Supplementary Methods, Tables and Figures

Processing and decay of 6S-1 and 6S-2 RNAs in *Bacillus subtilis*

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SUPPLEMENTARY METHODS

6S-1 RNA folding and pLNA annealing

In vitro-transcribed, 5'-^{32}P-endlabeled 6S-1 RNA (0.5 pmol), either in the presence or absence of chemically synthesized pLNA oligonucleotides (10 pmol; 14 nucleotides, sequence 5'-GTTCGGTCAAAACT-3', all positions LNA, 100 µM stock solution), was denatured/renatured in a volume of 4 to 8 µl 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) in a PCR machine to fold the RNA and anneal the pLNA, starting with 95°C, followed by 85°, 70°, 60°, 50° and 37°C, with a dwell time of 2 min at each temperature. Samples were then placed on ice.

5'-^{32}P-endlabeling of 6S-1 RNA

For 5'-monophosphorylated mature 6S-1 RNA, ~30 pmol of 5'-dephosphorylated 6S-1 RNA were combined with 3 µl [γ-^{32}P]-ATP (3000 Ci/mmol) and 1 µl T4 polynucleotide kinase (T4 PNK, 10 U/µl; Thermo Scientific) in 1 x T4 PNK buffer A [50 mM Tris-HCl (pH 7.6 at 25 °C), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine] in a final volume of 10 µl, followed by incubation for 1 h at 37°C and preparative denaturing PAGE. 5'-triphosphorylated pre-6S-1 RNA was synthesized by T7 transcription in the presence of [γ-^{32}P]-GTP. Samples (50 µl) contained 33 mM $MgCl_2$, 80 mM HEPES pH 7.5, 1 mM spermidine, 5 mM DTT, 0.05 U pyrophosphatase, ~2 µg of plasmid pUC18_T7_pre-6S-1 linearized with HindIII (or 0.25 µg of a corresponding PCR template encoding pre-6S-1 RNA with the native 5'-terminal A residue), 4 mM each ATP, CTP and UTP, 3 mM GTP, 3 µl [γ-^{32}P]-GTP (3000 Ci/mmol), 0.02% (v/v) DMSO and ~100 U T7 RNA polymerase. Incubation was carried out for 2.5 h at 37°C, followed by desalting and removal of [γ-^{32}P]-GTP on Sephadex G-25 columns, ethanol precipitation of the 5'-[γ-^{32}P]-GTP-endlabeled pre-6S-1 RNA eluate and subsequent denaturing PAGE purification.

Complex formation of 6S-1 RNA and RNA polymerase

5'-^{32}P-endlabeled 6S-1 RNA (0.5 pmol) was denatured/renatured as above (in the absence of pLNA). Buffer conditions were adjusted to 1x RNase Y reaction buffer (final concentration) using a 10x RNase Y buffer (200 mM Hepes, pH 7.5, 80 mM $MgCl_2$, 1 M NaCl). Then 5 pmol of *B. subtilis* σ^A-RNA polymerase holoenzyme (σ^A-RNAP, stock solution: 4.8 mg/ml, 11.3 µM; prepared as described in: Jimenez F, Avila J, Vinuela E, Salas M. 1974. Initiation of the transcription of φ29 DNA by *Bacillus subtilis* RNA polymerase. *Biochim Biophys Acta* **349:** 320–327. doi:10.1016/0005-2787(74)90119-1) was added and samples were inbubated for 30 min at 30°C, followed by ethanol precipitation. RNA pellets were dissolved in 8 µl TE buffer (per reaction).

RNase Y cleavage assays

Assays were conducted in 1x RNase Y reaction buffer (see above). To 8 µl 6S-1 RNA (0.5 pmol), either preincubated alone, after pRNA annealing or after complexation with σ^A-RNAP, 2 µl RNase Y (5 pmol/µl) were added. Samples were incubated for 10 min at 30°C and stopped by transfer to ice, followed by phenol extraction and ethanol precipitation. RNA pellets were dissolved in TE buffer (8 µl/reaction/0.5 pmol 6S-1 RNA) and mixed with 15 µl denaturing loading buffer, of which 7.5 µl were loaded onto a denaturing polyacrylamide gel.

Sequences of T7 transcripts used as markers in northern blot experiments

Extra G residues (small bold letters) at the 5'-end were introduced for efficient T7 transcription, those at the 3'-end (underlined) originated from the use of an HindIII site for cloning purposes and linearization of T7 transcription templates; capital letters: sequence-identical to native precursor or mature 6S-1 or 6S-2 RNAs, respectively.

