The Essential Function of *B. subtilis* RNase III Is to Silence Foreign Toxin Genes

Sylvain Durand, Laetitia Gilet, Ciarán Condon*

CNRS UPR 9073 (affiliated with Université Paris Diderot, Sorbonne Paris Cité), Institut de Biologie Physico-Chimique, Paris, France

Abstract

RNase III–related enzymes play key roles in cleaving double-stranded RNA in many biological systems. Among the best-known are RNase III itself, involved in ribosomal RNA maturation and mRNA turnover in bacteria, and Drosha and Dicer, which play critical roles in the production of micro (mi)–RNAs and small interfering (si)–RNAs in eukaryotes. Although RNase III has important cellular functions in bacteria, its gene is generally not essential, with the remarkable exception of that of *Bacillus subtilis*. Here we show that the essential role of RNase III in this organism is to protect it from the expression of toxin genes borne by two prophages, Skin and SPβ, through antisense RNA. Thus, while a growing number of organisms that use RNase III or its homologs as part of a viral defense mechanism, *B. subtilis* requires RNase III for viral accommodation to the point where the presence of the enzyme is essential for cell survival. We identify *txpA* and *yonT* as the two toxin-encoding mRNAs of Skin and SPβ that are sensitive to RNase III. We further explore the mechanism of RNase III–mediated decay of the *txpA* mRNA when paired to its antisense RNA *RatA*, both in *vivo* and *in vitro*.

Introduction

Ribonuclease III is a key enzyme for double-stranded (ds) RNA processing reactions in both bacterial and eukaryotic systems. In bacteria, it is best known for its role in ribosomal RNA maturation [1] and more recently has been shown to be involved in the regulation by small RNAs [2–4]. The enzyme was first identified and characterized for its roles in phage RNA (f2, T7 and lambda) processing in *E. coli* [for recent review, see [5]]. Recently, RNase III has shown to be involved in bacterial gene silencing by processing CRISPR RNAs, generated as part of a host defense mechanism against phage DNA in many species [6]. In eukaryotes, enzymes with RNase III domains, such as Dicer and Drosha, play fundamental roles in the processes of RNA interference and in the generation of microRNAs [7,8].

The recent influx of high resolution RNA transcriptome data has revealed a high level of antisense RNA transcription in many species and this has further stimulated interest in how cells deal with dsRNA on a larger scale. A recent study in *Staphylococcus aureus*, showed a relatively high level of pervasive transcription that is removed by RNase III [9]. Antisense RNAs to a large proportion of coding RNAs have also been revealed in *Helicobacter pylori*, *Synechocystis* and other organisms [10–12]. RNase III is the most likely candidate for controlling the level of dsRNA in these systems.

It has long been a mystery why RNase III, encoded by the *rnc* gene, is essential in *B. subtilis* [13]. It is possible to delete *rnc* in *E. coli* and other bacteria [14–16], although such mutations are often accompanied by a decrease in growth rate. Within the Firmicutes, it has been shown that the *mc* gene is non-essential in *S. aureus* [2]. Furthermore, an RNase III-encoding gene is naturally lacking from *Deinococcus radiodurans* [17] and throughout the archaean kingdom, although many archaea possess an enzyme of analogous function, called bulge-helix-bulge (BHB) or splicing endonuclease [18]. In eukaryotes, it has been shown that it is possible to delete the RNase III gene *rnt1* in yeast, albeit with severe growth defects [19]. The essential role of RNase III in *B. subtilis* is not related to its function in rRNA metabolism or in maturation of the scRNA, part of the essential 4.5S particle involved in co-translational insertion of proteins in the cellular membrane [13]. Indeed, only trace amounts of 30S rRNA precursor, much lower than in *E. coli*, are observed in the absence of RNase III in *B. subtilis*. This indicates that the enzymes that catalyze the final steps of rRNA maturation, RNase J1 [20], Mini-III [21] and RNase M5 [22], function efficiently without prior RNase III action.

We recently performed a tiling array analysis of *B. subtilis* strains depleted for RNase III and observed a surprisingly minor contribution of RNase III to the stability of many known antisense RNAs [23]. Furthermore, the effect of RNase III-depletion on many of the specific mRNAs tested was shown to be at the transcriptional level. The whole SigW regulon was up-regulated in this way under conditions of RNase III-deficiency, for example. One of the few RNAs tested that did show an effect on mRNA stability was the *txpA* mRNA. The *txpA* gene encodes a short (59 amino acids) hydrophobic peptide that causes cell lysis when overexpressed in *B. subtilis* [24]. It is part of a type I toxin/antitoxin (TA) system. TA systems were initially discovered as part of plasmid and transposon maintenance mechanisms and have...
Author Summary

RNase III–related enzymes play key roles in cleaving double-stranded RNA throughout biology. Some of the best-known enzymes are RNase III itself, involved in ribosomal RNA maturation and mRNA turnover in bacteria, and Drosha and Dicer, which catalyze the maturation of micro (mi)–RNAs and small interfering (si)–RNAs in eukaryotes. Although RNase III has important cellular functions in bacteria, its gene is generally not essential, with the remarkable exception of that of Bacillus subtilis. In this paper, we show that the essential role of RNase III in this organism is to protect it from the expression of toxin genes borne by two prophages, through antisense RNA. B. subtilis is thus one of a growing number of organisms that uses RNase III or its homologs as part of viral defense or viral accommodation mechanisms, in this case to the point where the presence of the enzyme is essential for cell survival.

more recently been found to be widespread on chromosomes, where they are thought to play important roles in adaptive responses to stress, including phenomena such as bacterial persistence and programmed cell death (for reviews, see [25,26]). Typically, the toxin is a relatively stable molecule that targets some fundamental cellular function, such as membrane integrity, DNA replication or provokes RNA degradation, while the antitoxin is an unstable entity that needs to be constantly synthesized to counteract the toxin. In type II TA systems, both toxin and antitoxins are proteins, coded in an operon under control of a single promoter, whereas in type I TA systems, the antitoxin is an antisense (as) RNA, expressed from its own promoter on the opposite strand to the toxin gene (for recent review, see [27]). The antitoxin for the txpA mRNA is the RatA RNA and the 3’ ends of the two RNAs overlap [24]. Overexpression of RatA leads to degradation of the txpA toxin RNA, by an unknown mechanism. In this study, we show that the essential role of RNase III in B. subtilis, is the degradation of two type I toxin-encoding mRNAs, the txpA mRNA and the yonT mRNA from the Skin and SPβ prophages, respectively. In the absence of these two prophages or these two toxin-encoding mRNAs, deletion of the RNase III gene is possible and the growth rate of the resulting strains is hardly affected. We probe the role of RNase III in the mechanism of RatA mediated degradation of txpA mRNA both in vivo and in vitro.

