan et al., 2009).

In contrast, RNA thermometers – temperature-sensitive non-coding RNA modules in 5′ UTRs of mRNAs – change their secondary structure in response to temperature shifts affecting the translation of the downstream gene (reviewed by Kortmann & Narberhaus, 2012). Three bacterial gene classes are influenced by thermoregulation: virulence, cold-shock and heat-shock genes (Narberhaus et al., 2006; Narberhaus, 2010). Typically, an increase in temperature destabilizes the RNA thermometer structure, liberates the Shine–Dalgarno (SD) sequence and allows formation of the translation initiation complex (Narberhaus, 2010). The so far unique cold-shock-regulated RNA thermometer is located in the 5′ UTR of cspA (cold-shock protein A). It was demonstrated that cspA mRNA is unstable and less efficiently translated at 37 °C than at lower temperatures (Giuliodori et al., 2010).

Binding of a small cis- or trans-encoded regulatory RNA (sRNA) can also cause alterations of the target RNA
structure that finally influence translation, stability or maturation of (m)RNA (e.g. see Brantl & Wagner, 1994; Heidrich et al., 2007; reviewed by Brantl, 2012a).

Currently, five types of toxin–antitoxin (TA) systems are known (reviewed by Brantl, 2012b). In type I TA modules, RNA antitoxins are small regulatory RNAs that inhibit expression of convergently or divergently transcribed toxin mRNAs by base-pairing interactions. These interactions cause translation inhibition and/or enhanced RNA degradation of the toxin mRNA (reviewed by Brantl & Jahn, 2015). TA systems were discovered on plasmids and chromosomes of bacteria and archaea. Whereas plasmid-encoded TA systems act as post-segregational killing systems (Gerdes & Wagner, 2007; Weaver, 2012), the biological role of chromosome-encoded TA systems is far less clear. In a few cases, a role in persister formation, chromosome stability, resistance against cell-wall inhibiting antibiotics or SOS-induced recycling of damaged RNA has been shown (reviewed by Brantl & Jahn, 2015). The Bacillus subtilis type I TA modules investigated so far are proposed to be required for maintenance of prophages and prophage-like regions (Durand et al., 2012a).

We previously reported on the type I TA module bsrG/SR4, which is located on the SPβ prophage of the B. subtilis chromosome. The bsrG mRNA is 294 nt long and encodes a toxic peptide of 38 aa, while the 180 nt antisense RNA SR4 overlaps the 3’ end of bsrG by 123 nt (Jahn et al., 2012). The deletion of sr4 or overexpression of bsrG causes cell lysis on agar plates. SR4 is a bifunctional antitoxin, as it promotes toxin mRNA degradation by an RNase III-dependent mechanism (Jahn et al., 2012) and, additionally, induces a conformational alteration around the bsrG SD sequence impeding ribosome binding (Jahn & Brantl, 2013). Recently, we have shown that the toxin BsrG is recruited to the cell membrane, but surprisingly neither induces pore formation nor causes energy starvation. Instead, it interferes with the cell envelope biosynthesis and provokes membrane invaginations that cause delocalization of the cell-wall synthesis machinery, which finally triggers autolysis (Jahn et al., 2015). The 3’–5’ exoribonuclease R and endonuclease Y are the major RNases involved in degradation of bsrG mRNA and SR4, whereas PNPase processes SR4 precursors into the mature RNA (Jahn et al., 2012). Interestingly, heat shock entails a significant reduction of the amount of bsrG mRNA, which could be attributed to a 3.5-fold shorter half-life at high temperatures (Jahn et al., 2012).

Here, we provide a combined in vitro and in vivo analysis of the heat-shock effect on bsrG mRNA. Experimental secondary structure probing was applied to determine the structure of bsrG mRNA at higher temperature in comparison with the previously published structure at 37 °C (Jahn & Brantl, 2013). The results revealed that the central region of bsrG mRNA unfolds into a structure containing more single-stranded stretches. Heat-shock experiments with different RNase deletion strains demonstrated that this altered secondary structure is more accessible to RNases Y and J1. In addition, calculation of the relative RNA amounts of bsrG mRNA and SR4 during growth revealed that bsrG mRNA is in excess over SR4 at the transition to stationary phase, suggesting that BsrG might play a role in stationary phase adaptation.

