

Quasi-essentiality of RNase Y in *Bacillus subtilis* is caused by its critical role in the control of mRNA homeostasis

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ABSTRACT

RNA turnover is essential in all domains of life. The endonuclease RNase Y (*rny*) is one of the key components involved in RNA metabolism of the model organism *Bacillus subtilis*. Essentiality of RNase Y has been a matter of discussion, since deletion of the *rny* gene is possible, but leads to severe phenotypic effects. In this work, we demonstrate that the *rny* mutant strain rapidly evolves suppressor mutations to at least partially alleviate these defects. All suppressor mutants had acquired a duplication of an about 60 kb long genomic region encompassing genes for all three core subunits of the RNA polymerase— α , β , β' . When the duplication of the RNA polymerase genes was prevented by relocation of the *rpoA* gene in the *B. subtilis* genome, all suppressor mutants carried distinct single point mutations in evolutionary conserved regions of genes coding either for the β or β' subunits of the RNA polymerase that were not tolerated by wild type bacteria. *In vitro* transcription assays with the mutated polymerase variants showed a severe decrease in transcription efficiency. Altogether, our results suggest a tight cooperation between RNase Y and the RNA polymerase to establish an optimal RNA homeostasis in *B. subtilis* cells.

INTRODUCTION

Among all organisms, bacteria are the ones multiplying most rapidly. Under optimal conditions, the model bacteria *Escherichia coli* and *Bacillus subtilis* have generation times

of 20 to 30 min. On the other hand, bacteria are exposed to a variety of changing environmental conditions, and due to their small size, the impact of environmental changes is particularly severe for bacterial cells. To adapt to these potentially rapidly changing conditions, bacteria have evolved a huge arsenal of systems to sense and respond to the environment. Especially in the competition between microorganisms, it is crucial that these responses are both rapid and productive. However, while regulatory events may be very rapid, there is an element of retardation in the system, and this is the stability of mRNA and protein molecules. If the continued activity of a protein may become harmful to the bacteria, it is important not only to prevent expression of the corresponding gene but also to take two important measures: (i) switch off the protein's activity and (ii) degrade the mRNA to exclude further production of the protein. The inactivation or even degradation of proteins is well documented in the model bacteria. For example, in both *E. coli* and *B. subtilis* the uptake of toxic ammonium is limited by a regulatory interaction of the ammonium transporter with GlnK, a regulatory protein of the PII family (1,2). Similarly, the uptake of potentially toxic potassium can be prevented by inhibition of potassium transporters at high environmental potassium concentrations, either by the second messenger cyclic di-AMP or by interaction with a dedicated modified signal transduction protein, PtsN (3–5). To prevent the accumulation of potentially harmful mRNAs, bacteria rely on a very fast mRNA turnover. Indeed, in *E. coli* and *B. subtilis* >80% of all transcripts have average half-lives of less than 8 min, as compared to about 30 min and 10 h in yeast or human cells, respectively (6–9). Thus, the mRNA turnover is much faster than the generation time. The high mRNA turnover rate in bacteria contributes to the fast adaptation even in rapidly growing cells. The rapid

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mRNA turnover is therefore a major factor to resolve the apparent growth speed-adaptation trade-off.

RNases are the key elements to achieve the rapid mRNA turnover in bacteria. These enzymes can degrade bulk mRNA in a rather unspecific manner, just depending on the accessibility of the RNA molecules as well as perform highly specific cleavages that serve to process an RNA molecule to its mature form. In all organisms, RNA degradation involves an interplay of endo- and exoribonucleases as well as other proteins such as RNA helicases that resolve secondary structures (10–13). Often, these proteins form a complex called the RNA degradosome. In *E. coli*, the RNA degradosome is organized around the essential endoribonuclease RNase E (14,15). RNase E consists of two parts, the N-terminal endoribonuclease domain that harbors the enzymatic activity and the C-terminal macromolecular interaction domain that serves as the scaffold for the degradosome components and is responsible for the binding of RNase E to the cell membrane (15,16). As mentioned above, RNase E is essential for viability of the bacteria. An analysis of the contributions of the two parts of RNase E to its essentiality revealed that the enzymatically active N-terminal domain is essential whereas the C-terminal interaction domain is dispensable (17). This suggests that the endoribonucleolytic attack on mRNA molecules is the essential function of RNase E, whereas the interaction with other degradosome components is not required for viability. This conclusion is supported by the fact, that the other components of the *E. coli* degradosome are also dispensable (14).

RNase E is widespread in proteobacteria, cyanobacteria, and actinobacteria, but absent from many firmicutes, ϵ -proteobacteria, or from bacteria of the *Deinococcus-Thermus* class. However, an efficient RNA-degrading machinery is important also for these bacteria to allow both rapid growth and adaptation. Indeed, these bacteria possess a different endoribonuclease, RNase Y (18,19). A depletion of RNase Y results in a 2-fold increase of the average mRNA half-life in *B. subtilis* (19). Similar to RNase E, RNase Y is a membrane protein, and it is capable of interacting with several proteins involved in RNA degradation. Among these proteins are the 5'-to-3' exoribonuclease RNase J1, polynucleotide phosphorylase, the RNA helicase CshA, the glycolytic proteins enolase and phosphofructokinase, and a protein complex composed of YaaT, YlbF and YmcA (18–23). Many of these interactions are likely to be transient as judged from the distinct localization of RNase Y and its interaction partners in the cell membrane and in the cytoplasm, respectively (24).

We are interested in the identification of the essential cellular components that are required for the viability of *B. subtilis* cells with the aim to construct strains that harbor only the minimal set of genes to fulfill the essential cellular functions (25–27). For *B. subtilis*, RNase Y and RNase J1 were originally described as being essential (18,19,28–30). Interestingly, these two RNases are also present in the most genome-reduced independently viable organism, *Mycoplasma mycoides* JCVI-syn3.0 (31). Both RNase J1 and RNase Y are involved in the processing and degradation of a large number of RNA molecules in *B. subtilis* (32–36). However, more recent studies demonstrated the possibility to delete the *rnjA* and *rny* genes, encoding the two

RNases (37,38) and the dispensability of RNase Y was confirmed in a global approach to inactivate all genes of *B. subtilis* (39).

Comprehensive knowledge on essential genes and functions is the key to construct viable minimal genomes. By definition, essential genes cannot be individually deleted in a wild type genetic background under standard growth conditions (25). In this study, we have addressed the essentiality of RNase Y in *B. subtilis*. While the *rny* gene could indeed be deleted, this was accompanied by the rapid acquisition of suppressor mutations that affect the transcription apparatus. We demonstrate that a strongly reduced transcription activity is required to allow stable growth of *B. subtilis* in the absence of RNase Y. Our results suggest that the accumulation of mRNA that cannot be degraded is the growth-limiting factor in strains lacking RNase Y.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

All *B. subtilis* strains used in this study are listed in Table 1. All strains are derived from the laboratory strain 168 (*trpC2*). *B. subtilis* and *E. coli* cells were grown in Lysogeny Broth (LB medium) (40). LB plates were prepared by addition of 17 g Bacto agar/l (Difco) to LB (40). The plasmids are listed in Table 2. Oligonucleotides are listed in Supplementary Table S1.

DNA manipulation and genome sequencing

B. subtilis was transformed with plasmids, genomic DNA or PCR products according to the two-step protocol (40,41). Transformants were selected on LB plates containing erythromycin (2 μ g/ml) plus lincomycin (25 μ g/ml), chloramphenicol (5 μ g/ml), kanamycin (10 μ g/ml), or spectinomycin (250 μ g/ml). Competent cells of *E. coli* were prepared and transformed following the standard procedure (40) and selected on LB plates containing kanamycin (50 μ g/ml). S7 Fusion DNA polymerase (Mobidiag, Espoo, Finland) was used as recommended by the manufacturer. DNA fragments were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). DNA sequences were determined by the dideoxy chain termination method (40). Chromosomal DNA from *B. subtilis* was isolated using the peqGOLD Bacterial DNA Kit (Peqlab, Erlangen, Germany). To identify the mutations in the suppressor mutant strains GP2503, GP2518, GP2636, GP2637, GP2912, GP2913 and GP3211 (see Table 1), the genomic DNA was subjected to whole-genome sequencing. Concentration and purity of the isolated DNA was first checked with a Nanodrop ND-1000 (PeqLab Erlangen, Germany) and the precise concentration was determined using the Qubit[®] dsDNA HS Assay Kit as recommended by the manufacturer (Life Technologies GmbH, Darmstadt, Germany). Illumina shotgun libraries were prepared using the Nextera XT DNA Sample Preparation Kit and subsequently sequenced on a MiSeq system with the reagent kit v3 with 600 cycles (Illumina, San Diego, CA, USA) as recommended by the manufacturer. The reads were mapped on the reference genome of *B. subtilis* 168 (GenBank accession number:

Table 1. *B. subtilis* strains used in this study

Strain	Genotype ^a	Source or reference
168	<i>trpC2</i>	Laboratory collection
CCB441	W168 Δ <i>rny::spc</i>	37
BSB1	Wild type	92
LK633	MO1099 <i>rpoE::aphA3 amyE::mIs</i>	62
LK1098	Δ <i>rpoE::aphA3</i>	LK633 → BSB1
BP351	<i>trpC2</i> Δ <i>greA::cat</i>	F. Commichau
GP2501 ^b	<i>trpC2</i> Δ <i>rny::spc</i>	CCB441 → 168
GP2503 ^b	<i>trpC2</i> Δ <i>rny::spc greA</i> (C374T – Ser125Leu) (<i>rrnW-rrnI</i>) ₂	Evolution of GP2501 at 22°C
GP2504	<i>trpC2</i> Δ <i>rny::spc greA</i> (G169T – Glu57Stop)	Evolution of GP2501 at 22°C
GP2518 ^b	<i>trpC2</i> Δ <i>greA::cat</i> Δ <i>rny::spc</i> (<i>rrnW-rrnI</i>) ₂	Evolution of GP2628 on LB agar at 37°C
GP2524	<i>trpC2</i> Δ <i>rny::ermC</i>	This work
GP2525	<i>trpC2 greA-3xflag spc</i>	pGP2542 → 168
GP2529	<i>trpC2</i> Δ <i>rny::ermC greA-3xflag spc</i>	GP2524 → GP2525
GP2538	<i>trpC2</i> Δ <i>rny::ermC greA</i> (Insertion A406)-3xflag <i>spc</i>	Evolution of GP2529 at 22°C
GP2539	<i>trpC2</i> Δ <i>rny::ermC greA</i> (Deletion A66)-3xflag <i>spc</i>	Evolution of GP2529 at 22°C
GP2542	<i>trpC2</i> Δ <i>recA::spc</i>	44
GP2614	<i>trpC2</i> Δ <i>cspD::aphA3</i>	This work
GP2615	<i>trpC2</i> Δ <i>cspD::aphA3</i> Δ <i>rny::spc</i>	GP2501 → GP2614
GP2628 ^b	<i>trpC2</i> Δ <i>greA::cat</i> Δ <i>rny::spc</i>	BP351 + GP2501 → 168
GP2636 ^b	<i>trpC2</i> Δ <i>rny::spc cspD</i> (G23A – Trp8Stop) (<i>rrnW-rrnI</i>) ₂	Evolution of GP2501 on LB agar at 37°C
GP2637 ^b	<i>trpC2</i> Δ <i>rny::spc adeR</i> (T163A – Tyr55Asn) <i>rpoE-Δ199–208</i> Δ <i>skin</i> (<i>rrnW-rrnI</i>) ₂	Evolution of GP2501 on LB agar at 22°C
GP2678	<i>trpC2</i> Δ <i>rny::spc RBS of cspD</i> (GGAGGA → GGAAGA)	Evolution of GP2501 on LB agar at 37°C
GP2901	<i>trpC2 rae1</i> (insertion T33)	This work
GP2902	<i>trpC2 dgk-rpoA-cat-yaaH</i>	This work
GP2903	<i>trpC2 dgk-rpoA-cat-yaaH</i> Δ <i>rpoA::aphA3</i>	This work
GP2904	<i>trpC2 dgk-rpoA-cat-yaaH</i> Δ <i>rpoA::aphA3</i> Δ <i>rny::spc</i>	GP2501 → GP2903
GP2907	<i>trpC2 rae1 P_{alf4}-gfp-ermC sigH</i>	This work
GP2909	<i>trpC2 dgk-rpoA-cat-yaaH</i> Δ <i>rpoA::aphA3</i> (<i>rae1 P_{alf4}-gfp-ermC sigH</i>)	GP2907 → GP2903
GP2910	<i>trpC2 dgk-rpoA-cat-yaaH</i> Δ <i>rpoA::aphA3</i> (<i>rae1 P_{alf4}-gfp-ermC sigH</i>) Δ <i>rny::spc</i>	GP2501 → 2909
GP2912 ^b	<i>trpC2 dgk-rpoA-cat-yaaH</i> Δ <i>rpoA::aphA3</i> Δ <i>rny::spc rpoC</i> (G263A – Arg88His) Δ <i>skin trnSL-Vall</i> (bp55T → C)	Evolution of GP2904 on LB agar at 37°C
GP2913 ^b	<i>trpC2 dgk-rpoA-cat-yaaH</i> Δ <i>rpoA::aphA3</i> (<i>rae1 P_{alf4}-gfp-ermC sigH</i>) Δ <i>rny::spc rpoB</i> (G3160T – Gly1054Cys) Δ <i>skin</i>	Evolution of GP2910 on LB agar at 37°C
GP2915	<i>trpC2 dgk-rpoA-cat-yaaH</i> Δ <i>rpoA::aphA3</i> (<i>rae1 P_{alf4}-gfp-ermC sigH</i>) Δ <i>rny::spc rpoC</i> (G134A – Gly45Asp)	Evolution of GP2910 on LB agar at 37°C
GP3210	<i>trpC2</i> Δ <i>rny::spc rpoE</i> (Insertion A88)	Evolution of GP2501 on LB agar at 22°C
GP3211 ^b	<i>trpC2</i> Δ <i>rny::spc</i> (<i>rrnW-rrnI</i>) ₂	Evolution of GP2501 at 37°C
GP3216	<i>trpC2</i> Δ <i>rpoE::aphA3</i>	LK1098 → 168
GP3217	<i>trpC2</i> Δ <i>rpoE::aphA3</i> Δ <i>rny::spc</i>	GP2501 → GP3216
GP3220	<i>trpC2 purT-rpoB</i> (partial)- <i>cat-mpr</i>	This work
GP3288	<i>trpC2 purT-rpoB-rpoC-spc-mpr</i>	This work
GP3289	<i>trpC2 dgk-rpoA-cat::yaaH purT-rpoB-rpoC-spc-mpr</i>	GP2903 → GP3288
GP3295	<i>trpC2 dgk-rpoA-cat::yaaH purT-rpoB-rpoC-spc-mpr</i> Δ <i>rny::ermC</i>	GP2524 → GP3289
GP3296	<i>trpC2 dgk-rpoA-cat::yaaH purT-rpoB-rpoC-spc-mpr</i> Δ <i>cspD::aphA3</i> Δ <i>rny::ermC</i>	GP2524 + GP2614 → GP3289
GP3297	<i>trpC2 dgk-rpoA-cat::yaaH purT-rpoB-rpoC-spc-mpr</i> Δ <i>rpoE::aphA3</i> Δ <i>rny::ermC</i>	GP2524 + GP3216 → GP3289

^aFor strains with suppressing point mutations the mutations are indicated using the one- and three letter code for nucleotide and amino acid substitutions, respectively.

^bThese strains were analyzed by whole genome sequencing.

NC_000964) (42). Mapping of the reads was performed using the Geneious software package (Biomatters Ltd., New Zealand) (43). Frequently occurring hitchhiker mutations (44) and silent mutations were omitted from the screen. The resulting genome sequences were compared to that of our in-house wild type strain. Single nucleotide polymorphisms were considered as significant when the total coverage depth exceeded 25 reads with a variant frequency of $\geq 90\%$. All identified mutations were verified by PCR amplification and Sanger sequencing. Copy numbers of amplified genomic regions were determined by dividing the mean coverage of the amplified regions by the mean coverage of the remaining genome as described previously (44,45).

Construction of deletion mutants

Deletion of the *rny*, *rpoA*, and *cspD* genes was achieved by transformation with PCR products constructed using oligonucleotides to amplify DNA fragments flanking the target genes and intervening antibiotic resistance cassettes as described previously (46–48). The identity of the modified genomic regions was verified by DNA sequencing.

Chromosomal relocation of the *rpoA* gene

To construct a strain in which the genes for the core subunits of RNA polymerase are genomically separated, we decided to place the *rpoA* gene between the *dgk* and *yaaH* genes, and

Table 2. Plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
pCD2	For overexpression of <i>B. subtilis</i> σ^A	53
pJOE8999	CRISPR-Cas9 vector	49
pBSURNAP	P_{T7} <i>rpoA rpoZ rpoE rpoY rpoB-rpoC-8xHis</i>	This work
pGP1331	Allows construction of triple FLAG-tag fusions	93
pGP2181	P_{T7} <i>rpoA rpoZ rpoE rpoY rpoB-rpoC*-8xHis</i> (RpoC-R88H)	This work
pGP2182	P_{T7} <i>rpoA rpoZ rpoE rpoY rpoB*-rpoC-8xHis</i> (RpoB-G1054C)	This work
pGP2542	pGP1331/ <i>greA-3xflag spc</i>	This work
pGP2825	pJOE8999/ <i>rpoC</i> (G263A)	This work
pGP2826	pJOE8999/ <i>rae1</i> (insertion T33)	This work
pRLG770	promoter vector	94
pRLG7558	pRLG770 with <i>B. subtilis</i> P_{veg} (-38/-1, +1G)	73
pRLG7596	pRLG770 with <i>B. subtilis</i> <i>rrnB</i> P1 (-39/+1)	73
pLK502	pRLG770 with <i>B. subtilis</i> P_{lvB} (-262/-1, +1GG)	This work

then to delete the original copy of the gene. First, the *rpoA* gene was fused in a PCR reaction with its cognate promoter and a chloramphenicol resistance gene at the 5' and 3' ends, respectively. In addition, the amplified *dgk* and *yaaH* genes were fused to this construct to direct the integration of the construct to the *dgk-yaaH* locus. The fusion of PCR products was achieved by overlapping primers. The final product was then used to transform *B. subtilis* 168. Correct insertion was verified by PCR amplification and sequencing. The resulting strain was *B. subtilis* GP2902. In the second step, the original *rpoA* gene was replaced by a kanamycin resistance gene as described above, leading to strain GP2903.

Chromosomal duplication of the *rpoBC* operon

To construct a strain carrying a duplication of the *rpoBC* operon, we inserted the operon in two steps into the genome of *B. subtilis* 168. First, the promoter and 5' part of the *rpoB* gene was fused in a PCR reaction with a chloramphenicol resistance gene at the 3' end as well as with the amplified *purT* and *mpr* genes to direct the integration of the construct to the *purT-mpr* locus. The fusion of the four PCR products was achieved by overlapping primers. The final product was then used to transform *B. subtilis* 168. Correct insertion was verified by PCR amplification and sequencing. The resulting strain was *B. subtilis* GP3220. In the second step, the cat gene was replaced by the 3' part of *rpoB* and the complete *rpoC* gene fused to a spectinomycin resistance gene using the previously introduced 5' part of *rpoB* and *mpr* to guide the homologous recombination. The resulting strain was GP3288. The correctness of the insert was verified by DNA sequencing.

Genome editing

Introduction of genetic changes in genes for RNA polymerase subunit RpoC or the non-essential RNase Rael at their native locus was attempted using CRISPR editing as described (49). Briefly, oligonucleotides encoding a 20 nucleotide gRNA with flanking *BsaI* sites and a repair fragment carrying mutations of interest with flanking *SfiI* restriction sites were cloned sequentially into vector pJOE8999 (49). The resulting plasmids pGP2825 and pGP2826 were used to transform recipient *B. subtilis* strain 168 and cells were plated on 10 μ g/ml kanamycin plates with 0.2% mannose. Transformation was carried

out at 30°C since replication of pJOE8999 derivatives is temperature-sensitive. The transformants were patched on LB agar plates and incubated at the non-permissive temperature of 50°C. The loss of the vector was verified by the inability of the bacteria to grow on kanamycin plates. The presence of the desired mutation in *rae1* or *rpoC* was checked via Sanger sequencing. While the desired mutation could be introduced into the *rae1* gene, this was not the case for *rpoC*.

Construction of the expression vector pBSURNAP

To facilitate the purification of different variants of *B. subtilis* RNA polymerase, we expressed and purified the core subunits of the RNA polymerase and the sigma factor separately in *E. coli*. For the expression of the core subunits, we cloned the corresponding *B. subtilis* genes into the backbone of a pET28a derivative as follows. The pRMS4 vector (a pET28a derivative, 50) containing *Mycobacterium smegmatis* RNA polymerase core subunit genes was used as a template to create an analogous vector containing the genes *rpoA*, *rpoZ*, *rpoE*, *rpoY* and *rpoBC*. The construct was designed to allow removal/substitution of each gene via unique restriction sites (Supplementary Figure S1). DNA encoding *rpoA*, *rpoZ*, *rpoE* and *rpoY* genes was cloned as one single fragment (purchased as Gene Art Strings from Invitrogen) via *XbaI* and *NotI* restriction sites. The *rpoB* and *rpoC* genes were amplified by PCR using genomic DNA of *B. subtilis* 168 as a template and inserted into the plasmid via *NotI* and *NcoI* or *NcoI* and *KpnI* restriction sites, respectively. The *rpoC* gene was inserted with a sequence encoding a 8xHis tag on the 3' end. The cloned construct was verified by DNA sequencing. The final vector, pBSURNAP, encodes a polycistronic transcript for expression of all six RNA polymerase core subunits. Expression is driven from an IPTG-inducible T7 RNAP-dependent promoter. Each gene is preceded by a Shine-Dalgarno sequence (AGGAG) except for *rpoC*. RpoB-RpoC are expressed as one fused protein connected by a short linker (nine amino acid residues) to decrease the possibility that *E. coli* subunits would mix with *B. subtilis* subunits as done previously for RNA polymerase from *Mycobacterium bovis* (51). The full sequence of pBSURNAP has been deposited in GenBank under Accession No. MT459825. The mutant alleles of *rpoB* and *rpoC* were amplified from the mutant strains GP2913 and GP2912 and introduced into pB-

SURNAP by replacing the wild type alleles as NotI/NcoI and NcoI/KpnI fragments, respectively. The resulting plasmids were pGP2181 (RpoC-R88H) and pGP2182 (RpoB-G1054C).

Purification of *B. subtilis* RNA polymerase from *E. coli* cells

For purification, *E. coli* BL21 carrying pBSURNAP or the plasmids specifying the mutant alleles was cultivated in LB medium containing kanamycin (50 µg/ml). Expression was induced by the addition of IPTG (final concentration 0.3 mM) to logarithmically growing cultures (OD₆₀₀ between 0.6 and 0.8), and cultivation was continued for 3 h. Cells were harvested and the pellets from 1 l of culture medium were washed in 50 ml buffer P (300 mM NaCl, 50 mM Na₂HPO₄, 3 mM β-mercaptoethanol, 1 mM PMSF, 5% glycerol) and the pellets were resuspended in 30 ml of the same buffer. Cells were lysed using a HTU DIGI-F Press (18 000 p.s.i., 138 000 kPa, two passes, G. Heinemann, Germany). After lysis, the crude extracts were centrifuged at 41 000 × *g* for 30 min at 4°C, and the RNA polymerase was purified from the supernatant via the His-tagged RpoC as described (52). The RNA polymerase-containing fractions were pooled and further purified by size exclusion chromatography. For this purpose, the complex was applied onto a HiLoad 16/600 Superdex 200 column (GE Healthcare) in buffer P. The buffer was filtered (0.2 µm filters) prior to protein separation on an Äkta Purifier (GE Healthcare). The fractions containing RNA polymerase were pooled and dialyzed against RNA polymerase storage buffer (50 mM Tris-HCl, pH 8.0, 3 mM β-mercaptoethanol, 0.15 M NaCl, 50% glycerol, 1:1000). The purified RNA polymerase was stored at -20°C.