Results

Some toxin-encoding mRNAs are stabilized in RNase III–depleted strains

In a recently performed analysis of B. subtilis strains depleted for the essential ribonucleases Rnase Y, J1 or III, we noticed a significant increase in the steady-state levels of the type I toxin mRNA txpA in strains depleted for Rnase III, while the RatA antitoxin RNA levels were elevated upon depletion of the single-strand specific Rnase Y [23]. This was an intriguing result, because we had anticipated that the levels of both sense and antisense partners would be sensitive to the double-strand specific enzyme RNase III. We confirmed the results of the tiling array experiment by Northern blot analysis at times after the addition of rifampicin to inhibit new transcription initiation and showed that the increased expression in both cases was due to increased RNA stability (Figure 1). In an Rnase J1 mutant, a degradation intermediate of the RatA asRNA accumulated. To determine whether this was a general phenomenon, we examined the expression patterns of two other suspected type I toxin/antitoxin cassettes, bsrH/as-bsrH, which lies adjacent to txpA/RatA on the chromosome, and bsg/as-bsg(SR4), which has recently been studied by the Braunl group [28]. In both cases, the asRNA was stabilized in strains depleted for the single-strand specific enzyme Rnase Y (Figures S1 and S2). However, while the bsg toxin mRNA was significantly stabilized in the Rnase III mutant, as seen by Jahn et al. [29], the bsrH toxin mRNA was not. Rather, it showed increased levels in the strain depleted for Rnase J1. Thus, even among related type I TA systems, turnover mechanisms differ.

The RNase III gene can be inactivated in strains lacking Skin and SPβ prophages

The fact that Rnase III is essential in B. subtilis, while the mc gene can be deleted in other species, suggests that the B. subtilis enzyme has a specific function that explains why it cannot be removed. We wondered whether this essential function might be related to its role in toxin mRNA turnover. Both the txpA/RatA and bsrH/as-bsrH cassettes belong to the prophage known as Skin, while the bsg/as-bsg(SR4) pair is found on the SPβ prophage. SPβ also contains a second known type I TA system named yonT/asyonT [27] and a TA pair that could be classified as type II, consisting of the precursor for the bacteriocidal sublancin peptide SunA and its immunity protein SunI [29]. Both of these toxin mRNAs are over-expressed in Rnase III depleted strains, yonT through increased mRNA stability and sunA through higher transcriptional levels (Figure 2 and Figure S3). The as-yonT transcript is highly stable regardless of whether Rnase III is present or not (Figure 2C).

Figure 1. The txpA and RatA RNAs are stabilized in strains depleted for Rnase III and Rnase Y, respectively. (A) Chromosomal context of the txpA/RatA toxin/antitoxin cassette present in the Skin prophage. (B) and (C) Northern blots performed on RNAs isolated at times (min) after rifampicin addition (150 μg/ml) in strains depleted for Rnase III (CCB288), Rnase Y (CCB294) and Rnase J1 (CCB034), probed for txpA and RatA, respectively. Northern were re-probed for S5 mRNA (S5) for normalization. Half-lives are given below each panel. The band labeled D in panel C (Rnase J1) is a degradation intermediate of RatA. Note that, in our hands, the txpA mRNA is about 45 nts longer than that proposed in [24] and consistent with the presence of a Rho-independent transcription terminator ~270 nts from the mapped transcription start site. The overlap between RatA and txpA is predicted to be ~120 nts.
doi:10.1371/journal.pgen.1003181.g001
Natural suppressors of the rnc::spc mutation have excised Skin

We next asked what was the nature of the few SpcR transformsants obtained with the wild-type and SPβ-deficient strains. Given the result above, it was conceivable that suppressor strains might be obtained by excision of the prophages under selective pressure. We therefore analyzed some of the SpcR transformsants obtained in wild-type and SPβ-deficient strains from a number of different experiments to determine their genotypes. First, none of about 100 colonies tested (23 wild-type; 84 SPβ) were CmR, indicating that their viability was not due to simultaneous transfer of the Psyl::cmr construct at amyE. We then examined the genotype of about 30 SpcR transformsants by multiplex colony PCR using oligonucleotide pairs specific for the mc, sigK and ypqP genes. The Skin and SPβ prophages interrupt the sigK and ypqP genes, respectively, and thus spontaneous excision events can be observed by restoration of these genes and the generation of 567 and 880 bp PCR products. An intact mc gene is indicated by a 347 bp PCR product. Three different classes of SpcR transformsants were obtained. A number of SpcR transformsants in wild-type strains retained an intact mc gene (10 of 19 tested; see Figure 4A for examples), indicating a background level of spontaneous spectinomycin resistance in this strain. This was also evident from the growth of a significant number of SpcR colonies in control transformations with water instead of CCB302 DNA (data not shown). All of the wild-type SpcR transformsants in which mc had been successfully inactivated (9 of 19 tested) had also excised the Skin prophage (see Figure 4A for examples). It is therefore possible to obtain suppressors of the mc::spc mutation by excising Skin. Curiously, none of the suppressors tested had excised SPβ, suggesting that both the two prophages, the presence of Skin is the most detrimental in an RncIII mutant strain. Over time, these suppressors show a pseudolysis phenotype on plates, however, suggesting all is not well in these strains. Almost all SpcR transformsants of the DSpβ strain had also excised Skin (13 of 14 tested; see Figure 4A for examples), confirming the idea that Skin is more deleterious than SPβ in strains lacking RNase III. The only suppressor that had not excised Skin (sup5) had a frame shift-mutation in the txpA gene (Figure 4B), suggesting that the TxpA peptide is the key Skin-encoded toxic moiety in the absence of RNase III.