### METHODS

**Enzymes.** Chemicals used were of the highest purity available. Firepol polymerase from Solis Biodyne, RNase T1 from Ambion, RNase T2 from Sigma and nuclease S1 from Thermo Scientific were used. Q5 high-fidelity DNA polymerase, T7 RNA polymerase and T4 polynucleotide kinase were from New England Biolabs.

**Strains and media.** TY medium served as complex medium for B. subtilis (Heidrich et al., 2006). Antibiotics were added at the following concentrations: 3 μg pheolmycin ml⁻¹, 12 μg kanamycin ml⁻¹, and 100 μg spectinomycin ml⁻¹. For the analysis of bsrG mRNA stability upon heat shock, B. subtilis BGI (WT), B. subtilis BG295 (∆mrr:: spec, Oussenko & Bechhofer, 2000) and B. subtilis BG119 (ApnApA:: kan, Wang & Bechhofer, 1996) as well as B. subtilis 503S (ArpH:: spec, Gimpel et al., 2010), B. subtilis 503P (AyhaM:: pheo, Gimpel et al., 2010), B. subtilis YDB (∆mrr:: spec), B. subtilis J1/DB (∆mrr:: spec) and their isogenic WT strain B. subtilis DB104 were used. The latter two deletion strains were constructed by transformation of B. subtilis DB104 with chromosomal DNA prepared from B. subtilis CCB434 (∆mrr:: spec) and B. subtilis CCB441 (∆mrr:: spec), respectively (Figaró et al., 2013).

**Preparation of total RNA, RNA gel electrophoresis, Northern blotting and determination of RNA half-lives.** Preparation of total RNA, RNA gel electrophoresis on 6 % denaturing polyacrylamide (PAA) gels and Northern blotting as well as the determination of RNA half-lives were carried out as described previously (Licht et al., 2005). For detection of bsrG mRNA and SR4, [γ-32P]UTP-labelled riboprobes were used, generated with T7 RNA polymerase on PCR-derived DNA fragments as described by Jahn et al. (2012). For the correction of loading errors, membranes were reprobed with [γ-32P]ATP-labelled oligonucleotide C767 specific for 5S rRNA. The oligonucleotides used are listed in Table 1.

**In vitro transcription and secondary structure analysis.** Full-length bsrG mRNA was synthesized in vitro by run-off transcription with T7 RNA polymerase from a PCR-generated DNA template (see Table 1) as described by Brantl & Wagner (1994). For usage in the structural analysis, bsrG mRNA was 5'-labelled with [γ-32P]ATP. All in vitro transcribed and labelled RNAs were purified from 6 % denaturing PAA gels. For secondary structure probing, 5'-labelled bsrG mRNA was incubated for 5 min at 37 °C or 55 °C to allow accurate folding. Subsequently, partial digestions with RNases T1 (1 U μl⁻¹) and T2 (22 U μl⁻¹) and nuclease S1 (100 U μl⁻¹) were carried out at 37 °C or 55 °C as described by Heidrich & Brantl (2003).

### RESULTS

**At transition from logarithmic to stationary growth phase, bsrG mRNA is in sevenfold excess over SR4**

Previously, we had shown that the heat-shock effect on bsrG mRNA was independent of SR4 (Jahn et al., 2012). Therefore, to investigate potential structural changes in WT bsrG mRNA upon heat shock, the bulk of bsrG
mRNA should not be sequestered by SR4. As a Δsr4 strain displays significant growth retardation and tends to mutate rapidly in liquid culture (Jahn et al., 2012), it is not applicable to additionally knock out different RNases. Therefore, we determined in which growth phase bsrG mRNA is in excess over SR4 in our WT strain DB104. To this fore, we determined in which growth phase to additionally knock out different RNases. There-
a slight preference for As. S1 is able to cut single-stranded RNA and DNA. The autoradiograms of the corresponding gels are shown in Fig. 2, and the results are summarized in Fig. 3 and compared with the structure previously determined at 37 °C (Jahn & Brantl, 2013). The large stem, P1, formed between the 5′ end of the RNA and part of its 3′ end as well as the terminator stem–loop SL4 remained unchanged. Furthermore, SL1 containing the SD sequence and the upper halves of SL2 and SL3 were not influenced. By contrast, the lower stems of SL2 and SL3, already separated from the upper stems by large internal loops, melted to give rise to an enormous inner loop comprising nt 54 to 230. This inner loop was now only interrupted between nt 90 and 130 by the shortened SL2 and nt 175 and 217 by the upper part of SL3.