The housekeeping sigma factor σ^A was overproduced from plasmid pCD2 (53) and purified as described (54).

In vitro transcription assays

Multiple round transcription assays were performed as described previously (55), unless stated otherwise. Initiation competent RNA polymerase was reconstituted using the core enzyme and saturating concentration of σ^A in dilution buffer (50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 50% glycerol) for 10 min at 30°C. Assays were carried out in 10 µl with 64 nM RNA polymerase holoenzyme and 100 ng plasmid DNA templates in transcription buffer containing 40 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 1 mM dithiothreitol (DTT), 0.1 mg/ml bovine serum albumin (BSA), 150 mM NaCl, and NTPs (200 µM ATP, 2,000 µM GTP, 200 µM CTP, 10 µM UTP plus 2 µM of radiolabeled [α -³²P]-UTP). The samples were preheated for 10 min at 37°C. The reaction was started by the addition of RNA polymerase and allowed to proceed for 20 min (30 min in the case of iNTP-sensing experiments) at 37°C. Subsequently, the reaction was stopped by the addition of 10 µl of formamide stop solution (95% formamide, 20 mM EDTA, pH 8.0). The samples were loaded onto 7 M urea-7% polyacrylamide gels. The gels were dried and exposed to Fuji MS phosphor storage screens, scanned with a Molecular Imager FX (BIORAD) and analyzed with Quantity One program (BIORAD).

Transcriptome analysis

Cells were grown in LB medium at 37°C to an OD₆₀₀ of 0.5 to 0.6. 5 ml samples of the cultures were added to 10 ml RNA-protect (Qiagen) and allowed to incubate for 5 min at room temperature, followed by centrifugation at 5000 × *g* for 10 min at 4°C. Pellets were quickly frozen in liquid nitrogen and stored at -80°C. A total of three independent biological replicates were included. The harvested pellets were resuspended in 800 µl RLT buffer (RNeasy Mini Kit, Qiagen) with β-mercaptoethanol (10 µl/ml) and cell lysis was performed using a laboratory ball mill. Subsequently 400 µl RLT buffer with β-mercaptoethanol (10 µl/ml) and 1200 µl 96% [v/v] ethanol were added. For RNA isolation, the RNeasy Mini Kit (Qiagen) was used as recommended by the manufacturer, but instead of RW1 buffer RWT buffer (Qiagen) was used to facilitate the isolation of RNAs smaller 200 nt. To determine the RNA integrity number (RIN) the isolated RNA was run on an Agilent Bioanalyzer 2100 using an Agilent RNA 6000 Nano Kit as recommended by the manufacturer (Agilent Technologies, Waldbronn, Germany). Remaining genomic DNA was removed by digesting with TURBO DNase (Invitrogen, ThermoFischer Scientific, Paisley, United Kingdom). The Pan-Prokaryotes riboPOOL kit v1 (siTOOLS BIOTECH, Planegg/Martinsried, Germany) was used to reduce the amount of rRNA-derived sequences. For sequencing, the strand-specific cDNA libraries were constructed with a NEBNext Ultra II directional RNA library preparation kit for Illumina (New England BioLabs, Frankfurt am Main, Germany). To assess quality and size of the libraries, samples were run on an Agilent Bioanalyzer 2100 using an Agilent High Sensitivity DNA Kit (Agilent Technologies, Waldbronn, Germany). Concentration of the libraries were determined using the Qubit® dsDNA HS Assay Kit as recommended by the manufacturer (Life Technologies GmbH, Darmstadt, Germany). Sequencing was performed by using the HiSeq4000 instrument (Illumina Inc., San Diego, CA, USA) using the HiSeq 3000/4000 SR Cluster Kit for cluster generation and the HiSeq 3000/4000 SBS Kit (50 cycles for sequencing in the single-end mode and running 1 × 50 cycles. Between 12.623.708 and 16.865.134 raw reads were generated for the samples. For quality filtering and removing of remaining adaptor sequences, Trimmomatic-0.39 (56) and a cutoff phred-33 score of 15 were used. The mapping of the remaining sequences was performed with the Bowtie (version 2) program (57) using the implemented end-to-end mode, which requires that the entire read aligns from one end to the other. First, surviving reads were mapped against a database consisting of tRNA and rRNA sequences of *B. subtilis* 168 and unaligned reads were subsequently mapped against the genome of *B. subtilis* 168. Differential expression analyses were performed with the BaySeq program (58). Genes with fold change in expression of ≥ 2.0 or ≤ -2.0 , a likelihood value of ≥ 0.9 , and an adjusted *P* value of ≤ 0.05 (the *P* value was corrected by the false discovery rate [FDR] on the basis of the Benjamini-Hochberg procedure) were considered differentially expressed. The raw reads have been deposited in the National Center for Biotechnology Information's (NCBI) Sequence Read Archive (SRA) under accession no. SRP274247. Functional and regulation information on the differen-

tially expressed genes was obtained from the *SubtiWiki* database (59).

RESULTS

Inactivation of the *rny* gene leads to evolution of suppressor mutations affecting transcription

RNase Y had been considered to be essential (18,28); however, two studies reported that the *rny* gene could be deleted from the genome (37,39). The deletion leads to severe growth defects and morphological changes (37). In an attempt to get a better understanding of the importance of RNase Y for *B. subtilis* physiology, we deleted the *rny* gene in the genetic background of *B. subtilis* 168. The colonies of the resulting strain, GP2501, were small and lysed rapidly. Moreover, the cells grew very slowly at low temperatures (below 22°C). However, we observed the appearance of suppressor mutants after a few days. By analysis of such mutants we wished to gain a better understanding of the growth-limiting problem of the *rny* mutant. For this purpose, we isolated suppressor mutants in different experimental setups. First, the *rny* mutant GP2501 was adapted to growth in liquid LB medium at 22°C since the *rny* mutants had a severe growth defect at low temperatures. After the adaptation experiment, the culture was plated at 22°C, and two colonies were isolated for further investigation. In addition to the adaptation experiment in liquid medium, we also evolved suppressors on solid LB agar plates both at 22°C and 37°C. We isolated two mutants under each condition (see Figure 1A).

Growth of the isolated strains was verified (Figure 1B, see also Supplementary Figures S2 and S3), and for each selection scheme, one mutant was analysed by whole genome sequencing. In all cases, this confirmed the deletion of the *rny* gene and revealed the presence of additional mutations. Strikingly, there was one feature common for all the suppressors tested, regardless of the isolation condition, which was not present in the progenitor strain GP2501: It was an identical genomic duplication of the approximately 60 kb long *ctsR-pdaB* region. This genomic segment is flanked by clusters of ribosomal RNA operons. Upstream of the duplicated region are the *rrnJ* and *rrnW* operons, and downstream the *rrnI*, *rrnH*, and *rrnG* operons (see Figure 2A). This duplicated region contains 76 genes encoding proteins of various functions, among them proteolysis (ClpC), signal transduction (DisA), RNA modification (YacO, TruA), RNases (MrnC, RaeI), translation factors (EF-G, IF-1, EF-Tu), several ribosomal proteins, and proteins involved in transcription (NusG, RpoA, RpoB, RpoC, SigH). Strikingly, the genes for all three main subunits of the RNA polymerase—*rpoA*, *rpoB* and *rpoC* were present in the duplicated region. The observation, that this duplication was observed irrespective of the selective condition used to isolate suppressor mutants suggests that the duplication is relevant to overcome the poor growth associated with the loss of RNase Y. However, in addition, for each selection scheme we found additional mutations that affect genes involved in transcription.

For the selection in liquid medium at 22°C, the suppressor mutant GP2503 had a point mutation that resulted in an amino acid substitution (S125L) in the *greA* gene encoding

a transcription elongation factor (60). For the other suppressor mutant (GP2504) isolated under the same selective conditions, we sequenced the *greA* gene to test whether it had also acquired a mutation in this gene. Indeed, we found a different mutation in *greA*, resulting in the introduction of a premature stop codon after E56. Moreover, we evolved two additional suppressor mutants applying this adaptive scenario, and both contained frameshift mutations in *greA* that resulted in premature stop codons after amino acid 23 and 137 (GP2539 and GP2538, respectively; see Table 1).

The strain isolated on LB plates at 22°C (GP2637) had a deletion of the skin element, an amino acid substitution (Y55N) in the AdeR activator protein (61), and a short internal deletion in the *rpoE* gene encoding the δ subunit of RNA polymerase, which resulted in a frameshift after residue G66 (54,62). For the second mutant isolated at 22°C (GP3210), we re-sequenced the *adeR* and *rpoE* genes. While the *adeR* gene was identical to the wild type, we found an insertion of an adenine residue after position 87 of *rpoE*, resulting in a frameshift after 29 amino acids and premature stop codon after 38 amino acids. Therefore, the *rpoE* but not the *adeR* mutation is likely to be required for the suppressor phenotype.

The suppressor evolved at 37°C on LB plates (GP2636) contained a mutation resulting in the introduction of a premature stop at the eighth codon of the *cspD* gene encoding an RNA binding protein which has transcription antitermination activity in *E. coli* (63,64). Sanger sequencing of the second suppressor isolated under the same condition (GP2678) also identified a mutation affecting *cspD*, but this time in its ribosomal binding site (GGAGGA → GGAAGA).

Taken together, the duplication of the *ctsR-pdaB* genomic region was accompanied by specific additional suppressor mutations affecting transcription in every single suppressor mutant analysed. These mutations result in the inactivation of the *greA* gene in liquid medium at 22°C, whereas the selective pressure on agar plates at 22°C and 37°C was directed at the inactivation of the RNA polymerase subunit RpoE or the RNA binding protein CspD, respectively (see Figure 1A). It is therefore tempting to speculate that the inactivation of these genes combined with the *ctsR-pdaB* genomic duplication is causative for the suppression.

In order to test whether the inactivation of the *greA*, *rpoE*, or *cspD* genes alone is sufficient for the suppression of the *rny* mutant strain, we constructed the corresponding double mutants. As both *rny* and *greA* mutants are defective in genetic competence (39), the *greA rny* double mutant was obtained by transforming the wild type strain 168 with DNA molecules specifying both deletions simultaneously (see Table 1). For the *greA* and *rpoE* deletions, the double mutants did not phenocopy the original suppressor mutants, instead the gene deletions conferred only partial suppression (see Figure 1B for the *rny greA* double mutant GP2628, and Supplementary Figure S2 for the *rny rpoE* double mutant GP3217). In the case of the *rny cspD* double mutant GP2615, complete suppression was observed (see Supplementary Figure S3). However, we cannot exclude that the mutant had already acquired the duplication of the *ctsR-pdaB* genomic region. Thus, we conclude that the suppression depends on both, the duplication of the *ctsR-pdaB*

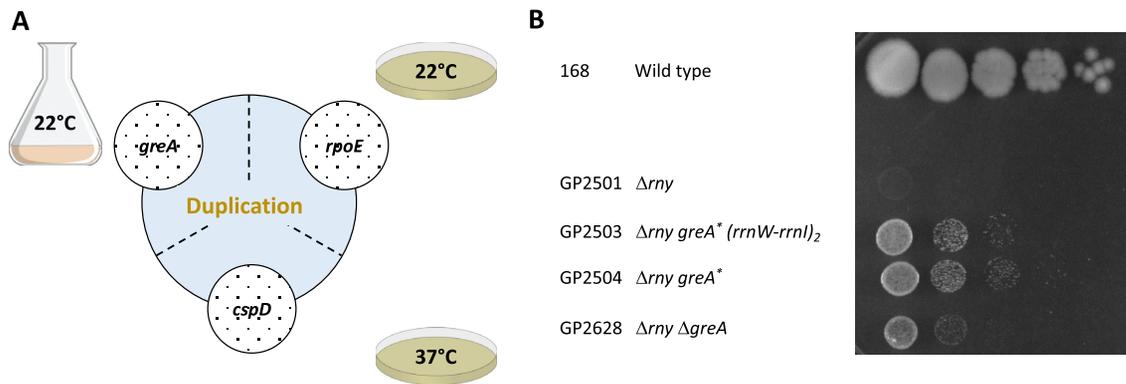


Figure 1. Suppressors of *rny* show increased growth at 22°C. (A) Schematic depiction of different single nucleotide polymorphisms identified in the initial suppressor screen and their overlap with the duplication of *ctsR-pdaB* region. (B) Serial drop dilutions comparing growth of the wild type strain 168, the *rny* mutant GP2501, its *greA* suppressors (GP2503, *greA* (Ser125Leu) (*rrnW-rrnI*)₂; GP2504, *greA* (Glu57Stop) and the *rny greA* double mutant GP2628 on LB-agar plate at 22°C. The *greA* suppressors are indicated with asterisks. The picture was taken after 2 days of incubation.