Expression of the txpA and yonT toxin mRNAs account for the lethality of the Skin and SPβ prophages in RncIII mutants

To determine which prophage genes were responsible for the toxic effects in the absence of RNase III, we constructed strains no longer expressing the different RncIII-sensitive toxin mRNAs. Expression of the txpA gene was abolished by making a markerless deletion of its -10 promoter region (txpA -10A) using the pMAD system [31] and confirmed by Northern blot (data not shown), while the bsrG, sunA and yonT genes were inactivated by antibiotic resistance cassettes (Table 5). Transformation of the txpA, sunA, bsrG or yonT single mutants, with CCB302 chromosomal DNA had little effect on the number of SpcR colonies or the SpcR/CmR ratio obtained overnight compared to wild-type (Figure 5). Based on the observation made above with the suppressor strain sup3, we assumed that TxpA was the relevant toxin in Skin and combined the txpA -10A mutation with that of each of the three toxin genes of SPβ. Only transformation of the txpA -10A yonT double mutant with CCB302 DNA yielded a similar SpcR/CmR colony ratio to strains lacking both Skin and SPβ prophages, suggesting these two toxin-encoding mRNAs are the primary determinants of cell lethality.
toxicity in the absence of RNase III. To confirm this, we compared the growth rates of the ΔSkin ΔSP bΔrnc and ΔtxpA -10 ΔyonT Δrnc strains with those of their parental strains lacking prophages or toxin genes. Deletion of the mc gene in these two genetic contexts had no significant effect on doubling time in LB medium (Table 1), confirming that the major role of RNase III in B. subtilis under these growth conditions is to protect against the expression of these two foreign toxin-encoding mRNAs.

The degradation pathway of RatA

We decided to further investigate the mechanism of RatA-induced degradation of txpA by RNase III, by studying the degradation pathways of the individual RNAs and the RatA/txpA complex. We were also curious about the sensitivity of the RatA antitoxin to the single-strand specific enzyme RNase Y, as we had expected that both toxin and antitoxin RNAs would be sensitive to RNase III.

The stabilization of RatA under conditions of RNase Y depletion suggested that the initial endonucleolytic cleavage of this asRNA is performed by RNase Y. When RNase J1 was depleted, a number of 3' proximal RatA degradation intermediates accumulated on high resolution gels, clustered around 130 nts and 75 nts in length (Figure S4A). This suggests that the major cleavage site by RNase Y and entry point for RNase J1 is around nt 90 of RatA, just upstream of the region of complementarity with txpA. The accumulation of the 75-nt species could either be due to the stabilization of the downstream product of a secondary endonucleolytic cleavage by an unknown enzyme or due to the incomplete depletion of RNase J1 and a stalling of the small amounts of remaining enzyme at the base of secondary structure. The 5' ends of the 130-nt and 75-nt species were mapped by primer extension to nts 91 and to two clusters of bands around nts 97–99, and nts 145–147, respectively (Figure S5), both at the base of secondary structures (see below).

In the absence of PNPase, the major 3' 5' exoribonuclease in B. subtilis, a n, 80 nt 5' proximal species accumulated (Figure S6), also consistent with an initial cleavage by RNase Y around nt 90 and a difficulty of the remaining 3' 5' exoribonucleases in degrading past stem loop 2 (see below). A model for the degradation pathway of RatA is shown in Figure 6A, with cleavage by RNase Y around nt 90 giving access to both PNPase and RNase J1.

Deletions of the mc gene were selected in strains lacking Skin and SP b prophages.

(A) Agar plates showing colony growth after transformation of wild-type (WT) strains and strains lacking up to three prophages with 5 μg CCB302 (mc::spc amyE::PxyI-rnc Cm) chromosomal DNA, selected for spectinomycin (left panel) or chloramphenicol (right panel) resistance. (B) Histograms showing transformation efficiencies (number of SpcR colonies/number CmR colonies) for the different prophage deficient strains.

doi:10.1371/journal.pgen.1003181.g003

Figure 3. The mc gene can be deleted in strains lacking Skin and SP b prophages. (A) Agar plates showing colony growth after transformation of wild-type (WT) and ΔSP b ΔSkin strains with 5 μg CCB302 (mc::spc amyE::PxyI-rnc Cm) chromosomal DNA, selected for spectinomycin (left panel) or chloramphenicol (right panel) resistance. (B) Histograms showing transformation efficiencies (number of SpcR colonies/number CmR colonies) for the different prophage deficient strains.

doi:10.1371/journal.pgen.1003181.g003

Figure 4. Suppressors of the mc::spc mutation lack the Skin prophage. (A) Agarose gel showing multiplex PCR analysis of mc::spc suppressors in wild-type (WT) and ΔSP b strains. A PCR product corresponding to the reconstituted yapP and sigK genes is indicative of excision of the SP b and Skin prophages, respectively. Spontaneous SpcR strains show a PCR product for the mc gene, while successfully deleted mc strains do not give a corresponding PCR product. A DNA marker (bp) is shown in the lane labeled M. (B) Sequence comparison of the txpA gene in wild-type (wt) and the sup5 mutant shown in panel A.

doi:10.1371/journal.pgen.1003181.g004

Figure 5. The mc gene can be deleted in strains lacking TxpA and YonT toxins. Histogram showing transformation efficiencies (number of SpcR colonies/number CmR colonies) for strains lacking different toxin genes or mRNAs.

doi:10.1371/journal.pgen.1003181.g005

Figure 6. The degradation pathway of RatA is shown in Figure 6A, with cleavage by RNase Y around nt 90 giving access to both PNPase and RNase J1.
promoter was deleted. Surprisingly, the degradation pattern of RatA did not change; we observed the same stabilization of RatA in the absence of RNase Y and the accumulation of the same 3’ proximal degradation intermediates of around 130 nts and 75 nts in cells depleted for RNase J1 (Figure S4B). There were two possible explanations for this result; either the structure of RatA does not change significantly upon hybridization to txpA, or RatA is produced in large excess over txpA, in which case the degradation pathway observed in wild-type cells is primarily that of RatA alone, rather than the RatA/txpA hybrid. Quantitative Northern blotting using known quantities of in vitro transcribed RatA and txpA, showed that RatA is present in about 15-fold excess over txpA in wild-type cells (Figure S7), in favor of the latter hypothesis.