T7 transcripts using linearized plasmid DNA templates

M(arker) 6S-1 RNA (197 nt)

5‘-
ggAGUCCUGAUGUGUUAGUUGUACACCUAGUUUUGACCGAACAUUUUUUUGAUUUGGGAGCCCGCAUU
UUUAAAUGGCGUACAUGCCUCUUUUCAUUCGGUAAAGAGGACUUACAAGAUUUAAAAGAGGGCACCCA
CCUGCUGAGAGCGGGUUCAAAACAAAGGAAAGCUGCAACGGCACUAUUGGGACUUUAagcu-
3‘(linearized with HindIII)

M pre-6S-1 RNA (208 nt)

5‘-
ggAAGUUAAAUAAAGUCCUGAUGUGUUAGUUGUACACCUAGUUUUGACCGAACAUUUUUUUGAUUUGG
GAGCCCGCAUUUUUAAAUGGCGUACAUGCCUCUUUUCAUUCGGUAAAGAGGACUUACAAGAUUUAAAA
GAGGGCACCCACCUGCUGAGAGCGGGUUCAAAACAAAGGAAAGCUGCAACGGCACUAUUGGGACUUUA
agcu-3‘ (linearized with HindIII)

M 5‘-6S-1 RNA (92 nt):

5‘-
ggAGUCCUGAUGUGUUAGUUGUACACCUAGUUUUGACCGAACAUUUUUUUGAUUUGGGAGCCCGCAUU
UUUAAAUGGCGUACAUGCCaagcu-3‘(linearized with HindIII)

M 3‘-6S-1 RNA (90 nt):

5‘-
ggCUUACAAGAUUUAAAAGAGGGCACCCACCUGCUGAGAGCGGGUUCAAAACAAAGGAAAGCUGCAAC
GGCACUAUUGGGACUUUAagcu-3‘(linearized with HindIII)

M 6S-2 RNA (209 nt):

5‘-
gGAAGCUACUUUGUGCGUAUUGUUAAUUAAGUUUUAACCUUUAAGCUGUAAUAGGGAGUUUUAUAUCG
AAGCCGGAAUGUCAUGCCUCCAUUGCAGAGGACGACAGGAACGUUUCUGCAAACACCCACUUUGAGGA
GUGGUGGUUUAAAACUUUCUUACUUGCAUACGGCAAGACGUAGCGAAAGCCAUUUCCUUUUCGGAAAU
aagcu-3‘(linearized with HindIII)

M 6S-2 RNA (146 nt):

5‘-
gGAAGCUACUUUGUGCGUAUUGUUAAUUAAGUUUUAACCUUUAAGCUGUAAUAGGGAGUUUUAUAUCG
AAGCCGGAAUGUCAUGCCUCCAUUGCAGAGGACGACAGGAACGUUUCUGCAAACACCCACUUUGAGGA
GUGGUGGUUU -3‘(linearized with DraI)

M 6S-2 RNA (111 nt):

5‘-
gGAAGCUACUUUGUGCGUAUUGUUAAUUAAGUUUUAACCUUUAAGCUGUAAUAGGGAGUUUUAUAUCG
AAGCCGGAAUGUCAUGCCUCCAUUGCAGAGGACGACAGGAACG-3‘ (linearized with AclI)

M 6S-2 RNA (217 nt):

5‘ –
gGAAGCUACUUUGUGCGUAUUGUUAAUUAAGUUUUAACCUUUAAGCUGUAAUAGGGAGUUUUAUAUCG
AAGCCGGAAUGUCAUGCCUCCAUUGCAGAGGACGACAGGAACGUUUCUGCAAACACCCACUUUGAGGA
GUGGUGGUUUAAAACUUUCUUACUUGCAUACGGCAAGACGUAGCGAAAGCCAUUUCCUUUUCGGAAAU
GGCUUUUUAagcu-3‘ (linearized with HindIII)

T7 transcripts using PCR templates

M 6S-2 RNA (194 nt):
5‘-
gGAAGCUACUUUGUGCGUAUUGUUAAUUAAGUUUUAACCUUUAAGCUGUAAUAGGGAGUUUUAUAUCG
AAGCCGGAAUGUCAUGCCUCCAUUGCAGAGGACGACAGGAACGUUUCUGCAAACACCCACUUUGAGGA
GUGGUGGUUUAAAACUUUCUUACUUGCAUACGGCAAGACGUAGCGAAAGCCAUUUCCU-3‘

M 6S-2 RNA (190 nt):
5‘-
gGAAGCUACUUUGUGCGUAUUGUUAAUUAAGUUUUAACCUUUAAGCUGUAAUAGGGAGUUUUAUAUCG
AAGCCGGAAUGUCAUGCCUCCAUUGCAGAGGACGACAGGAACGUUUCUGCAAACACCCACUUUGAGGA
GUGGUGGUUUAAAACUUUCUUACUUGCAUACGGCAAGACGUAGCGAAAGCCAUU-3‘

M 6S-2 RNA (129 nt):
5‘-
gGAAGCUACUUUGUGCGUAUUGUUAAUUAAGUUUUAACCUUUAAGCUGUAAUAGGGAGUUUUAUAUCG
AAGCCGGAAUGUCAUGCCUCCAUUGCAGAGGACGACAGGAACGUUUCUGCAAACACCCACU-3‘