**The refolded bsrG mRNA can be degraded by RNases J1 and Y**

To investigate which RNases are responsible for the rapid degradation of the refolded bsrG mRNA, we performed heat-shock experiments with knockout strains for the major endoribonuclease Y, RNase J1, which can act as endo- and 5′–3′ exoribonuclease, and the 3′–5′ exoribonucleases PnPα, R, Rph and YhaM in comparison with the isogenic WT strains. DB104 was used as WT strain for RNases Y, J1, Rph and YhaM, whereas BG1 served as WT strain for RNases R and PnPα. We did not analyse an RNase III deletion strain because we have shown before that the double-strand-specific RNase III does not affect the half-life of bsrG mRNA or SR4 individually, but only their duplex (Jahn et al., 2012). In 2012, only depletion strains for RNases Y and J1 were available, which were used to calculate the half-life of bsrG mRNA in the absence of these RNases (Jahn et al., 2012). As it was found later that both RNases are not essential (Figaro et al., 2013), we used in a control experiment the now available deletion strains for RNases Y and J1 to repeat our half-life determinations for these two RNases and the isogenic WT strain DB104 at 37 °C. As shown in Fig. 4(a), the deletion of RNase J1 had only a minor effect (the half-life decreased from 47 to 33 min), but the absence of RNase Y led to a twofold decrease of the bsrG mRNA half-life from 47 to 22 min. Moreover, the half-life of bsrG mRNA in the WT strain at 37 °C was, with 47 min, higher than measured before (Jahn et al., 2012). This can only be explained by a different composition of the complex TY medium, for which we used a new batch of yeast extract. The *B. subtilis* degradosome contains endo- and phosphofructokinase. As it was shown that, for example, citrate modulates *B. subtilis* enolase activity, glycolytic enzymes like Eno and Pfkα may act as sensors of nutritional stress and coordinate this with the RNA degrading machinery (Newman et al., 2012).

Furthermore, we compared the degradation pattern of bsrG RNA between WT and Δrny and ΔrnjA strains, respectively (Fig. 4b). At least one RNase Y cut occurs, as an ~210 nt band is missing only in the Δrny strain, but present in the WT and the ΔrnjA strains. The destabilization of bsrG RNA reflected in the shorter half-life seems to be due to an overlap of a destabilizing cut and an indirect effect that might mask the RNase Y-dependent decay. Furthermore, an ~109 nt band is significantly stronger in the ΔrnjA strain compared with the two other strains, indicating that this ~109 nt processing product is apparently degraded exonucleolytically by RNase J1.

For the heat-shock experiment, cultures were grown at 37 °C and upon transition to stationary phase (when bsrG mRNA is in excess over SR4) shifted to 48 °C. Time samples were withdrawn after 5, 10, 20, 40 and 60 min (Fig. 5a), and total RNA was prepared and subjected to Northern blotting. As shown in Fig. 5(b), no alterations were observed in strains deficient for 3′–5′ exoribonucleases PnPα, R, YhaM or Rph. In these strains, the amount of bsrG mRNA decreased as rapidly as in the isogenic WT strain. By contrast, in the strains lacking RNase Y or RNase J1, only within the first 5–10 min, a certain reduction in the bsrG mRNA amount was visible, whereas afterwards no further decrease occurred. The initial reduction might be due to a general adaptation to higher temperature. These results demonstrate that the major endonuclease RNase Y and the endo/5′–3′-exonuclease RNase J1 are involved in rapid degradation of the refolded RNA upon heat shock.