**Degradation of txpA is dependent on both RatA and RNase III**

In wild-type cells, turnover of txpA is primarily dependent on RNase III. The fact that RatA is present in large excess over txpA in this strain suggests that, in the case of txpA, we primarily measured an effect of enzyme depletion on the RatA/txpA hybrid. We failed in attempts to make a deletion of the -10 region of the RatA promoter to study the degradation of wild-type txpA in the absence of RatA in vivo, presumably because of TxpA toxicity. To circumvent this problem, we made an untranslated derivative of the txpA mRNA by changing its start codon to AAG. This mutation falls outside of the region of RatA/txpA complementarity and is not anticipated to affect the secondary structure of txpA (see below) or the ability of these two RNAs to form a duplex. Like the wild-type txpA mRNA, the AUG→AAG mRNA was stabilized in strains depleted for RNase III (Figure 7), showing that the degradation of this non-translated derivative is still RNase III dependent. The difference in half-life compared to that measured with the wild-type txpA mRNA in Figure 1 (11.5 mins vs. >20 mins) is most likely explained by different RNase III depletion efficiencies in the two experiments.

We were able to successfully replace the ratA promoter region with an antibiotic resistance cassette in the context of the txpA [AUG→AAG] mutation, showing that the peptide rather than the mRNA is toxic in *B. subtilis*, and allowing us to study the txpA mRNA independently of RatA. In the absence of RatA, the txpA [AUG→AAG] mRNA was highly stabilized, regardless of whether or not RNase III was present. These experiments show that the rapid turnover of the txpA mRNA is dependent on both RatA and RNase III; in the absence of either one, the txpA mRNA is extremely stable.

**txpA and RatA form an extended hybrid that is a substrate for RNase III cleavage**

To study the degradation mechanisms of RatA and txpA further, we turned to *in vitro* experiments. We made 5’ labeled RatA and txpA RNAs in *vivo* using T7 RNA polymerase and first probed the secondary structures of the individual RNAs and the RatA/txpA hybrid using the single-stranded endonuclease activity of RNase J1. We have previously shown that this property of RNase J1 can be exploited to determine both known and unknown RNA folding patterns [32]. The structure probing data are shown in Figures S8, S9, and S10 and the predicted secondary structures of the individual RatA and txpA RNAs and the RatA/txpA hybrid are shown in Figure 6. The RatA asRNA forms four stem-loop structures (labeled SL1–4) in addition to the transcription terminator (ter). The txpA RNA forms up to six helical structures (SL1–6) in addition to the terminator in *vivo*. The significantly reduced intensity of RNase J1 cleavages between nts 137 and 169 of RatA in the RatA/txpA hybrid (Figure S8) is consistent with an extended duplex comprising most of the last 120 nts of each RNA. The duplex does not appear to extend all the way to the 3’ end however, as an RNase J1 hypersensitive region appears in rats corresponding to the downstream strand of the RatA terminator sequence (marked with an asterisk in Figure S8B) when RatA is complexed with txpA.

Incubation of the txpA mRNA with purified RNase III revealed a major cleavage site at nts 96–98 and a corresponding cleavage site at nts 218–220, consistent with cleavage on both sides of the helical structure SL4 by RNase III (Figure 8A). When txpA was hybridized to RatA, these cleavages disappeared completely and different set of RNase III cleavage sites was seen between nts 170–179 of txpA. Thus, binding of RatA changes the structure of txpA, creating new RNase III-sensitive sites. Corresponding cleavages were seen between nts 198–206 of the RatA RNA (Figure 8B), suggesting that both sense and antisense partners are simultaneously cleaved by RNase III. A second pair of RNase III cleavage sites was observed at nts 217/218 of txpA and nt 159 of RatA.

Since the RatA asRNA is a substrate for RNase Y in *in vivo*, we also subjected both RNAs to RNase Y cleavage in *vivo*. Incubation of the RatA mRNA with purified RNase Y, revealed a number of minor sites of endonucleolytic cleavage, indicative of a relatively relaxed specificity for this enzyme in *vivo* (Figure 8B). The site most relevant to the pathway seen in *vivo* is likely to be that at nt 93, close to the major site of RNase J1 access to the RatA RNA in *vivo* (nt 91). Upon hybridization to txpA, the in *vivo* cleavage by RNase Y at nt 93 was considerably weaker while that at nt 103, immediately upstream of the duplex, was significantly enhanced. Furthermore, the clusters of cleavages from nt 137 to 159 were lost, consistent with the creation of an extended RatA/txpA duplex in this region. Although a relatively prominent RNase Y cleavage site was observed at nt 201 of the txpA mRNA alone (Figure 8A), this site is in the region of complementarity to RatA and is not likely to exist very much *in vivo* under conditions of a large excess of RatA. Indeed, cleavage at this site no longer occurs when txpA is hybridized to RatA in *vivo*.

**Discussion**

In this paper we have determined that the essential function for RNase III in *B. subtilis* is to protect it from the expression of toxin genes borne by the Skin and SPF prophages, via antisense RNA. The cleavage of double-stranded RNA by RNase III-related enzymes as part of host defense mechanisms against virus infection is well-documented. The role played by Dicer in the generation of siRNAs in the process of RNA interference, for example, is a fundamental component of this innate viral defense system (for recent review, see [33]). A recent study has also shown that the CRISPR system, initially characterized as a self...
contained defense mechanism against prokaryotic plasmids and phages, uses the host enzyme RNase III to help generate the short protective crRNAs in some bacteria [6]. Although B. subtilis does not contain a CRISPR cassette, our data shows that it nonetheless relies on RNase III to protect it from prophage gene expression, through antisense RNA.