M 6S-2 RNA (115 nt):
5‘-
gGAAGCUACUUUGUGCGUAUUGUUAAUUAAGUUUUAACCUUUAAGCUGUAAUAGGGAGUUUUAUAUCG
AAGCCGGAAUGUCAUGCCUCCAUUGCAGAGGACGACAGGAACGUUUC-3‘

M 6S-2 RNA (100 nt):
5‘-
gGAAGCUACUUUGUGCGUAUUGUUAAUUAAGUUUUAACCUUUAAGCUGUAAUAGGGAGUUUUAUAUCG
AAGCCGGAAUGUCAUGCCUCCAUUGCAGAGGA-3‘

M 6S-2 RNA (95 nt):
5‘-
gGAAGCUACUUUGUGCGUAUUGUUAAUUAAGUUUUAACCUUUAAGCUGUAAUAGGGAGUUUUAUAUCG
AAGCCGGAAUGUCAUGCCUCCAUUGCA-3‘

M 6S-2 RNA (91 nt):
5‘-
gGAAGCUACUUUGUGCGUAUUGUUAAUUAAGUUUUAACCUUUAAGCUGUAAUAGGGAGUUUUAUAUCG
AAGCCGGAAUGUCAUGCCUCCAU-3‘

SUPPLEMENTARY FIGURES

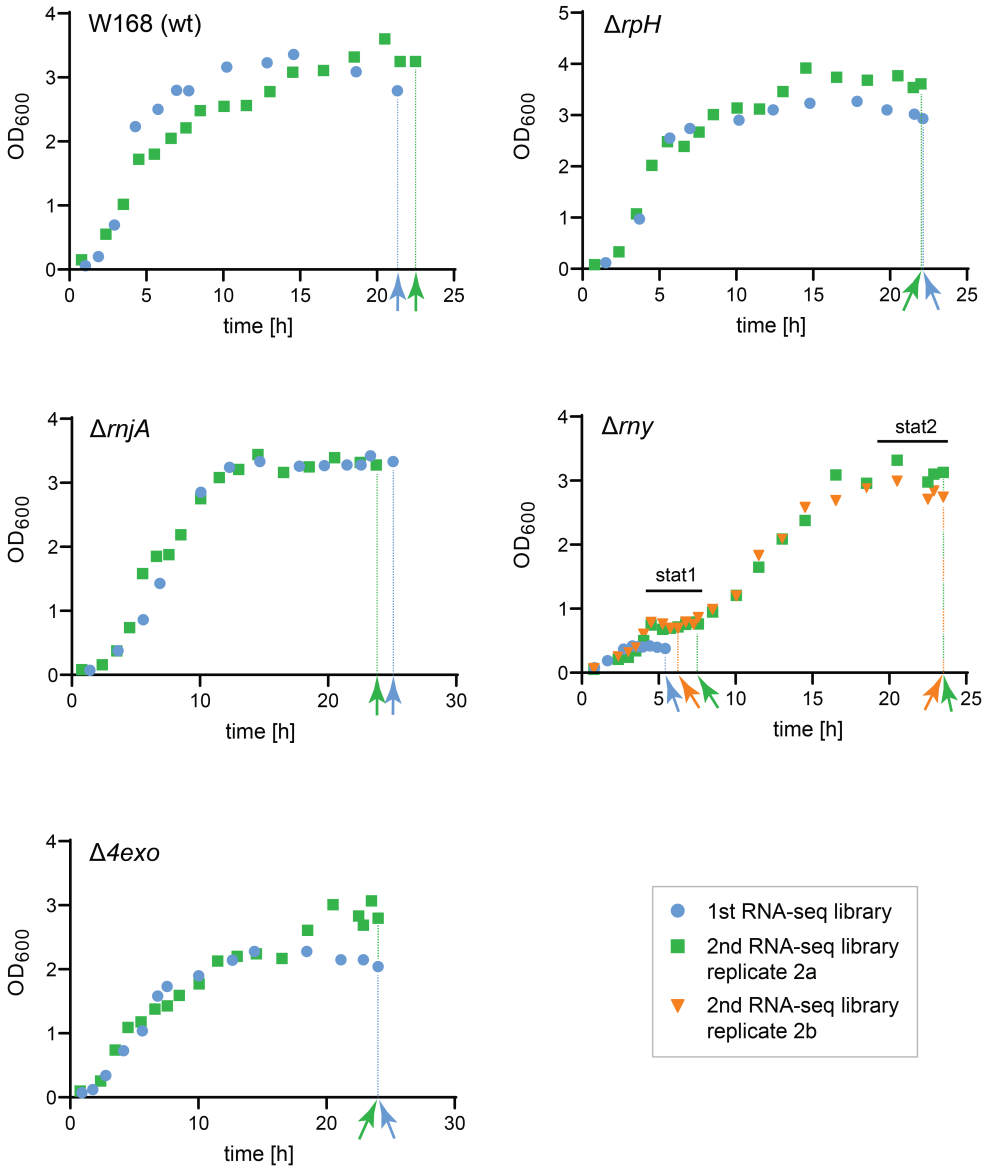


Fig. S1: Growth curves of the *B. subtilis* W168 (wt) strain and derivative strains with RNase gene knockouts. The time points of cell harvest for total RNA preparation for the two replicate RNA-seq libraries are indicated by the vertical dotted lines and arrows; wt: OD_{600} = 2.79 (21.33 h) and 3.25 (22.5 h); Δ*rph*: OD_{600} = 2.93 (22.13 h) and 3.61 (22 h); Δ*rnjA*: OD_{600} = 3.33 (25.07 h) and 3.28 (23.75 h); Δ*rph*_Δ*rnr*_Δ*yhaM*_Δ*pnp* (= Δ*4exo*): OD_{600} = 2.04 (24.03 h) and 2.8 (24.02 h). The Δ*rny* strain showed a biphasic growth behavior with intermediate growth stagnation (phase stat1); therefore, cells were harvested at this intermediate growth arrest as well as at the final stationary phase (phase stat2): OD_{600} = 0.378 (5.42 h), 0.763 (7.57 h) and 0.694 (6.25 h) for replicates RNA-seq 1, RNA-seq 2a and RNA-seq 2b of stat1 phase; OD_{600} = 3.13 (23.5 h) and 2.74 (23.5 h) for replicates RNA-seq 2a and 2b of stat2 phase.