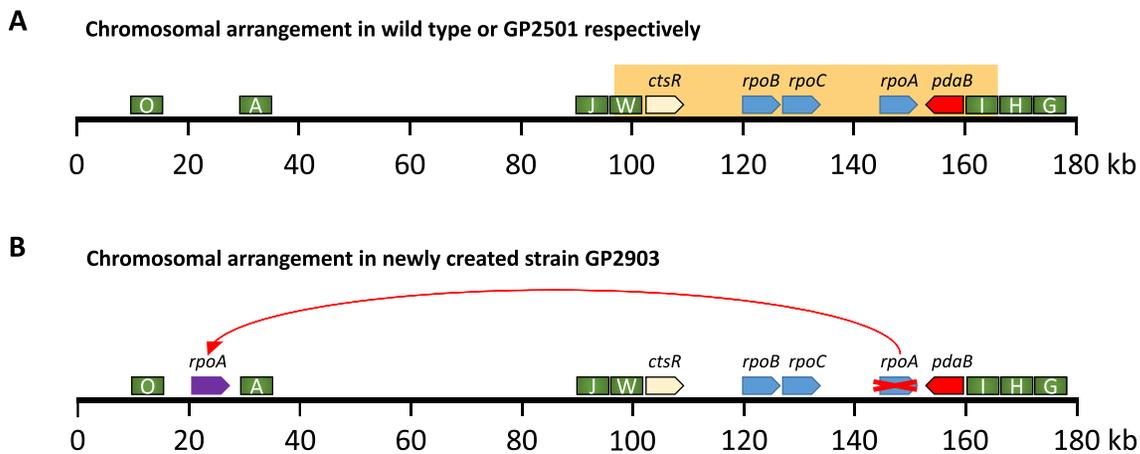


Figure 2. Genomic organization of the duplicated genomic region. (A) Schematic representation of the first 180 kb of the *B. subtilis* chromosome. The orange box indicates the duplicated region in the suppressors of *rny* strain GP2501. rRNA operons are depicted as green rectangles, RNA polymerase genes *rpoA*, *rpoB*, *rpoC* as blue arrows, the *ctsR* and *pdaB* genes are shown in yellow and red, respectively. (B) Chromosomal relocation of the *rpoA* gene. For the color code, see above; the relocated *rpoA* is shown as a purple arrow.

region and the concomitant mutations that inactivate genes involved in transcription.

Transcriptome analysis of the *rny* mutant and a suppressor strain

As mentioned above, the deletion of *greA* allowed only partial suppression of the growth defect caused by the loss of RNase Y. However, the *rny greA* double mutant GP2628 eventually gave rise to a better suppressing mutant, GP2518. Whole genome sequencing of this strain revealed that in addition to the *greA* deletion it had only acquired the duplication of the *ctsR-pdaB* genomic region. Again, this highlights the relevance of the combination of the *greA* deletion and the *ctsR-pdaB* duplication for suppression.

To get insights into the global consequences of the suppressing mutations, we compared the transcriptomes of the wild type strain 168, the *rny* mutant GP2501, and the suppressor mutant GP2518 by RNA-Seq analysis. We identified 1102 genes (corresponding to about 25% of all genes of *B. subtilis*) with at least two-fold differential expression in

the Δrny strain GP2501 as compared to the wild type 168. It should be noted that the number of differentially expressed genes is likely to be underestimated, since about 50% of all genes are not or only very poorly expressed during vegetative growth (27,65). The *rny* gene is encoded within an operon with the *ymdB* gene (66); however, there was no polar effect on the expression of *ymdB*, suggesting that the observed changes are a direct result of the loss of RNase Y.

From the dataset mentioned above, 587 and 515 genes were down- and upregulated, respectively, in the *rny* strain. The most severe difference (more than 100-fold decrease) was observed for the *yxkC* gene. This gene codes for protein of unknown function and is part of the σ^D regulon (67). Interestingly, 14 out of the 30 most strongly downregulated genes are σ^D dependent (see Supplementary Table S2). This may be the result of the reduced expression of the *sigD* gene itself. Since σ^D controls the expression of many genes responsible for motility as well as peptidoglycan autolysins (*lytA*, *lytB*, *lytC*, *lytD* and *lytF*) this reduced expression of target genes might cause the disordered cell wall of the *rny* deletion strain (37). Among the most strongly upregulated

genes (see Supplementary Table S2), many are members of the general stress response factor σ^B regulon. Another set of upregulated genes is controlled by the sporulation specific sigma factors σ^F and σ^G , whose genes are also >4-fold upregulated. This is especially striking taking into account that the *rny* mutant strain is not able to form spores (37).

Importantly, we wanted to test whether the suppressor mutant had restored a wild type-like expression of genes that were affected by the loss of RNase Y. We found 461 genes with differential expression between the suppressor mutant GP2518 and the *rny* mutant GP2501. Of these, however, only some were returned towards the expression levels of the wild type (176 genes, see Supplementary Table S3), while for others, the mRNA levels were even more distant from the wild type. In total 115 genes that were upregulated in the *rny* strain showed reduced expression in the suppressor mutant. On the other hand, also 61 genes which were downregulated in the *rny* mutant, had increased their expression again in the suppressor mutant GP2518 (see Supplementary Table S3). Among these genes with restored expression, four (*murAA*, *tagA*, *tagB*, *ywpB*) are essential, and only the expression of *ywpB* encoding an enzyme of fatty acid biosynthesis is 2.4-fold reduced in the *rny* mutant. This weak regulation suggests that fatty acid biosynthesis is not the growth-limiting factor for the *rny* mutant. In contrast, many of these genes with (partially) restored expression belong to prophage PBSX or are required for rather specific metabolic pathways. In conclusion, the evaluation of the genes which had their expression restored as a result of the suppressing mutations did not give a clear clue to the reason of suppression.

Genomic separation of the genes encoding the core subunits of RNA polymerase

As mentioned above, the region duplicated in all suppressor mutants contained genes encoding RNA modification enzymes, translation factors, ribosomal proteins, RNases, and proteins involved in transcription. MrnC and Rae1 are RNase Mini-III required for the maturation of 23S rRNA and ribosome-associated A site endoribonuclease, respectively (68,69). As our suppressor screen identified additional mutations related to transcription, we assumed that the translation-specific RNases encoded in this region might not be relevant for the suppression of the *rny* deletion. Therefore, we hypothesized that the duplication of the genes encoding the main three subunits of RNA polymerase made a major contribution to the selective advantage provided by the duplication.

To test the idea that simultaneous duplication of all three genes for the RNA polymerase core subunits is key for the suppression of the loss of RNase Y, we decided to interfere with this possibility. The duplicated region is located between two highly conserved *rmn* gene clusters which may facilitate the duplication event (see Figure 2A). Therefore, we attempted to separate the core RNA polymerase genes by relocating the *rpoA* gene out of this genomic region flanked by the *rmn* operons. We assumed that if RNA polymerase was indeed key to the original suppression, such a duplication would not be likely in the new background with relocated *rpoA*, since simultaneous duplication of all

three RNA polymerase subunit genes would be disabled there. For this purpose, the *rpoA* gene kept under the control of its natural promoter P_{rpsJ} was placed between the *dgk* and *yaaH* genes, and the original copy of *rpoA* was deleted (see Figure 2B, Materials and Methods for details). We then compared the growth of the wild type strain 168 and the strain with the relocated *rpoA* GP2903 using a drop-dilution assay. No differences were observed, thus excluding a possible negative impact of the *rpoA* relocation on *B. subtilis* physiology (see Supplemental Figure S4).

Strain GP2903 was then used to delete the *rny* gene, and to isolate suppressor mutants. Indeed, even with the genomically separated RNA polymerase genes, suppressor mutations appeared upon the deletion of the *rny* gene encoding RNase Y. There were three possibilities for the outcome of the experiment. First, the same genomic region as in the original suppressors might duplicate thus falsifying our hypothesis that the simultaneous duplication of all three genes encoding the core subunits of RNA polymerase is required for suppression. Second, both regions containing the *rpoA* and *rpoBC* genes might be duplicated. Third, in the new genetic background completely new suppressing mutations might evolve. Two of these suppressor mutants were subjected to whole genome sequencing. None of them had the duplication of the *ctsR-pdaB* region as in the original suppressors. Similarly, none of the mutants had the two regions containing the *rpoA* and the *rpoBC* genes duplicated. Instead, both mutants had point mutations in the RNA polymerase subunit genes that resulted in amino acid substitutions (GP2912: RpoC, R88H; GP2913: RpoB, G1054C; see Table 1). A mutation affecting RNA polymerase was also evolved in one strain (GP2915) not subjected to whole genome sequencing. In this case, the mutation resulted in an amino acid substitution (G45D) in RpoC.

An analysis of the localization of the amino acid substitutions in RpoB and RpoC revealed that they all affect highly conserved amino acid residues (see Figure 3A). G1054 of RpoB and G45 of RpoC are universally conserved in RNA polymerases in all domains of life, and R88 of RpoC is conserved in the bacterial proteins. This high conservation underlines the importance of these residues for RNA polymerase function. The mutations G45D and R88H in RpoC affect the N-terminal β' zipper and the zinc-finger like motif of the β' subunit, respectively, that are required for the processivity of the elongating RNA polymerase (70,71). G1054C in RpoB is located in the C-terminal domain of the β subunit that is involved in transcription termination (72). In the three-dimensional structure of RNA polymerase, these regions of the β and β' subunits are located in close vicinity opposite to each other in the region of the RNA exit channel which guides newly transcribed RNA out of the enzyme (see Figure 3B, 71), and they are both in direct contact with DNA (70).