**DISCUSSION**

Our data reported here provide experimental proof of our previous hypothesis that heat shock leads to a structural change in bsrG mRNA that permits RNases to attack the now extended single-stranded regions more efficiently. Here, we show that the bsrG mRNA indeed unfolds at higher temperatures in its central part producing a large internal loop. The *in vivo* experiments with various RNase knockout strains demonstrate that in particular the major endoribonuclease RNase Y (Shahbabi et al., 2009) is involved in degradation of the toxin RNA under heat-shock conditions. In addition, RNase J1 (Even et al., 2005), which was first discovered as an endoribonuclease but later found to also possess a 5′–3′ exoribonuclease activity (reviewed by Laalami et al., 2014), was required too. This is interesting insofar as our previous half-life experiments with different RNase knockout strains did not reveal a measurable effect of an *rnjA* depletion on bsrG mRNA stability. The repetition of the half-life calculation at 37 °C with the *rnjA* deletion strain used for the heat-shock experiment yielded only a minor effect of RNase J1 on bsrG mRNA (Fig. 4a). Surprisingly, with the new RNase Y deletion strain we observed a twofold-decreased half-life at 37 °C, which was not observed with the RNase Y depletion strain in 2012 (Jahn et al., 2012). As reported previously, decreased RNA half-lives upon deletion of RNase Y or J1 can be explained by indirect effects or stabilizing cuts by the corresponding RNases (Durand et al., 2012b; Laalami et al., 2013). In the case...
Fig. 2. Secondary structure probing of bsrG mRNA with different RNases at 55 °C. In vitro-synthesized, purified, 5'-labelled WT bsrG mRNA1–296 (Table 1) was subjected to limited cleavage with the RNases indicated at 37 °C and 55 °C as described in Methods. The digested RNAs were separated on 8 % denaturing gels. Dilutions used for T1, T2 and S1 are indicated above the corresponding lanes. Autoradiograms and nucleotide positions are depicted. C, Control without RNase treatment; L, alkaline ladder; T1L, T1 digestion under denaturing conditions. Regions of altered cleavages at 55 °C are denoted by lines.
of *bsrG* mRNA, the latter can be excluded, as the size of full-length *bsrG* mRNA in the WT is identical to that in the RNase Y deletion strain. However, the comparison of the degradation pattern of *bsrG* mRNA between WT and Δ*nrn* or Δ*mja* strains yielded at least one RNase Y-dependent cleavage product, an ~210 nt band that was missing only in the Δ*nrn* strain (asterisk in Fig. 4b). Thus, RNase Y is still involved in the degradation of *bsrG* mRNA at 37 °C as observed previously (Jahn et al., 2012), but the effect is masked by indirect effects resulting in destabilization of *bsrG* mRNA in the Δ*nrn* strain. Although we had identified RNase R as the main 3′–5′ exoribonuclease in degradation of *bsrG* mRNA and SR4, this RNase – as well as the other 3′ exoribonucleases PnP, RP and YhaM, which did not affect the toxin RNA half-life at 37 °C – does not seem to participate in the rapid toxin RNA decay upon heat shock.

Interestingly, RNase Y and RNase J1 have practically the same effect on the stability of the full-length *bsrG* mRNA upon heat shock. Three different possibilities are conceivable. Firstly, RNase Y might cleave very close to the 5′ end and the resulting almost full-length 5′ end after pyrophosphate removal and ‘breathing’ of the 5′ end structure at high temperature. Thirdly, RNase Y and RNase J1 might act independently and

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Fig. 3. Altersations of the *bsrG* mRNA structure upon heat shock. The secondary structures of *bsrG* mRNA at 37 °C (left, structure previously reported by Jahn & Brantl, 2013) and 55 °C (right). All cleavage sites depicted are taken from Fig. 2. Major (filled symbols) and minor (open symbols) cuts are indicated (see box). The long double-stranded helix P1 and the four main stem–loops SL1 to SL4 are denoted. The SD sequence of the *bsrG* ORF is boxed, and the start and stop codons are shaded in grey.
Fig. 4. Stability of bsrG mRNA in WT and isogenic Δrny (RNase Y) and ΔrnjA (RNase J1) strains. (a) Half-lives were determined as described by Licht et al. (2005). Samples were taken at the times indicated after addition of 200 μg rifampicin ml⁻¹. [α-³²P]UTP-labelled riboprobes for bsrG mRNA and SR4 were used. Reprobing was performed with [γ-³²P]ATP-labelled oligonucleotide C767 specific for 5S rRNA. Autoradiograms of the Northern blots are shown; C, control. Half-lives indicated are the mean of three independent determinations. (b) Cleavage pattern of bsrG mRNA. Northern blots as in (a) taken from RNA prepared from transition state cultures. Asterisk denotes fragment derived potentially from RNase Y cleavage, triangle shows fragment stabilized in the absence of RNase J1. The bold asterisk and the filled triangle indicate alterations in comparison with the two other strains. M, Size marker.
produce one or several endonucleolytic cuts in the large unfolded region of \(bsrG\) mRNA upon heat shock. Consequently, the remaining stable \(bsrG\) mRNA in each single mutant would represent a population that is not susceptible to degradation by the remaining endoribonuclease. Currently, we cannot distinguish between the last two possibilities/hypotheses.