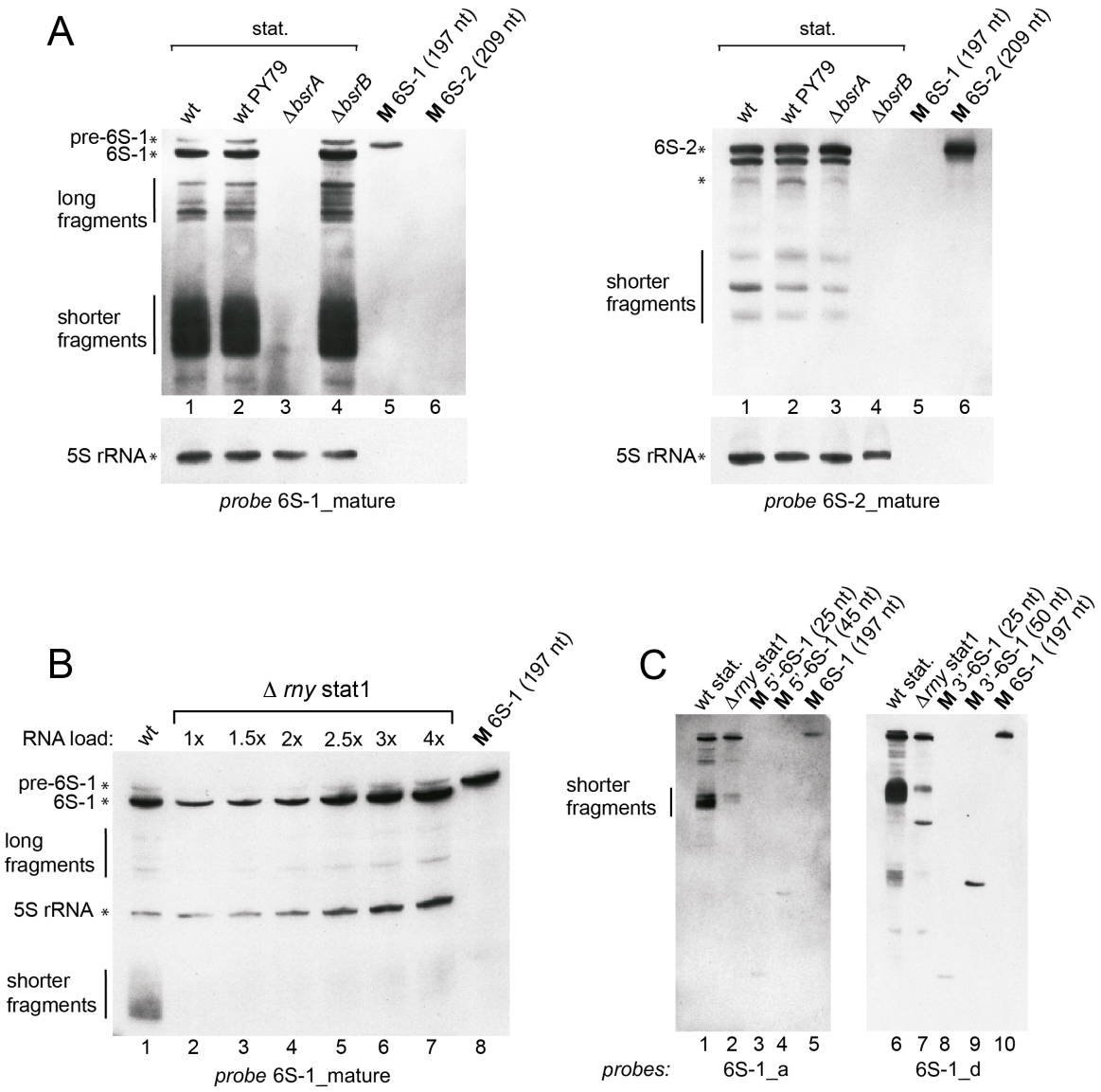
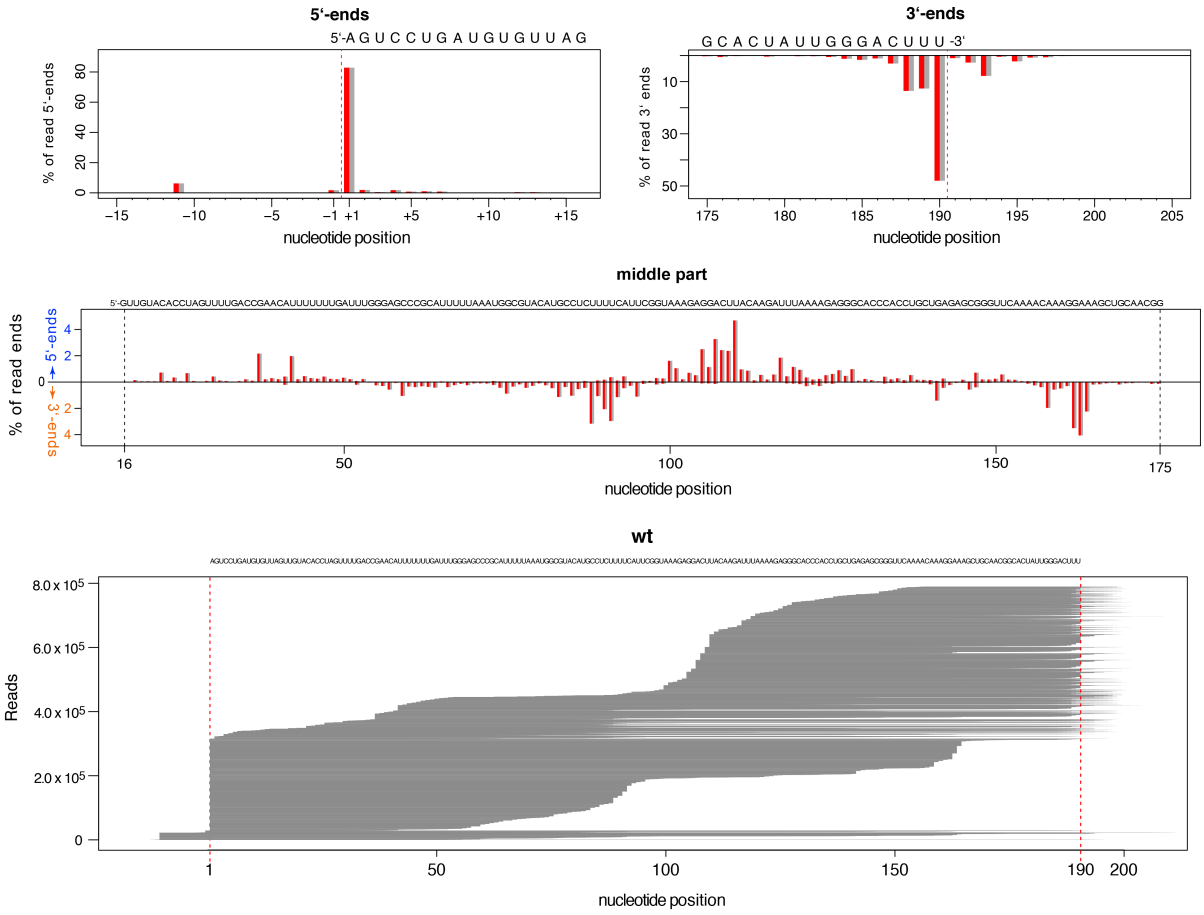


Fig. S2: (**A**) Analysis of the 6S-1/2 RNA specificity of full-length probes used in northern blot experiments. To demonstrate that the 6S-1 (left panel) and 6S-2 (right panel) RNA-specific full-length probes exclusively detected their target RNA or fragments thereof, we included total RNAs from *B. subtilis* strain PY79 and derived mutants with deletion of the 6S-1 (Δ*bsrA*) or 6S-2 (Δ*bsrB*) RNA gene. In both panels, lanes 3 and 4 indicate that all northern blot signals obtained with the full-length probes are specific for the respective 6S RNA. Total RNA was isolated from stationary (stat.) cells. (**B**) Adjustment of total RNA from *Δrny* stat1 cells to that of the wt strain to visualize equal amounts of full-length 6S-1 RNA (RNA load: one- to fourfold relative to RNA from the wt strain, lane 1); this was the case when we loaded ~2.5-fold more total RNA from *Δrny* stat1 cells relative to total RNA from the parental wt (W168) strain in the genuine stationary phase (see Fig. S1). The purpose of this experiment was to demonstrate that the shorter fragments are indeed depleted relative to the wt strain in this intermediate growth stagnation phase originally thought to represent the terminal stationary phase of *Δrny* bacteria. (**C**) Northern blot analysis (12% denaturing PAGE) of 6S-1