The fact that several independent mutations affecting RNA polymerase were obtained in the suppressor screen strongly supports the idea that RNA polymerase is key for the suppression. As the mutations affect highly conserved residues, they are likely to compromise the enzyme's activity. Based on the structural information, the mutations might weaken RNA polymerase-nucleic acid interactions and therefore, destabilize the transcription elongation com-

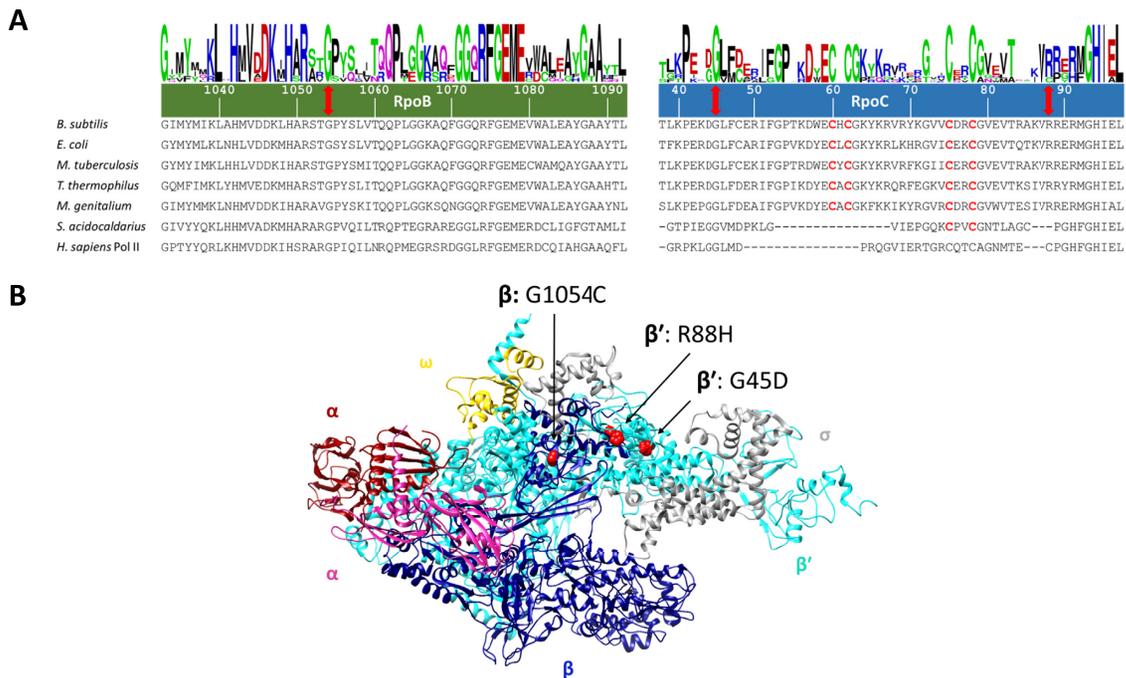


Figure 3. Suppressor mutations in RNA polymerase localize to evolutionary conserved regions. (A) Multiple sequence alignment of RpoB and RpoC sequences from various species, the numbering of amino acid residues is based on the *B. subtilis* sequence. The positions of mutations are indicated with red double head arrows, conserved cysteines involved in Zn-finger formation are shown in red. Logos were created as described (95). Abbreviations: *B. subtilis*, *Bacillus subtilis*; *E. coli*, *Escherichia coli*; *M. tuberculosis*, *Mycobacterium tuberculosis*; *T. thermophilus*, *Thermus thermophilus*; *M. genitalium*, *Mycoplasmata genitalium*; *S. acidocaldarius*, *Sulfolobus acidocaldarius*; *H. sapiens*, *Homo sapiens*. (B) Localization of the mutations (indicated as red spheres) in the RNA polymerase shown at their corresponding position in the structure of *T. thermophilus* (PDB ID: 1HW7; 96). The two α subunits are shown in dark red and violet, respectively, the β subunit is shown in dark blue, β' in cyan, ω in gold and the σ subunit is shown in grey. The image was created using UCSF Chimera (97).

plex which may result in increased premature termination and reduced RNA polymerase processivity. However, RNA polymerase is essential, therefore the mutations cannot inactivate the protein completely.

Establishing the *rpoB* and *rpoC* mutations in wild type background

Based on the essentiality of transcription, we expected that the mutations in *rpoB* and *rpoC* that we have identified in the suppressor screen with the *rny* mutant and genomically separated RNA polymerase genes might adjust some of the properties of RNA polymerase. To study the consequences of these mutations for the RNA polymerase and hence also for the physiology of *B. subtilis*, we decided to introduce one of them (RpoC-R88H) into the wild type background of *B. subtilis* 168. For this purpose, the CRISPR/Cas9 system designed for use in *B. subtilis* was employed (49). As a control, we used the same procedure to introduce a mutation in the *rae1* gene, which is located nearby on the chromosome. Although this system readily allowed the introduction of a frameshift mutation (introduction of an extra T after 32 bp) in *rae1* (strain GP2901), we failed to isolate genome-edited clones expressing the RpoC-R88H variant in multiple attempts. This failure to construct the RpoC-R88H variant in the wild type background suggests that the properties of the protein are altered in a way that is incompatible with the presence of an intact RNA degradation machine.

Mutated RNA polymerases have highly decreased activity *in vitro*

Since our attempts to study the effect of the mutations *in vivo* failed, we decided to test the properties of the mutant RNA polymerases using *in vitro* transcription. *B. subtilis* RNA polymerase is usually purified from a strain expressing His-tagged RpoC (52). However, the loss of competence of the *rny* mutant and the lethality of the *rpoC* mutation in the wild type background prevented the construction of a corresponding strain. To solve this problem, we used an approach to purify *B. subtilis* RNA polymerase from *E. coli* that had been applied successfully before for RNA polymerase of *Mycobacterium smegmatis* (50). Briefly, plasmid pBSURNAP containing genes *rpoA*, *rpoB*, *rpoC*, *rpoE*, *rpoY* and *rpoZ* for the RNA polymerase subunits under control of an IPTG inducible promoter was constructed in a way that each individual gene for a subunit could be cleaved out using unique restriction sites and replaced with its mutant counterpart, yielding pGP2181 (RpoC-R88H) and pGP2182 (RpoB-G1054C) (for details of the construction, see Materials and Methods). The variant RNA polymerases were expressed in *E. coli* BL21 and purified via affinity chromatography and subsequent size exclusion chromatography.

We purified the wild type and two mutant RNA polymerases (RpoC-R88H and RpoB-G1054C) and assessed their activity by *in vitro* transcription on three different templates, containing well-studied promoters of the *veg* and

ilvB genes and the P1 promoter of the *rrnB* operon (73,74). In agreement with previous results on wild type RNA polymerase (75), this enzyme performed well on all three substrates. In contrast, the mutated variants of RNA polymerase exhibited a drastic decrease of transcription activity on all three promoters; for the RpoB-G1054C variant the transcripts were only barely detectable (Figure 4A).

On many promoters, including the P1 promoter of the *rrnB* operon, *B. subtilis* RNA polymerase is sensitive to the concentration of the first transcribed nucleotide both *in vitro* and *in vivo* (73). This prompted us to compare the response of the wild type and the RpoC-R88H variant RNA polymerases to different concentrations of GTP, the initiation NTP for the *rrnB* P1 transcript. As described before, transcription with the wild type enzyme increased gradually in response to the GTP concentration (73). In contrast, the mutated variant was saturated with a relatively low GTP concentration, suggesting that this important regulatory mechanism is not functional here (see Figure 4B).

Taken together, our results suggest that a reprogramming of the properties of RNA polymerase as indicated by a substantial reduction in RNA polymerase activity and its altered ability to be regulated by iNTPs allows the suppressor mutants to overcome the loss of RNase Y.

A pre-existing duplication of the genomic region containing *rpoA* and *rpoBC* is fixed in response to the deletion of *rny*

The screen for suppressor mutations that facilitate growth of strains lacking RNase Y yielded two classes of mutants: the first set harboured mutations in genes involved in transcription (*greA*, *rpoE* or *cspD*) in addition to a duplication of the chromosomal region encoding the core subunits of RNA polymerase. The second class had point mutations affecting the β or β' subunits of RNA polymerase that result in strongly decreased transcription activity. At a first glance, these results seem to be conflicting. Considering RNA degradation as the function of RNase Y, it seemed plausible that the selective pressure caused by deletion of *rny* would result in alleviating the stress from mRNA accumulation. This seems to be the case in the second class of suppressors (see above), whereas the reason for the duplication seems to be less obvious. Importantly, this duplication was always accompanied by one of the other aforementioned mutations reducing transcription efficiency. In an attempt to determine the order of the evolutionary events in these suppressors we established a method to detect the presence of the duplication without whole genome sequencing. For this, we made use of a pair of oligonucleotides that binds to the *pdaB* and *ctsR* genes giving a product of about 10 kb, if the region is duplicated or amplified but no product in the absence of duplication or amplification (see Figure 5A). This PCR product was very prominent for the strain GP2636 that is known to carry the duplication. However, a band was also observed in the wild type strain 168, indicating that the duplication is present in a part of the population independent from the selective pressure exerted by the *rny* deletion (Figure 5B).

It is well-established that genomic duplications or amplifications occur frequently in bacterial populations, even in the absence of selective pressure (76). In *Salmonella ty-*

phimurium, *rrn* operons have been shown to be a hotspot of gene duplications or amplifications (77). Since evolution of such a genomic duplication is dependent on homologous recombination, we performed the PCR also on the *recA* mutant GP2542, which is defective in homologous recombination and thus unable to amplify chromosomal regions (44,45). Indeed, in this case we did not obtain even a faint band. Interestingly, the genomic duplication can also be observed in cells having the core subunits of RNA polymerase at distinct genomic regions (GP2903). For the derived suppressor mutant GP2912 that carries a point mutation in *rpoC*, the band indicating the presence of the duplication was also detectable by PCR analysis although the duplication could not be detected by whole genome sequencing. This apparent discrepancy is most easily resolved by assuming that the duplication was present only in a small subpopulation (as observed for the wild type strain) and therefore only detectable by the very sensitive PCR assay.

Obviously, the different genomic and genetic backgrounds of the *rny* mutants generate distinct selective forces: While the duplication is not fixed in strains with separated *rpo* genes, it seems to become fixed in the suppressor mutants that have the *rpo* genes in one genomic region. To investigate the order of evolutionary events, we cultivated the *rny* mutant strain GP2501 for 75 h and monitored the status of the *rpoA-rpoBC* chromosomal region by PCR (see Figure 5B). The initial sample for the *rny* mutant GP2501 that was used for the experiment, already revealed the presence of the duplication in a small sub-population similar to the wild type strain. This supports the finding that the duplication is present irrespective of any selection. The band corresponding to the duplicated *pdaB-ctsR* region became more and more prominent in the course of the experiment, after 75 h it was comparable to the signal obtained with strain GP2636 that carries the duplication. As a control, we also amplified the genomic region of the *rny* gene. In the wild type strain, this PCR product has a size of 2.5 kb, whereas the replacement of *rny* by a spectinomycin resistance gene resulted in a product of 2 kb. Importantly, the intensity of this PCR product did not change during the course of the evolution experiment, thus confirming that the increased intensity of the product for the *pdaB-ctsR* region represents the spread of the duplication in the bacterial population. To verify the duplication and to check for the presence of accompanying mutations, we subjected genomic DNA of the strain obtained in this evolution experiment after 75 h (GP3211) to whole genome sequencing. The sequencing confirmed presence of the duplication, but did not reveal any additional suppressor mutation. Based on this result, we can assume that upon deletion of *rny* the bacteria first fixed the duplication of the *pdaB-ctsR* region and then, later, may acquire the point mutations affecting *greA*, *rpoE*, or *cspD*.

The duplication of genes encoding the subunits of RNA polymerase is not sufficient for suppression

In the investigation of suppressor mutants, we have found suppressor mutants that exhibited severely reduced RNA polymerase activity as well as suppressor mutants with increased copy number of core RNA polymerase subunit

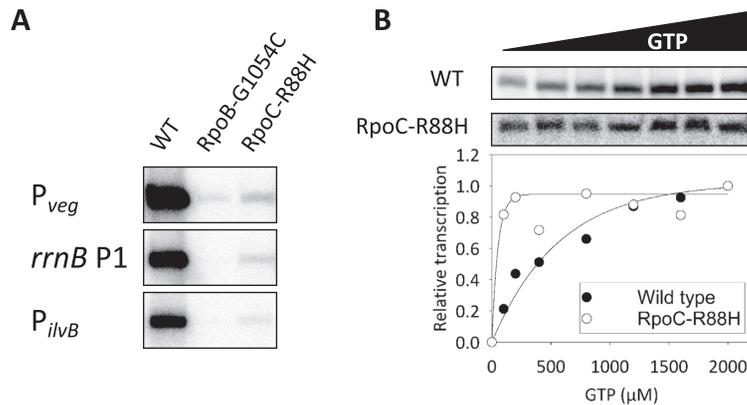


Figure 4. Comparison of transcriptional activity between RNA polymerase variants. (A) The RNA polymerase variants (64 nM) were reconstituted with saturating concentrations of σ^A (1:10). Holoenzymes were used to initiate transcription on three promoters as indicated. A representative image from three independent experiments is shown. (B) Transcription from the *rrnB* P1 promoter in dependence on increasing concentration of iNTP (GTP). The intensity of the transcripts generated by RNA polymerase containing RpoC-R88H was adjusted for better visibility. The relative activity of this mutant RNA polymerase was 2.5% of the wild type RNA polymerase at 2,000 μ M GTP. Representative primary data are shown. The graph shows the averages of two replicates normalized for maximal transcription of each polymerase (set as 1).