The unfolding of \(bsrG\) mRNA at higher temperature resembles at a first glance that of an RNA thermometer at 42 °C compared with 37 °C (e.g. Narberhaus, 2010). However, unfolding of an RNA thermometer permits the ribosomal 30S subunit to bind to the previously sequestered SD region and, hence, activates gene expression. By contrast, restructuring of the \(bsrG\) toxin mRNA allows the single-strand-specific RNases J1 and Y to gain access to the hitherto predominantly double-stranded RNA and, therefore, prevents gene expression. As the toxin mRNAs in all the other confirmed or predicted type I TA systems on the \(B. subtilis\) chromosome [\(txnA\) mRNA (Durand et al., 2012a), \(bsrG\) mRNA (Jahn & Brantl, 2013; this report), \(bsrE\) mRNA (Meißner et al., 2016)] have tightly closed SD sequences that prevent ribosome binding, toxin expression under normal conditions is very unlikely. However, even tightly closed G+C-rich RNA double-strands can 'breathe' from time to time, allowing a ribosomal 30S subunit to slide in and start translation. Under these conditions, the corresponding RNA antitoxins bind to the toxin mRNAs' 3' ends and promote their degradation (reviewed by Brantl & Jahn, 2015). As we show in Fig. 1, \(bsrG\) mRNA is only in excess over SR4 at the transition to stationary growth phase. Therefore, only during this transition phase would a temperature increase affect toxin expression. Heat shock might, therefore, present a third safety mechanism that could preclude an untimely production of BsrG.

Another type I TA system from \(B. subtilis\), \(bsrE/SR5\), resembles \(bsrG/SR4\) in that the \(bsrE\) toxin mRNA has a similar secondary structure to \(bsrG\) mRNA, and the toxin/antitoxin-binding pathway also involves three subsequent steps with similar kinetic parameters (Meißner et al., 2016). Furthermore, this TA system responds to multiple stress factors, among them heat shock, under which
the half-life of bsrE RNA was significantly reduced, too (Müller et al., 2016). Therefore, we propose that upon temperature shift, bsrE RNA unfolds similarly to bsrG mRNA, allowing endonucleases to access extended single-stranded regions. To date, only in a few cases have biological functions or targets of chromosome-encoded type I toxin been established. Whereas type I toxins present in the cytoplasm can act as RNases (e.g. E. coli SymE; reviewed by Kawano, 2012) or DNases (e.g. E. coli RalA; Guo et al., 2014), those located in the membrane either induce pores into the cell membrane (e.g. E. coli Hok; reviewed by Gerdes & Wagner, 2007), affect DNA recombination (E. coli DinQ; Weel-Sneve et al., 2013) or interfere with cell envelope synthesis (E. faecalis Fst; reviewed by Weaver, 2012). In other cases, like E. coli TisB, a role in persister formation has been demonstrated (e.g. see Dörr et al., 2010; reviewed by Wagner & Unoson, 2012). Persisters are dormant cells that have ceased to replicate, but can resuscitate when conditions improve (e.g. when no antibiotic is present any more). Previously, we had speculated that the type I toxins from the B. subtilis prophage regions might provide a linkage to metabolism and/or be important under stress conditions, e.g. when certain nutrients are exhausted or oxygen becomes limiting (Durand et al., 2012a). This is the case when cells enter stationary phase. As bsrG mRNA is in excess over SR4 at transition state, we hypothesize that BsrG toxin synthesis might occur in a subpopulation of cells, inducing cell lysis to reduce the consumption of oxygen and nutrients and, consequently, ensuring the survival of the remaining cells. However, detailed investigations at the single-cell level are needed to further pursue this issue.

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