RNA cleavage products smaller than 90 nucleotides derived from the 5'-end (probe 6S-1_a) or 3'-end (probe 6S-1_d; see Fig. 1 of the main manuscript) in Δ*rny* stat1 cells compared with stationary (stat.) wt cells. To adjust the 6S-1 RNA levels, a 2.5-fold higher amount of total RNA from Δ*rny* stat1 cells relative to the W168 strain was loaded (lanes 2 and 7 *vs.* 1 and 6). As size markers, DNA oligonucleotides were loaded (sequence-identical to nt 1-25, 1-45 or 1-50 nt of 6S-1 RNA). The shown blots are representative of 2 individual blots each performed with independent RNA preparations.

A, replicate 1, 6S-1 wt



B, replicate 2, 6S-1 wt

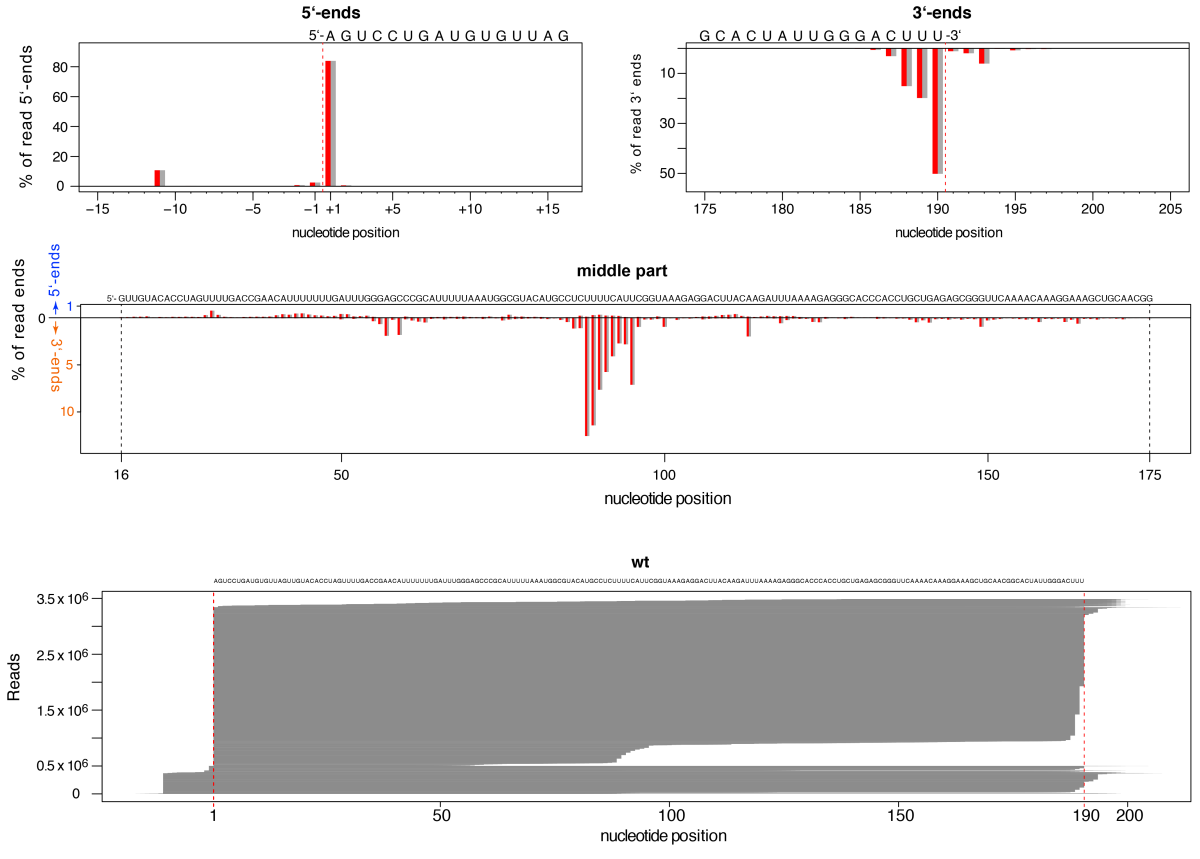


Fig. S3: RNA-seq analysis of 6S-1 RNA processing and decay in the parental wt (W168) strain. (**A, B**) RNA-seq experiments (replicates) 1 and 2, respectively. Top panels: bar diagrams that indicate the fraction (in %) of reads having their 5'-end (bars pointing upward) or 3'-end (bars pointing downward) at this nucleotide position in the library. Second row panels: enlargement of the central 6S-1 RNA region without the 5'- and 3'-terminal portions. In these presentations, the gray bars, of equal height as the red bars, represent the wt strain as well; the gray bars were bioinformatically always included for the purpose of illustrating the differences between the wt and individual RNase knockout strains. Bottom panels: read coverage along the pre-6S-1 RNA sequence.