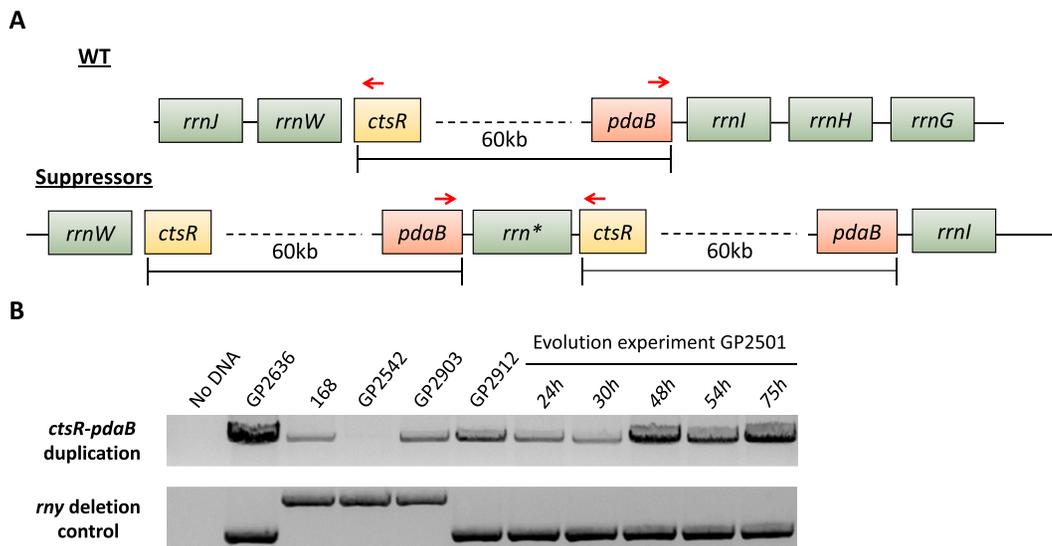


Figure 5. Duplication of the *ctsR-pdaB* region in suppressors of the Δrny mutant GP2501. (A) Schematic representation of the *ctsR-pdaB* region and its duplication in suppressors of GP2501. In the suppressors, a chimeric *rrn* operon (shown as *rrn**) is located between the *pdaB* and *ctsR* genes. The binding sites of the oligonucleotides used for the PCR detection of the duplication is indicated by red arrows. (B) Upper panel: The PCR product obtained by PCR using primers binding to *pdaB* and *ctsR* genes indicating presence of the duplication. Lower panel: The PCR product for the amplification of the *rny* region. Note the 5 μ l of the PCR product were loaded in the upper panel, and 1 μ l in the lower panel.

genes. In the latter mutants, one might expect that the increased copy number of RNA polymerase core subunit genes would result even in increased transcription, which seems to be in contradiction to the other set of suppressors. To address this obvious discrepancy, we decided to construct a strain carrying two copies of the genes encoding the RNA polymerase core subunits and compared the effect of *rny* deletion in strains with one or two copies of the *rpoA*, *rpoB*, and *rpoC* genes. For the duplication of the core subunit genes, we first assembled a second chromosomal copy of the *rpoBC* operon adjacent to the *purT* gene (see Material and Methods). Into the resulting strain GP3288, we inserted the second copy of the *rpoA* gene and then deleted

the *rny* gene (see Table 1). Growth of the resulting strains was compared on LB plates. As shown in Figure 6, the duplication of the RNA polymerase core subunit genes did not overcome the severe growth defect of the *rny* deletion mutant. Even the additional deletion of *cspD* or *rpoE*, which were found to be inactivated in the original suppressor mutants that carry the duplication of the *pdaB-ctsR* region, did not restore growth (see Figure 6). This observation shows that the duplication of the genes for the RNA polymerase α , β , and β' subunits is not sufficient for suppression of the *rny* deletion, and suggests that one or more additional genes that are encoded in the *pdaB-ctsR* region are involved in the suppression as well.

168	Wild type
GP2501	Δrny
GP3289	2 x <i>rpoABC</i>
GP3295	2 x <i>rpoABC</i> Δrny
GP3296	2 x <i>rpoABC</i> Δrny $\Delta cspD$
GP3297	2 x <i>rpoABC</i> Δrny $\Delta rpoE$

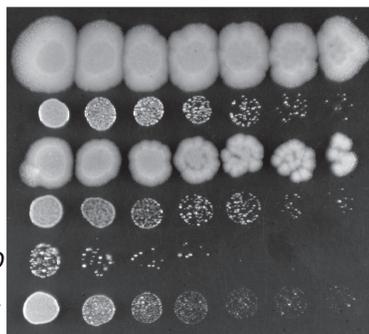


Figure 6. The duplication of the genes for core RNA polymerase is not sufficient to compensate for the loss of RNase Y. Serial drop dilutions comparing growth of the wild type strain 168, the *rny* mutant GP2501, and of the strains carrying the duplication of the *rpoA*, *rpoB* and *rpoC* genes on LB-agar plate at 37°C. The picture was taken after 2 days of incubation.

DISCUSSION

RNases E and Y are the main players in RNA degradation in *E. coli* and *B. subtilis*, respectively. Recently, it has been estimated that about 86% of all bacteria contain either RNase E or RNase Y (or, sometimes, both) supporting the broad relevance of these two enzymes (13). While RNase E of *E. coli* is essential (78), conflicting results concerning the essentiality of RNase Y have been published (18,28,29,37,39). In this study, we have examined the properties of *B. subtilis* mutants lacking RNase Y due to deletion of the corresponding *rny* gene. We observed that the *rny* mutant grew poorly, and rapidly acquired secondary mutations that suppressed, at least partially, the growth defect caused by the deletion of the *rny* gene. Thus, we conclude that RNase Y is in fact quasi-essential (31) for *B. subtilis*, since the mutant cannot be stably propagated on complex medium without acquiring suppressor mutations.

A lot of effort has been devoted to the understanding of the reason(s) of the (quasi)-essentiality of RNases E and Y for *E. coli* and *B. subtilis*, respectively. Initially, it was assumed that the essentiality is caused by the involvement of these RNases in one or more key essential processing event(s) that may affect the mRNAs of essential genes as has been found for *B. subtilis* RNase III and *E. coli* RNase P (33–35,79,80). However, such a target was never identified. Instead, different conclusions were drawn from suppressor studies with *E. coli rne* mutants lacking RNase E: some studies reported suppression by the inactivation or overexpression of distinct genes, such as *deaD* encoding a DEAD-box RNA helicase and *ppsA* encoding phosphoenolpyruvate synthetase, respectively (81,82). In addition, the processing and degradation of the essential stable RNAs, such as tRNAs and rRNAs was shown to be an essential function of RNase E (83). Yet another study suggested that mRNA turnover is the growth-limiting factor of the *E. coli rne* mutant (78). The results presented here lend strong support to the idea that the main task of RNase Y in *B. subtilis* is the control of intracellular mRNA concentration via the initiation of mRNA degradation. The transcriptome analysis with the *rny* mutant and a suppressor mutant revealed that only a limited number of genes shows restored expression in the suppressor mutant. Moreover, most of these genes are part of the prophage PBSX or encode very specific

metabolic functions. In addition, irrespective of the conditions used in the different suppressor screens, we identified a coherent set of mutations that resulted in improved growth of the *B. subtilis rny* mutant. The initial mutants carry a duplication of the chromosomal region that contains the genes for the core subunits of RNA polymerase (RpoA, RpoB, RpoC) and point mutations in *greA*, *rpoE*, and *cspD* that all affect transcription. If this duplication was prevented by genomically separating the RNA polymerase genes, we found suppressor mutants affecting the core subunits of RNA polymerase which result in strongly compromised transcription activity. Taken together, these findings suggest that the (quasi)-essentiality of RNases E and Y is related to their general function in initiating mRNA turnover rather than to the processing of specific RNA species. This idea is further supported by three lines of evidence: First, mutations that mimic a stringent response and therefore reduce RNA polymerase activity suppressed the growth defect of a *rne* mutant. Second, artificial expression of RNase Y or of the ribonucleases RNase J1 or J2 from *B. subtilis* partially suppressed the *E. coli* strain lacking RNase E, but only under specific growth conditions (84,85), and third, RNase E can functionally replace RNase Y (86).

With the initiation of global mRNA degradation as the (quasi)-essential function of RNases E and Y in *E. coli* and *B. subtilis*, respectively, one might expect that the overexpression of other RNases might compensate for their loss. By analogy, such a compensation has been observed for the essential DNA topoisomerase I of *B. subtilis*, which could be replaced by overexpression of topoisomerase IV (44). However, in all the seven suppressor mutants analysed by whole genome sequencing (Table 1), we never observed a mutation affecting any of the known RNases of *B. subtilis*. Similarly, no such compensatory mutations resulting from overexpression of other cognate RNases have been found in suppressor screens for *E. coli* RNase E. While RNase Y does not have a paralog in *B. subtilis*, *E. coli* possesses the two related RNases E and G. However, not even the overexpression of RNase G allowed growth of an *E. coli rne* mutant (87,88) suggesting that RNase G has a much more narrow function than RNase E and that none of the other RNases in either bacterium is capable of initiating global mRNA degradation.

An interesting result of this study was the apparent contradiction between the isolation of suppressor mutants with increased copy number of core RNA polymerase subunit genes in one setup, intuitively suggesting increased transcription activity, and the isolation of mutants that exhibited severely reduced RNA polymerase activity in the other setup. We therefore tested with a theoretical model whether duplication of the core subunits leads to abortive incomplete complexes, as the composition of the RNA polymerase complex might be perturbed by the duplication of the core. The model confirmed that perturbing the stoichiometry of the transcription machinery may result in a strong reduction of the fraction of core RNA polymerases that assemble a functional complex. However, our experimental data demonstrate that duplication of the RNA polymerase core genes is not sufficient for suppression indicating that other factors encoded in the *pdaB-ctsR* chromosomal region are required to compensate for the loss of RNase Y.

In each organism, an optimal trade-off between RNA synthesis and degradation must be adjusted to allow optimal growth. Obviously, the loss of the major RNA decay-initiating enzyme will bring this adjustment out of equilibrium. This idea is supported by the observation that reduced RNA degradation in *B. subtilis* is accompanied by the acquisition of mutations that strongly reduce transcription activity of the RNA polymerase. In fact, the reduction of activity was so strong that it was not tolerated in a wild type strain with normal RNA degradation. This indicates that the suppressor mutants have reached a new stable equilibrium between RNA synthesis and degradation, which, however, is not optimal as judged from the reduced growth rates of the suppressor mutants as compared to the wild type strain. It has already been noticed that generation times and RNA stability are directly related (9,89). This implies that a stable genetic system requires a balance between transcription and RNA degradation to achieve a specific growth rate. In bacteria, rapid growth requires high transcription rates accompanied by rapid RNA degradation. The association between RNA polymerase and components of the RNA degrading machinery, as shown for *B. subtilis* and *Mycobacterium tuberculosis* might be a factor to achieve this coupling between RNA synthesis and degradation (90,91).