The mode of action of the TxpA and YonT toxins is not known. Both encode short peptides of 59 and 58 amino acids, respectively, with

Figure 6. Secondary structures of RatA, txpA, and the RatA/txpA hybrid. Stem-loop (SL) structures and transcription terminators (ter) are labeled. Strong RNase III cleavages sites are shown as thick red arrows and minor sites as thin red arrows. Strong RNase Y cleavage sites are shown as black arrows and minor sites as grey arrows. The main degradation pathway in vivo is represented by RNase Y cleavage (blue scissors), followed by degradation of the upstream and downstream fragments by PNPase (green Pacman symbol) and RNase J1 (orange Pacman symbol), respectively. (A) Secondary structure of RatA. (B) Secondary structure of txpA. (C) Secondary structure of RatA/txpA hybrid.

doi:10.1371/journal.pgen.1003181.g006

Figure 7. Degradation of txpA (AUG→AAG) mRNA by RNase III is RatA-dependent. Northern blots performed on RNA isolated at times (min) after rifampicin addition in (A) strain CCB467 txpA (AUG→AAG) and (B) strain CCB468 txpA (AUG→AAG) PratA::ery, depleted or not for RNase III. Northerns were re-probed for 5S rRNA (5S) for normalization. Half-lives are given below each panel.

doi:10.1371/journal.pgen.1003181.g007
little sequence homology between them. Both have hydrophobic N-termini, predicted to form a transmembrane domain [27], and hydrophilic C-termini; the last 20 amino acids of YonT are very highly positively charged (75% arginine or lysine residues). TxpA has been shown to cause cell-lysis in *B. subtilis* [24] and YonT to cause growth arrest upon induction in *E. coli* [27]. We had previously noticed that TxpA (previously known as YqdB) and YonT have the two strongest Shine-Dalgarno (SD) sequences in *B. subtilis* [34], with 11–12 possible base-pairs with the anti-SD 3' end of 16S rRNA. It is not known what effect this has on toxin translation levels; while a very strong SD should help ribosome recruitment, these ribosomes may have difficulty escaping the SD. The observation that suppressor colonies, obtained upon transformation of either wild-type or *ΔSP*b cells with the *rnc::spc* construct, have excised the Skin prophage or mutated TxpA, suggests that TxpA is the more toxic of the two peptides (it also has the stronger match to the anti-SD in 16S rRNA).

Paradoxically, we were able to obtain suppressor strains lacking only the Skin prophage, but for optimal transformation efficiency with the *mcz::spc* construct, it was necessary to remove both Skin and SPβ. Similarly, one of the suppressor strains had a frame-shift mutation in *txpA*, yet the transformation efficiency of the *txpA−10A* mutant was much lower than that of the *txpA yonT* double mutant. We cannot rule out the possibility that the suppressors have additional mutations that affect the levels or expression of SPβ genes (see below). However, it is also important to consider that the pressure on the cell to produce colonies is not the same in both cases. In the transformation efficiency assay, cells are additionally being asked to go through the process of becoming competent, a complex developmental program put in place by starving cells. Although the specific effect of competence on toxin gene expression is not known, expression of *yonT* has recently been shown to reach a peak about 30 mins after glucose starvation [35] and this could have a significant effect on the number of colonies recovered in the transformation assay. The fact that the *Δskin SPβ* suppressors of the *mcz::spc* mutation show a pseudolysis phenomenon on plates suggests that they are not completely healthy, consistent with the idea that the lack of both prophages and their encoded toxins is the optimal configuration for growth and survival in the absence of RNase III.

![Figure 8. Cleavage of RatA, *txpA*, and the RatA/*txpA* hybrid by RNase III and RNase Y.](http://www.plosgenetics.org/doi/fig/10.1371/journal.pgen.1003181.g008)
The Bechhofer laboratory has previously isolated two suppressor strains (BG322 and BG323) in which the *mc* gene was successfully inactivated [13]. We also examined both of these strains for the presence of the Skin and SPβ prophages. Both BG322 and BG323 have excised Skin, but appear to have different propensities to excise SPβ during growth (Figure S11). BG323 consistently has a greater proportion of cells lacking SPβ than BG322, but only a minor fraction of cells in freshly plated colonies of either strain have lost SPβ (data not shown), suggesting that this prophage is not stably cured and that these strains have additional mutations that influence their ability to excise SPβ.

We performed *in vivo* and *in vitro* experiments to probe the mechanism of RatA-mediated destabilization of txpA mRNA catalyzed by RNase III. We showed that degradation of a nontranslated derivative of txpA (AUG→AAG) is dependent on both RatA and RNase III in *vivo* and that hybridization of the txpA and RatA RNAs generates a highly sensitive substrate for RNase III in *vivo*. We also determined the secondary structures of the two individual RNAs and the RatA/txpA hybrid. The apical loops of SL3 and the transcription terminator of RatA are complementary to those of the terminator of txpA and SL6, respectively (Figure 6).

The formation of sense/antisense RNA complexes is often initiated through loop-loop or ‘kissing’ interactions [36,37] and RatA and txpA can potentially use the same mechanism to initiate hybrid formation. The extent to which the duplex extends in either direction from the initial interaction site depends on the RNAs in question. For the copA/copT sense/antisense pair, involved in copy number control of the plasmid R1, the duplex does not extend very far from the initial interaction site before getting trapped in a four-way junction with side-by-side helices [37]. The RatA/txpA duplex, on the other hand, seems to extend over most of the 120 nucleotides of complementarity between the two RNAs.

The formation of an extended duplex between RatA and txpA generates a substrate for RNase III and this is the primary mechanism of txpA control in *vivo*. Indeed, we can calculate that RatA remains in about a 3.5 fold excess over txpA in *vivo* strains depleted for RNase III; despite this continued excess of RatA, the increased levels of the txpA mRNA are toxic to *B. subtilis*, presumably because the txpA mRNA can still be translated when paired to RatA. The control of *txpB* by *av-*bga (SR4A) also occurs at the level of RNA turnover, mediated by RNase III [28], while the control of the type I TA system of *Enterococcus faecalis*, Fst, occurs primarily at the translational level [38]. The products of txpA cleavage by RNase III appear to be degraded primarily by PNPase, judging from the accumulation of many 5′ proximal txpA fragments in a *txpB* mutant strain in *vivo* (Figure S6B). The txpA mRNA is also a good substrate for RNase III in *vivo* even in the absence of RatA, through the formation of the double-stranded helix SL4 (Figure 6). SL4 can no longer form when PNPase is depleted due to PNP overexpression from a pLP-based plasmid [10].

Depletion of RNase III also leads to an accumulation of the yonT mRNA and a longer species that encodes both YonT and YoyJ. A ~100-nt asRNA to yonT was detected previously by Northern blot [27] and we have seen this species is stable even in the presence of RNase III (Figure 2C). A recent tiling array analysis suggests this asRNA also overlaps the beginning of the YoyJ reading frame (Figure 2A) and may therefore control the expression of both genes. YoyJ encodes an 83 amino acid protein of unknown function.