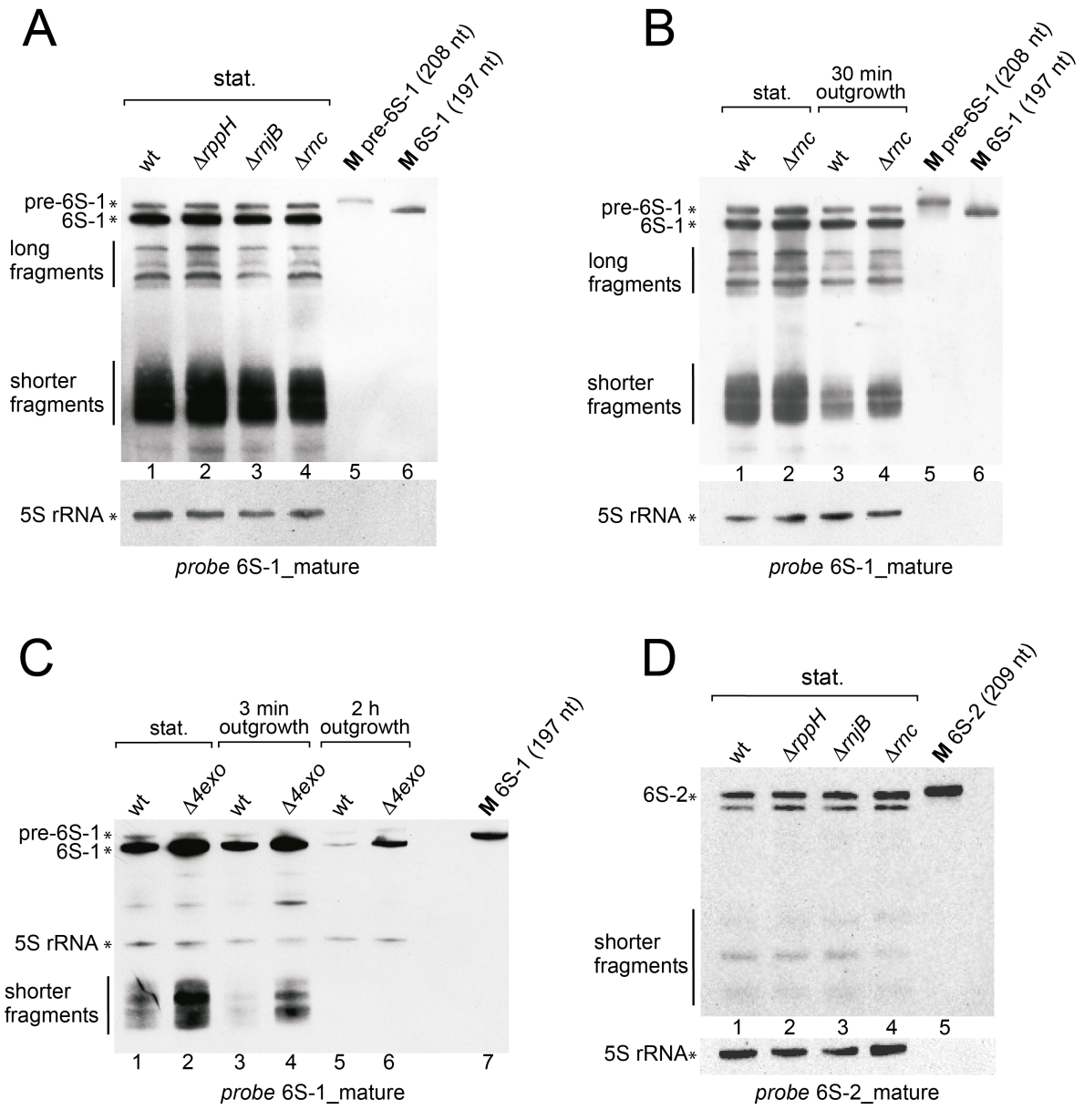
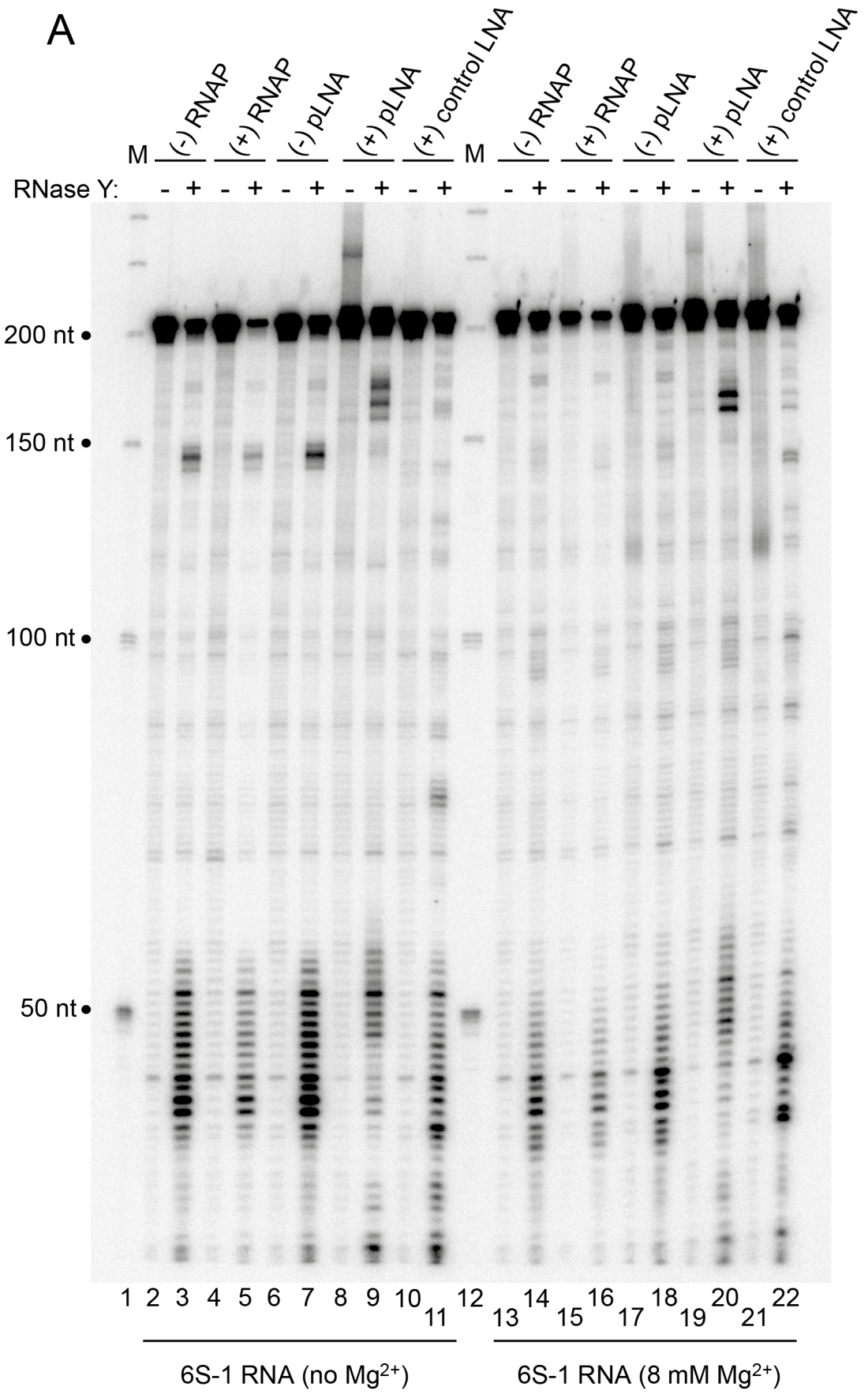