In conclusion, our study suggests that the initiation of mRNA degradation to keep the equilibrium between RNA synthesis and degradation is the function of RNase Y that makes it quasi-essential for *B. subtilis*. In addition to RNase Y, RNase J1 is also quasi-essential for this bacterium. In the future, it will be interesting to understand the reasons behind the critical role of this enzyme as well in order to get a more comprehensive picture of the physiology of RNA metabolism.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

- Coutts,G., Thomas,G., Blakey,D. and Merrick,M. (2002) Membrane sequestration of the signal transduction protein GlnK by the ammonium transporter AmtB. *EMBO J.*, **21**, 536–545.
- Detsch,C. and Stülke,J. (2003) Ammonium utilization in *Bacillus subtilis*: transport and regulatory functions of NrgA and NrgB. *Microbiology*, **149**, 3289–3297.
- Corrigan,R.M., Campeotto,I., Jeganathan,T., Roelofs,K.G., Lee,V.T. and Gründling,A. (2013) Systematic identification of conserved bacterial c-di-AMP receptor proteins. *Proc. Natl. Acad. Sci. U.S.A.*, **110**, 9084–9089.
- Gundlach,J., Krüger,L., Herzberg,C., Turdiev,A., Poehlein,A., Tascón,I., Weiß,M., Hertel,D., Daniel,R., Hänel,I. *et al.* (2019) Sustained sensing in potassium homeostasis: Cyclic di-AMP controls potassium uptake by KimA at the levels of expression and activity. *J. Biol. Chem.*, **294**, 9605–9614.
- Lee,C.R., Cho,S.H., Yoon,M.J., Peterkofsky,A and Seok,Y.J. (2007) *Escherichia coli* enzyme IANtr regulates the K⁺ transporter TrkA. *Proc. Natl. Acad. Sci. U.S.A.*, **104**, 4124–4129.
- Geisberg,J.V., Moqtaderi,Z., Fan,X., Ozsolak,F. and Struhl,K. (2014) Global analysis of mRNA isoform half-lives reveals stabilizing and destabilizing elements in yeast. *Cell*, **156**, 812–824.
- Hambraeus,G., von Wachenfeldt,C. and Hederstedt,L. (2003) Genome-wide survey of mRNA half-lives in *Bacillus subtilis* identifies extremely stable mRNAs. *Mol. Gen. Genomics*, **269**, 706–714.
- Bernstein,JA., Lin,P.H., Cohen,S.N. and Lin-Chao,S. (2004) Global analysis of *Escherichia coli* RNA degradosome function using DNA microarrays. *Proc. Natl. Acad. Sci. U.S.A.*, **101**, 2758–2763.
- Yang,E., van Nimwegen,E., Zavolan,M., Rajewsky,N., Schroeder,M., Magnasco,M. and Darnell,J.E. (2003) Decay rates of human mRNAs: correlation with functional characteristics and sequence attributes. *Genome Res.*, **13**, 1863–1872.
- Lehnik-Habrink,M., Lewis,R.J., Mäder,U. and Stülke,J. (2012) RNA degradation in *Bacillus subtilis*: an interplay of essential endo- and exoribonucleases. *Mol. Microbiol.*, **84**, 1005–1017.
- Durand,S., Tomasini,A., Braun,F., Condon,C. and Romby,P. (2015) sRNA and mRNA turnover in Gram-positive bacteria. *FEMS Microbiol. Rev.*, **39**, 316–330.
- Redder,P. (2018) Molecular and genetic interactions of the RNA degradation machineries in Firmicutes bacteria. *Wiley Interdiscip. Rev. RNA*, **9**, e1460.
- Tejada-Arranz,A., de Crécy-Lagard,V. and de Reuse,H. (2020) Bacterial RNA degradosomes: molecular machines under tight control. *Trends Biochem. Sci.*, **45**, 42–57.
- Carpousis,A.J. (2007) The RNA degradosome of *Escherichia coli*: an mRNA-degrading machine assembled on RNase E. *Annu. Rev. Microbiol.*, **61**, 71–87.
- Mackie,G.A. (2013) RNase E: at the interface of bacterial RNA processing and decay. *Nat. Rev. Microbiol.*, **11**, 45–57.
- Khemici,V., Poljak,L., Luisi,B.F. and Carpousis,A.J. (2008) The RNase E of *Escherichia coli* is a membrane-binding protein. *Mol. Microbiol.*, **70**, 799–813.
- Kido,M., Yamanaka,K., Mitani,T., Niki,H., Ogura,T. and Hiraga,S. (1996) RNase E polypeptides lacking a carboxyl-terminal half

- suppress a mukB mutation in *Escherichia coli*. *J. Bacteriol.*, **178**, 3917–3925.
18. Commichau, F.M., Rothe, F.M., Herzberg, C., Wagner, E., Hellwig, D., Lehnik-Habrink, M., Hammer, E., Völker, U. and Stülke, J. (2009) Novel activities of glycolytic enzymes in *Bacillus subtilis*: interactions with essential proteins involved in mRNA processing. *Mol. Cell. Proteomics*, **8**, 1350–1360.
 19. Shahbadian, K., Jamali, A., Zig, L. and Putzer, H. (2009) RNase Y, a novel endoribonuclease, initiates riboswitch turnover in *Bacillus subtilis*. *EMBO J.*, **28**, 3523–3533.
 20. Lehnik-Habrink, M., Newman, J., Rothe, F.M., Solovyova, A.S., Rodrigues, C., Herzberg, C., Commichau, F.M., Lewis, R.L. and Stülke, J. (2011) RNase Y in *Bacillus subtilis*: a natively disordered protein that is the functional equivalent to RNase E from *Escherichia coli*. *J. Bacteriol.*, **193**, 5431–5441.
 21. Newman, J.A., Hewitt, L., Rodrigues, C., Solovyova, A.S., Harwood, C.R. and Lewis, R.J. (2012) Dissection of the network of interactions that links RNA processing with glycolysis in the *Bacillus subtilis* degradosome. *J. Mol. Biol.*, **416**, 121–136.
 22. Salvo, E., Alabi, S., Liu, B., Schlessinger, A. and Bechhofer, D.H. (2016) Interaction of *Bacillus subtilis* polynucleotide phosphorylase and RNase Y: structural mapping and effect on mRNA turnover. *J. Biol. Chem.*, **291**, 6655–6663.
 23. deLoughery, A., Dengler, V., Chai, Y. and Losick, R. (2016) Biofilm formation by *Bacillus subtilis* requires an endoribonuclease-containing multisubunit complex that controls mRNA levels for the matrix gene repressor SinR. *Mol. Microbiol.*, **99**, 425–437.
 24. Cascante-Estapa, N., Gunka, K. and Stülke, J. (2016) Localization of components of the RNA-degrading machine in *Bacillus subtilis*. *Front. Microbiol.*, **7**, 1492.
 25. Commichau, F.M., Pietack, N. and Stülke, J. (2013) Essential genes in *Bacillus subtilis*: a re-evaluation after ten years. *Mol. Biosyst.*, **9**, 1068–1075.
 26. Reuß, D.R., Commichau, F.M., Gundlach, J., Zhu, B. and Stülke, J. (2016) The blueprint of a minimal cell: *MiniBacillus*. *Microbiol. Mol. Biol. Rev.*, **80**, 955–987.
 27. Reuß, D.R., Altenbuchner, J., Mäder, U., Rath, H., Ischebeck, T., Sappa, P.K., Thürmer, A., Guérin, C., Nicolas, P., Steil, L. et al. (2017) Large-scale reduction of the *Bacillus subtilis* genome: consequences for the transcriptional network, resource allocation, and metabolism. *Genome Res.*, **27**, 289–299.
 28. Kobayashi, K., Ehrlich, S.D., Albertini, A., Amati, G., Andersen, K.K., Arnaud, M., Asai, K., Ashikaga, S., Aymerich, S., Bessières, P. et al. (2003) Essential *Bacillus subtilis* genes. *Proc. Natl. Acad. Sci. U.S.A.*, **100**, 4678–4683.
 29. Hunt, A., Rawlins, J.P., Thomaidis, H.B. and Errington, J. (2006) Functional analysis of 11 putative essential genes in *Bacillus subtilis*. *Microbiology*, **152**, 2895–2907.
 30. Mathy, N., Bénard, L., Pellegrini, O., Daou, R., Wen, T. and Condon, C. (2007) 5'-to-3' exoribonuclease activity in bacteria: role of RNase J1 in rRNA maturation and 5' stability of mRNA. *Cell*, **129**, 681–692.
 31. Hutchison, C.A. 3rd, Chuang, R.Y., Noskov, V.N., Assad-Garcia, N., Deerinck, T.J., Ellisman, M.H., Gill, J., Kannan, K., Karas, B.J., Ma, L. et al. (2016) Design and synthesis of a minimal bacterial genome. *Science*, **351**, aad6253.
 32. Mäder, U., Zig, L., Kretschmer, J., Homuth, G. and Putzer, H. (2008) mRNA processing by RNases J1 and J2 affects *Bacillus subtilis* gene expression on a global scale. *Mol. Microbiol.*, **70**, 183–196.
 33. Lehnik-Habrink, M., Schaffer, M., Mäder, U., Diethmaier, C., Herzberg, C. and Stülke, J. (2011) RNA processing in *Bacillus subtilis*: Identification of targets of the essential RNase Y. *Mol. Microbiol.*, **81**, 1459–1473.
 34. Durand, S., Gilet, L., Bessières, P., Nicolas, P. and Condon, C. (2012) Three essential ribonucleases – RNase Y, J1 and III – control the abundance of a majority of *Bacillus subtilis* mRNAs. *PLoS Genet.*, **8**, e1002520.
 35. Laalami, S., Bessières, P., Rocca, A., Zig, L., Nicolas, P. and Putzer, H. (2013) *Bacillus subtilis* RNase Y activity *in vivo* analysed by tiling microarrays. *PLoS One*, **8**, e54062.
 36. deLoughery, A., Lalanne, J.B., Losick, R. and Li, G.-W. (2018) Maturation of polycistronic mRNAs by the endoribonuclease RNase Y and its associated Y-complex in *Bacillus subtilis*. *Proc. Natl. Acad. Sci. U.S.A.*, **115**, E5585–E5594.
 37. Figaro, S., Durand, S., Gilet, L., Cayet, N., Sachse, M. and Condon, C. (2013) *Bacillus subtilis* mutants with knockouts of the genes encoding ribonucleases RNase Y and RNase J1 are viable, with major defects in cell morphology, sporulation, and competence. *J. Bacteriol.*, **195**, 2340–2348.
 38. Šíková, M., Wiedermannová, J., Převořovský, M., Barvík, I., Sudzinová, P., Kofroňová, O., Benada, O., Šanderová, H., Condon, C. and Krásný, L. (2020) The torpedo effect in *Bacillus subtilis*: RNase J1 resolves stalled transcription complexes. *EMBO J.*, **39**, e102500.
 39. Koo, B.M., Kritikos, G., Farelli, J.D., Todor, H., Tong, K., Kimsey, H., Wapinski, I., Galardini, M., Cabal, A., Peters, J.M. et al. (2017) Construction and analysis of two genome-scale deletion libraries for *Bacillus subtilis*. *Cell Syst.*, **4**, 291–305.
 40. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) In: *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, NY.
 41. Kunst, F. and Rapoport, G. (1995) Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. *J. Bacteriol.*, **177**, 2403–2407.
 42. Barbe, V., Cruveiller, S., Kunst, F., Lenoble, P., Meurice, G., Sekowska, A., Vallenet, D., Wang, T., Moszer, I., Médigue, C. and Danchin, A. (2009) From a consortium sequence to a unified sequence: the *Bacillus subtilis* 168 reference genome a decade later. *Microbiology*, **155**, 1758–1775.
 43. Kearsse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C. et al. (2012) Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, **28**, 1647–1649.
 44. Reuß, D.R., Faßhauer, P., Mroch, P.J., Ul-Haq, I., Koo, B.M., Pöhlein, A., Gross, C.A., Daniel, R., Brantl, S. and Stülke, J. (2019) Topoisomerase IV can functionally replace all type 1A topoisomerases in *Bacillus subtilis*. *Nucleic Acids Res.*, **47**, 5231–5242.
 45. Dormeyer, M., Lübke, A.L., Müller, P., Lentjes, S., Reuß, D.R., Thürmer, A., Stülke, J., Daniel, R., Brantl, S. and Commichau, F.M. (2017) Hierarchical mutational events compensate for glutamate auxotrophy of a *Bacillus subtilis* *glcC* mutant. *Environ. Microbiol. Rep.*, **9**, 279–289.
 46. Guérout-Fleury, A.M., Shazand, K., Frandsen, N. and Stragier, P. (1995) Antibiotic-resistance cassettes for *Bacillus subtilis*. *Gene*, **167**, 335–336.
 47. Youngman, P. Use of transposons and integrational vectors for mutagenesis and construction of gene fusions in *Bacillus subtilis*. In: Hardwood, C.R. and Cutting, S.M. (eds). *Mol. Biol. Methods for Bacillus*. Wiley, NY, pp. 221–266.
 48. Wach, A. (1996) PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. *Yeast*, **12**, 259–265.
 49. Altenbuchner, J. (2016) Editing of the *Bacillus subtilis* genome by the CRISPR-Cas9 system. *Appl. Environ. Microbiol.*, **82**, 5421–5427.
 50. Kouba, T., Pospíšil, J., Hnilicová, J., Šanderová, H., Barvík, I. and Krásný, L. (2019) The core and holoenzyme forms of RNA polymerase from *Mycobacterium smegmatis*. *J. Bacteriol.*, **201**, e00583-18.
 51. Czyz, A., Mooney, R.A., Iaconi, A. and Landick, R. (2014) Mycobacterial RNA polymerase requires a U-tract at intrinsic terminators and is aided by NusG at suboptimal terminators. *mBio*, **5**, e00931-14.
 52. Qi, Y. and Hulett, F.M. (1998) Pho-P and RNA polymerase sigma A holoenzyme are sufficient for transcription of Pho regulon promoters in *Bacillus subtilis*: PhoP-P activator sites within the coding region stimulate transcription *in vitro*. *Mol. Microbiol.*, **28**, 1187–1197.
 53. Chang, B.Y. and Doi, R.H. (1990) Overproduction, purification, and characterization of *Bacillus subtilis* RNA polymerase sigma A factor. *J. Bacteriol.*, **172**, 3257–3263.
 54. Juang, Y.L. and Helmann, J.D. (1994) The delta subunit of *Bacillus subtilis* RNA polymerase. An allosteric effector of the initiation and core-recycling phases of transcription. *J. Mol. Biol.*, **239**, 1–14.
 55. Wiedermannová, J., Sudzinová, P., Koval, T., Rabatinová, A., Šanderová, H., Ramaniuk, O., Rittich, Š., Dohnálek, J., Fu, Z., Halada, P. et al. (2014) Characterization of HelD, an interacting partner of RNA polymerase from *Bacillus subtilis*. *Nucleic Acids Res.*, **42**, 5151–5163.