It is not clear what the role TxA or YonT play in the biology of the prophage. They may simply be part of a prophage maintenance system, by killing *B. subtilis* cells that do not constantly synthesize their asRNAs. However, they could also play a role in linking prophage biology to the physiology of the cell. It has recently been shown, for example, that levels of the *bogG* toxin mRNA of SPβ are decreased about 10-fold at 48°C [28]. Interestingly, both *txpA* and *yonT* show high expression levels under conditions of glucose exhaustion [35]. TxA expression is additionally sensitive to both high and low phosphate concentrations, while *yonT* is induced in the presence of mitomycin C, which induces the SOS response. It will be interesting to determine whether these conditions affect either the behavior of the prophage or the host-cell in a toxin-dependent manner. In a recent study, deletion of the Skin transcriptional repressor (SkinR) was shown to cause cell death through overexpression of two proteins YqAM and YqAH, which bind to DnaA and DnaC, respectively [39]. The Skin prophage appears thus to have a variety of options that allow it to slow or halt the growth of its host cell.

**Materials and Methods**

**Construction of bacterial strains**

The *B. subtilis* strains used in this study were derivatives of W168 or 168 *tp-*. Strains lacking SPβ, Skin and PBSX prophages (168 *tp- background*) have been described previously [30] and were a kind gift from J.M. van Dijl. A Skin-less derivative of W168 was kindly constructed by P. Stragier and named CCB297.

Strain CCB302 (mc::spc amyE::pX-reC Cm) was constructed as follows. The *mc* gene was amplified from *B. subtilis* chromosomal DNA using oligos CCB316/817 (Table S1), digested with SpeI and cloned in pX [40] cleaved with the same enzymes. The resulting plasmid, pX- *mc*, was integrated in the *amyE* locus of Skin-less strain CCB297 to create CCB298. The *mc* gene of CCB298 was then interrupted by a spectinomycin resistance cassette using chromosomal DNA from strain BG324 [13] to create strain CCB302. Growth of this strain is xylose-dependent.

Strains CCB304 (mg4pMUTIN-rnja), CCB285 (mc::spc amyE::pX-spac-reC Cm) and CCB294 (mc::spc amyE::pPac-agC Cm) have been described previously [20,23]. RNase deletions were performed as in [20,23].

Strain CCB363 (SPβ::PSPID::spB kan) was constructed by transforming W168 with chromosomal DNA from MO4738 SPβ::PSPID::spB kan *spoIIIE::TeC*, a kind gift from P. Stragier. This chromosomal DNA was also used to transform CCB297 to create CCB364 ASkin SPβ::PSPID::spB kan.

Strain CCB325 (*txpA-10A*) was made by markerless mutation of the -10 promoter region of *txpA* on the W168 chromosome using plasmid pMAD-I according to [31]. Plasmid pMAD-I was constructed as follows. Overlapping upstream and downstream fragments containing the *txpA-10* promoter deletion were amplified using oligo pairs CC907/908 and CC909/910, respectively. The overlapping fragments were then assembled in a new PCR reaction with CC907 and CC910, digested with BamHI and cloned in pMAD [31], a kind gift from M. Debarbouillé.

Strain CCB361 *bogG::kan* was constructed by building a PCR fragment containing upstream and downstream regions of the *bogG* gene flanking a kanamycin resistance cassette over by overlapping PCR, using oligos CC1015–1020 (Table S1), and transforming in W168. Chromosomal DNA from this strain was used to transform strain CCB325 to create CCB365 *txpA-10A bogG::kan*.

Strain CCB377 *sunA::kan* was made by transforming W168 by chromosomal DNA from a *sunA::kan* strain, kindly provided by P. Stragier.
and RatA transcripts were transcribed with a 5′ OH group (using an 6-fold excess of guanosine over GTP) to facilitate 5′ to 3′ polymerase (Ambion) using PCR templates with integrated T7 transcription signals. RNAs were synthesized with a 5′ OH group and cloned in pMAD.

In vitro RNase cleavage assays

The purification and assay of RNase III has been described previously [32]. The txpA and RatA transcripts were transfected into E. coli BL21 CodonPlus cells to yield strain CCE192.

The purification and assay of RNase III has been described previously [32]. The txpA and RatA transcripts were transfected into E. coli BL21 CodonPlus cells to yield strain CCE192.

Supporting Information

Figure S1  The as-bsrH RNA shows similar RNase sensitivity to RatA, but the bsrH mRNA is insensitive to RNase III depletion. (A) Chromosomal context of the bsrH/as-bsrH toxin/antitoxin cassette present in the skin prophage. (B) and (C) Northern blots performed on RNAs isolated at times (min) after rifampicin addition (150 μg/ml) in strains depleted for RNase III (CCB288), RNase Y (CCB294) and RNase J1 (CCB304), probed for bsrH and as-bsrH, respectively. Northern blots were re-probed for 5S rRNA (5S) for normalization. Half-lives are given below each panel. The band labeled D in panel C (RNase J1) is a degradation intermediate of as-bsrH.

Figure S2  The bsrG and as-bsrG (SR4) RNAs are stabilized in strains depleted for RNase III and RNase Y, respectively. (A) Chromosomal context of the bsrG/as-bsrG toxin/antitoxin cassette present in the SP8 prophage. (B) and (C) Northern blots performed on RNAs isolated at times (min) after rifampicin addition (150 μg/ml) in strains depleted for RNase III (CCB288), RNase Y (CCB294) and RNase J1 (CCB304), probed for bsrG and as-bsrG, respectively. Northern blots were re-probed for 5S rRNA (5S) for normalization. Half-lives are given below each panel.

Figure S3  The sunI mRNA is overexpressed in a strain depleted for RNase III. (A) Chromosomal context of the sunI-sunA locus present in the SP8 prophage. (B) Northern blots performed on RNA isolated at times (min) after rifampicin addition in strains depleted for RNase III (CCB288). The Northern blot was re-probed for 5S rRNA (5S) for normalization. Half-lives are given below each panel.