56. Bolger, A.M., Lohse, M. and Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, **30**, 2114–2120.
57. Langmead, B. and Salzberg, S. (2012) Fast gapped-read alignment with Bowtie 2. *Nat. Methods*, **9**, 357–359.
58. Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L. and Wold, B. (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat. Methods*, **5**, 621–628.
59. Michna, R.M., Commichau, F.M., Tödter, D., Zschiedrich, C.P. and Stülke, J. (2014) SubtiWiki – a database for the model organism *Bacillus subtilis* that links pathway, interaction and expression information. *Nucleic Acids Res.*, **42**, D692–D698.
60. Kusuya, Y., Kurokawa, K., Ishikawa, S., Ogasawara, N. and Oshima, T. (2011) Transcription factor GreA contributes to resolving promoter-proximal pausing of RNA polymerase in *Bacillus subtilis* cells. *J. Bacteriol.*, **193**, 3090–3099.
61. Lin, T.-H., Wei, G.-T., Su, C.-C. and Shaw, G.-C. (2012) AdeR, a PucR-type transcription factor, activates expression of L-alanine dehydrogenase and is required for sporulation of *Bacillus subtilis*. *J. Bacteriol.*, **194**, 4995–5001.
62. Rabatinová, A., Šanderová, H., Matějčková, J.J., Korelusová, J., Sojka, L., Barvík, I., Popoušková, V., Sklenář, V., Židek, L. and Krásný, L. (2013) The δ subunit of RNA polymerase is required for rapid changes in gene expression and competitive fitness of the cell. *J. Bacteriol.*, **195**, 2603–2611.
63. Graumann, P., Wendrich, T.M., Weber, M.H.W., Schröder, K. and Marahiel, M.A. (1997) A family of cold shock proteins in *Bacillus subtilis* is essential for cellular growth and for efficient protein synthesis at optimal and low temperature. *Mol. Microbiol.*, **25**, 741–756.
64. Bae, W., Xia, B., Inoye, M. and Severinov, K. (2000) *Escherichia coli* CspA-family RNA chaperones are transcription antiterminators. *Proc. Natl. Acad. Sci. U.S.A.*, **97**, 7784–7789.
65. Rasmussen, S., Nielsen, H.B. and Jarmer, H. (2009) The transcriptionally active regions in the genome of *Bacillus subtilis*. *Mol. Microbiol.*, **73**, 1043–1057.
66. Diethmaier, C., Pietack, N., Gunka, K., Wrede, C., Lehnik-Habrink, M., Herzberg, C., Hübner, S. and Stülke, J. (2011) A novel factor controlling bistability in *Bacillus subtilis*: the YmdB protein affects flagellin expression and biofilm formation. *J. Bacteriol.*, **193**, 5997–6007.
67. Serizawa, M., Yamamoto, H., Yamaguchi, H., Fujita, Y., Kobayashi, K., Ogasawara, N. and Sekiguchi, J. (2004) Systematic analysis of SigD-regulated genes in *Bacillus subtilis* by DNA microarray and Northern blotting analyses. *Gene*, **329**, 125–136.
68. Redko, Y., Bechhofer, D.H. and Condon, C. (2008) Mini-III, an unusual member of the RNase III family of enzymes, catalyses 23S ribosomal RNA maturation in *Bacillus subtilis*. *Mol. Microbiol.*, **68**, 1096–1106.
69. Leroy, M., Piton, J., Gilet, L., Pellegrini, O., Proux, C., Coppée, J.-Y., Figaro, S. and Condon, C. (2017) Rael/YacP, a new endoribonuclease involved in ribosome-dependent mRNA decay in *Bacillus subtilis*. *EMBO J.*, **36**, 1167–1181.
70. Nudler, E., Avetisova, E., Markovtsov, V. and Goldfarb, A. (1996) Transcription processivity: protein-DNA interactions holding together the elongation complex. *Science*, **273**, 211–217.
71. Nudler, E. (2009) RNA polymerase active center: the molecular engine of transcription. *Annu. Rev. Biochem.*, **78**, 335–361.
72. Clerget, M., Jin, D.J. and Weisberg, R.A. (1995) A zinc-binding region in the beta' subunit of RNA polymerase is involved in antitermination of early transcription of phage HK022. *J. Mol. Biol.*, **248**, 768–780.
73. Krásný, L. and Gourse, R.L. (2004) An alternative strategy for bacterial ribosome synthesis: *Bacillus subtilis* RNA polymerase rRNA transcription regulation. *EMBO J.*, **23**, 4473–4483.
74. Krásný, L., Tiserová, H., Jonák, J., Rejman, D. and Šanderová, H. (2008) The identity of the transcription +1 position is crucial for changes in gene expression in response to amino acid starvation in *Bacillus subtilis*. *Mol. Microbiol.*, **69**, 42–54.
75. Sojka, L., Kouba, T., Barvík, I., Šanderová, H., Maderová, Z., Jonák, J. and Krásný, L. (2011) Rapid changes in gene expression: DNA determinants of promoter regulation by the concentration of the transcription initiating NTP in *Bacillus subtilis*. *Nucleic Acids Res.*, **39**, 4598–4611.
76. Andersson, D.I. and Hughes, D. (2009) Gene amplification and adaptive evolution in bacteria. *Annu. Rev. Genet.*, **43**, 167–195.
77. Anderson, P. and Roth, J. (1981) Spontaneous tandem genetic duplications in *Salmonella typhimurium* arise by unequal recombination between rRNA (rrn) cistrons. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 3113–3117.
78. Hammarlöf, D.L., Bergman, J.M., Garmendia, E. and Hughes, D. (2015) Turnover of mRNAs is one of the essential functions of RNase E. *Mol. Microbiol.*, **98**, 34–45.
79. Mohanty, B.K., Agrawal, A. and Kushner, S.R. (2020) Generation of pre-tRNAs from polycistronic operons is the essential function of RNase P in *Escherichia coli*. *Nucleic Acids Res.*, **48**, 2564–2578.
80. Durand, S., Gilet, L. and Condon, C. (2012) The essential function of *B. subtilis* RNase III is to silence foreign toxin genes. *PLoS Genet.*, **8**, e1003181.
81. Tamura, M., Kers, J.A. and Cohen, S.N. (2012) Second-site suppression of RNase E essentiality by mutation of the *dead* RNA helicase in *Escherichia coli*. *J. Bacteriol.*, **194**, 1919–1926.
82. Tamura, M., Honda, N., Fujimoto, H., Cohen, S.N. and Kato, A. (2016) PpsA-mediated alternative pathway to complement RNase E essentiality in *Escherichia coli*. *Arch. Microbiol.*, **198**, 409–421.
83. Sulthana, S., Basturea, G.N. and Deutscher, M.P. (2016) Elucidation of pathways of ribosomal RNA degradation: an essential role for RNase E. *RNA*, **22**, 1163–1171.
84. Himabindu, P. and Anupama, K. (2017) Decreased expression of stable RNA can alleviate the lethality associated with RNase E deficiency in *Escherichia coli*. *J. Bacteriol.*, **199**, e00724-16.
85. Tamura, M., Kageyama, D., Honda, N., Fujimoto, H. and Kato, A. (2017) Enzymatic activity necessary to restore the lethality due to *Escherichia coli* RNase E deficiency is distributed among bacteria lacking RNase E homologues. *PLoS One*, **12**, e0177915.
86. Laalami, S., Cavauiolo, M., Roque, S., Chagneau, C. and Putzer, H. (2021) *Escherichia coli* RNase E can efficiently replace RNase Y in *Bacillus subtilis*. *Nucleic Acids Res.*, **49**, 4643–4654.
87. Deana, A. and Belasco, J.G. (2004) The function of RNase G in *Escherichia coli* is constrained by its amino and carboxyl termini. *Mol. Microbiol.*, **51**, 1205–1217.
88. Chung, D.-H., Min, Z., Wang, B.-C. and Kushner, S.R. (2010) Single amino acid changes in the predicted RNase H domain of *Escherichia coli* RNase G lead to complementation of RNase E deletion mutants. *RNA*, **16**, 1371–1385.
89. Rustad, T.R., Minch, K.J., Brabant, W., Winkler, J.K., Reiss, D.J., Baliga, N.S. and Sherman, D.R. (2013) Global analysis of mRNA stability in *Mycobacterium tuberculosis*. *Nucleic Acids Res.*, **41**, 509–517.
90. Delumeau, O., Lecoine, F., Muntel, J., Guillot, A., Guédon, E., Monnet, V., Hecker, M., Becher, D., Polard, P. and Noirot, P. (2011) The dynamic partnership of RNA polymerase in *Bacillus subtilis*. *Proteomics*, **11**, 2992–3001.
91. Płociński, P., Macios, M., Houghton, J., Niemiec, E., Płocińska, R., Brzostek, A., Słomka, M., Dziadek, J., Young, D. and Dziembowski, A. (2019) Proteomic and transcriptomic experiments reveal an essential role of RNA degradosome complexes in shaping the transcriptome of *Mycobacterium tuberculosis*. *Nucleic Acids Res.*, **47**, 5892–5905.
92. Nicolas, P., Mäder, U., Dervyn, E., Rochat, T., Leduc, A., Pigeonneau, N., Bidnenko, E., Marchadier, E., Hoebeke, M., Aymerich, S. et al. (2012) Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. *Science*, **335**, 1103–1106.
93. Lehnik-Habrink, M., Pförtner, H., Rempeters, L., Pietack, N., Herzberg, C. and Stülke, J. (2010) The RNA degradosome in *Bacillus subtilis*: Identification of CshA as the major RNA helicase in the multi-protein complex. *Mol. Microbiol.*, **77**, 958–971.
94. Ross, W., Thompson, J.F., Newlands, J.T. and Gourse, R.L. (1990) *E. coli* Fis protein activates ribosomal RNA transcription *in vitro* and *in vivo*. *EMBO J.*, **9**, 3733–3742.
95. Crooks, G.E., Hon, G., Chandonia, J.-M. and Brenner, S.E. (2004) WebLogo: a sequence logo generator. *Genome Res.*, **14**, 1188–1190.
96. Vassylyev, D.G., Sekine, S., Laptchenko, O., Lee, J., Vassylyeva, M.N. and Borukhov, S. (2002) Crystal structure of a bacterial RNA polymerase holoenzyme at 2.6 Å resolution. *Nature*, **417**, 712–719.
97. Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C. and Ferrin, T.E. (2004) UCSF Chimera – a visualization system for exploratory research and analysis. *J. Comput. Chem.*, **25**, 1605–1612.