Figure S4  The degradation profile of RatA is identical in wild-type strains and in strains no longer expressing txpA. (A) High resolution (5% polyacrylamide) Northern blots performed on RNAs isolated from wild-type cells at times (min) after rifampicin addition (150 μg/ml) in strains depleted for RNase III (CCB288), RNase Y (CCB294) and RNase J1 (CCB304). Migration positions (in nt) of an RNA marker are given to the right of the figure. (B) High resolution Northern blots performed on RNAs isolated from txpA -10Δ cells (CCB323) and txpA -10Δ cells depleted for RNase III (CCB348), RNase Y (CCB338) and RNase J1 (CCB337). Half-lives are given below each panel.

Figure S5  Mapping of 5′ ends of RatA intermediates that accumulate in strains depleted for RNase J1. Primer extension assay using oligo CC758 (Table S1) on 15 μg of total RNA isolated from wild-type strains (WT) and strain CCB034 (Table S2) grown in the presence and absence of IPTG. A sequence reaction performed with the same oligo on a PCR template of the txpA/RatA region (oligos CC795/796; Table S1) is shown to the left. The sequence is labeled as its reverse complement to facilitate direct reading.

Figure S6  RatA and txpA degradation intermediates accumulate in the absence of PNPase. Northern blots of RNA isolated from wild-type (WT), PNPase (SSB1030), RNase R (CCB021) and RNase PH (CCB308) mutants (Table S2) probed with (A) oligo CC862 (Table S1) specific for the 5′ end of RatA and (B) oligo CC861 (Table S1) specific for the 5′ end of txpA.

Figure S7  RatA is present in excess over txpA in wild-type cells. Quantitative Northern blot loaded with known quantities (in pg) of

The sequence is labeled as its reverse complement to facilitate direct reading.
in vitro transcribed txpA and RatA RNAs, and either 5 or 15 μg of total RNA isolated from wild-type cells.

(TIF)

Figure S8 Structure probing of RatA RNA and RatA/txpA hybrid. In vitro transcribed 5’-labeled RatA RNA (0.5 pmol) alone hybridized to a 2-fold excess of unlabeled txpA were incubated with 0.6 μg RNase J1 for 2 or 5 minutes and loaded on a 5% polyacrylamide/urea gel. The RatA RNA was also digested with RNase T1 (Ambion) under denaturing conditions at the dilutions shown to reveal migration positions of G residues. A DNA size standard (in nts) is shown in the lane labeled M. (A) short migration (B) long migration with same samples.

(TIF)

Figure S9 Structure probing of txpA RNA and txpA/RatA hybrid. In vitro transcribed and 5’-labeled txpA RNA (0.5 pmol) alone hybridized to a 2-fold excess of unlabeled RatA were incubated with 0.6 μg RNase J1 for 2 or 5 minutes and loaded on a 5% polyacrylamide/urea gel. The 5’-labeled txpA RNA was also digested with RNase T1 (Ambion) under denaturing conditions at the dilutions shown to reveal migration positions of G residues. A DNA size standard (in nts) is shown to the right. (A) short migration (B) long migration with same samples.

(TIF)

Figure S10 Summary of structure probing data for RatA, txpA and txpA/RatA hybrids. Mapped RNase J1 cleavages (arrowheads) on the best-fitting secondary structures of (A) RatA (B) txpA and (c) the txpA/RatA hybrid. Overlapping sequences of RatA and txpA are shown in red and green, respectively. The Shine-Dalgarno (SD) sequence, initiation and termination codons of txpA are shown in blue.

(TIF)

Table S1 Oligonucleotides used in this study. Non-hybridizing sequences are in lower case letters.

(DOC)

Table S2 B. subtilis strains used in this study.

(DOC)

Acknowledgments

We thank lab members for helpful discussion and S. Figaro for the purification of RNase Y. We thank S. Duperrier and P. Stragier for the construction of the W168 skinless strain and J. M. van Dijl for the snaA::kan strain and strains lacking prophages. We also thank D. Bechhofer for strains BG322–324 and M. Déharboulle for plasmid pMD.

Author Contributions

Conceived and designed the experiments: SD CC. Performed the experiments: SD LG. Analyzed the data: SD CC. Contributed reagents/materials/analysis tools: SD LG CC. Wrote the paper: CC SD.

References

1. Nikolaev N, Silengo L, Schlesinger D (1973) A role for ribonuclease 3 in processing of ribosomal ribonucleic acid and messenger ribonucleic acid precursors in Escherichia coli. J Biol Chem 248: 7967–7969.

2. Huntziger E, Boisvert S, Savaea C, Benito Y, Grossmann T, et al. (2005) Staphylococcus aureus RNAIII and the endoribonuclease III coordinateately sygA gene expression. EMBO J 24: 824–835.

3. Viegas SC, Silva JJ, Saramago M, Domingues S, Arraiano CM (2011) Regulation of the small regulatory RNA Mca of bacilli, the ribonuclease III dependent pathway. Nucleic Acids Res 39: 2911–2920.

4. Opylka JA, Fozo EM, Hemm MR, Storz G (2011) RNase III participates in GadA-dependent cleavage of the gadGadW mRNA. J Mol Biol 406: 29–43.

5. Nicholson AW, editor (2011) Ribonuclease III and the role of double-stranded RNA processing in bacterial systems. Berlin: Springer-Verlag. 269 p.

6. Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, et al. (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 471: 602–607.

7. Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409: 363–366.

8. Lee Y, Ahn C, Han J, Choi H, Kim J, et al. (2003) The nuclear RNase III in Saccharomyces cerevisiae. EMBO J 22: 415–419.

9. LasI, Toleo-Arana A, Dobin A, Villanueva M, de los Mozos IR, et al. (2011) Genome-wide antisense transcription drives mRNA processing in bacteria. Proc Natl Acad Sci U S A 108: 20172–20177.

10. Sharma CM, Hoffmann S, Darfeuille F, Reignier J, Findeiss S, et al. (2011) Experimentally anchored map of transcriptional start sites in the model cyanobacterium Synechocystis sp. PCC6803. Proc Natl Acad Sci U S A 108: 2124–2129.

11. Georg J, Hess WR (2011) cis-antisense RNA, another level of gene regulation in cyanobacteria. Microbiol Mol Biol Rev 75: 286–300.

12. Xue S, Calvin K, Li H (2006) RNA recognition and cleavage by a splicing endonuclease. Science 312: 906–910.

13. Chaufour G, Rotondo G, Legrain P, Jacquier A (1998) Processing of a dicistronic small nuclear RNA precursor by the RNA endonuclease Rnt1. EMBO J 17: 3726–3737.

14. Britton RA, Wen T, Schafer L, Pellegrini O, Uicker WC, et al. (2007) Maturation of the 3’ end of B. subtilis ssa168 RNA by the essential ribonuclease YkG/RNase J1. Mol Microbiol 63: 127–138.

15. Redko Y, Bechhofer DH, Condon C (2008) Mini-I, an unusual member of the RNase III family of enzymes, catalyses 23S ribosomal RNA maturation in B. subtilis. Mol Microbiol 66: 1096–1106.

16. Viegas SC, Piráirer V, Sitka A, Silva JJ, Vogel J, et al. (2007) Characterization of the role of ribonucleases in Salmoneal small RNA decay. Nucleic Acids Res 35: 7651–7664.

17. Cordon G, Putzer H (2002) The phylogenetic distribution of bacterial ribonucleases. Nucleic Acids Res 30: 5339–5346.

18. Kogak KL, Li H (2006) RNA recognition and cleavage by a splicing endonuclease. Science 312: 906–910.

19. Chanfreau G, Rotondo G, Legrain P, Jacquier A (1998) Processing of a dicistronic small nucleolar RNA precursor by the RNA endonuclease Rnt1. EMBO J 17: 3726–3737.

20. Britton RA, Wen T, Schafer L, Pellegrini O, Uicker WC, et al. (2007) Maturation of the 3’ end of B. subtilis ssa168 RNA by the essential ribonuclease YkG/RNase J1. Mol Microbiol 63: 127–138.

21. Jahn N, Preis H, Wiedemann C, Brantl S (2012) BsrG/SR4 from Bacillus subtilis is a long-range RNA endonuclease in the initiation step of RNA interference. Nature 409: 363–366.

22. Condon C, Brechmier-Baey D, Belchev B, Grunberg-Manago M, Putzer H (2001) Identification of the gene encoding the 5S ribosomal RNA maturase in Bacillus subtilis. Nature 415: 242–253.

23. Durand S, Gillet I, Nicolas P, Besüères P, Condon C (2012) Three essential ribonucleases, RNase Y, J1 and III, control the abundance a majority of B. subtilis mRNAs. PLoS Genet doi:10.1371/journal.pgen.1002528.

24. Sivagami JM, Perkins JB, Losick R (2005) Small untranslated RNA antitoxin in Bacillus subtilis. J Bacteriol 187: 6641–6650.

25. Van Melder L (2010) Toxin-antitoxin systems: why so many, what for? Curr Opin Microbiol 13: 701–705.

26. Yamauchi Y, Inouye M (2011) Regulation of growth and death in Escherichia coli by toxin-antitoxin systems. Nat Rev Microbiol 9: 779–790.

27. Fozo EM, Makarova KS, Shabalina SA, Yutin N, Koonin EV, et al. (2010) Identification of the gene encoding the 5S ribosomal RNA maturase in Bacillus subtilis. Nature 58: RNA is dispensable for ribosome function. RNA 7: 242–253.

28. Condon C, Brechmier-Baey D, Belchev B, Grunberg-Manago M, Putzer H (2001) Identification of the gene encoding the 5S ribosomal RNA maturase in Bacillus subtilis. Nature 415: 242–253.

29. Dostov JV, Koewen TR, Scharich AK, Reis CR, Ensing HT, et al. (2009) Immunity to the bacteriocin sublincan 168 is determined by the Sunl (YodF) protein of Bacillus subtilis. Antimicrob Agents Chemother 53: 651–661.
30. Westers H, Doerenbos R, van Dijl JM, Kabel J, Flanagan T, et al. (2003) Genome engineering reveals large dispensable regions in *Bacillus subtilis*. Mol Biol Evol 20: 2076–2090.

31. Arnaud M, Chastanet A, Debarbouille M (2004) New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. Appl Environ Microbiol 70: 6887–6891.

32. Daou-Chabo R, Condon C (2009) RNase J1 endonuclease activity as a probe of RNA secondary structure. RNA 15: 1417–1423.

33. Umbach JL, Cullen BR (2009) The role of RNAi and microRNAs in animal virus replication and antiviral immunity. Genes Dev 23: 1151–1164.

34. Daou-Chabo R, Mathy N, Benard L, Condon C (2009) Ribosomes initiating translation of the *hbs* mRNA protect it from 5’–3’ exoribonucleolytic degradation by RNase J1. Mol Microbiol 71: 1538–1550.

35. Nicolas P, Mader U, Dervyn E, Rochat T, Leduc A, et al. (2012) Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. Science 335: 1103–1106.

36. Persson C, Wagner EG, Nordstrom K (1990) Control of replication of plasmid R1: formation of an initial transient complex is rate-limiting for antisense RNA–target RNA pairing. EMBO J 9: 3777–3785.

37. Kolb FA, Enggalen HM, Slagter-Jager JG, Ehresmann B, Ehresmann C, et al. (2000) Progression of a loop-loop complex to a four-way junction is crucial for the activity of a regulatory antisense RNA. EMBO J 19: 5905–5913.

38. Shokeen S, Patel S, Greenfield TJ, Brinkman C, Weaver KE (2008) Translational regulation by an intramolecular stem-loop is required for intermolecular RNA regulation of the *par* addiction module. J Bacteriol 190: 6076–6083.

39. Kimura T, Amaya Y, Kobayashi K, Ogasawara N, Sato T (2010) Repression of *sigK* intervening (skin) element gene expression by the CI-like protein SknR and effect of SknR depletion on growth of *Bacillus subtilis* cells. J Bacteriol 192: 6209–6216.

40. Kim L, Mogk A, Schumann W (1996) A xylose-inducible *Bacillus subtilis* integration vector and its application. Gene 181: 71–76.

41. Condon C, Pellegrini O, Mathy N, Benard L, Redko Y, et al. (2008) Assay of *Bacillus subtilis* ribonucleases in vitro. Methods Enzymol 447: 277–308.