Fig. S4: Northern blot analysis of 6S-1 and 6S-2 RNA decay in other *B. subtilis* RNase knockout strains. (**A**) Analysis of 6S-1 RNA-specific signals using total RNAs from stationary phase cells, either from the parental wt (W168) strain (lane 1), or derivative strains with a knockout of RNA pyrophosphohydrolase (Δ*rppH*, lane 2), RNase J2 (Δ*rnjB*, lane 3) or RNase III (Δ*rnc*, lane 4). (**B**) 6S-1 RNA-specific blots using total RNAs from the wt and the Δ*rnc* strain either isolated from stationary phase cells (lanes 1 and 2) or outgrowth cells (lanes 3 and 4; RNA extraction 30 min after dilution of stationary phase cells in fresh medium). (**C**) 6S-1 RNA-specific blots using total RNAs from the wt and the quadruple mutant (Δ*4exo* = Δ[*rph rnr yhaM pnp*]) strain, either isolated from stationary phase cells (lanes 1 and 2) or outgrowth cells (lanes 3-6; RNA extraction 3 or 120 min after dilution of stationary phase cells in fresh medium). (**D**) Lanes 1 to 4: as in panel A, but using probe 6S-2_mature (Table 1 of the main manuscript). The shown blots are representative of 7 (panel A), 8 (panel B), 6 (panel C) or 11 (panel D) individual blots using 2-4 independent RNA preparations. For T7 transcripts used as markers in lanes 5 and 6 of panels A and B, lane 7 of panel C and lane 5 of panel D, see Supplementary Methods above.


A
M
(-) RNAP
(+) RNAP
(-) pLNA
(+) pLNA
(+) control LNA
M
(-) RNAP
(+) RNAP
(-) pLNA
(+) pLNA
(+) control LNA
RNase Y: - + - + - + - + - + - + - + - + - + - +
200 nt
150 nt
100 nt
50 nt
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
6S-1 RNA (no Mg2+)
6S-1 RNA (8 mM Mg2+)

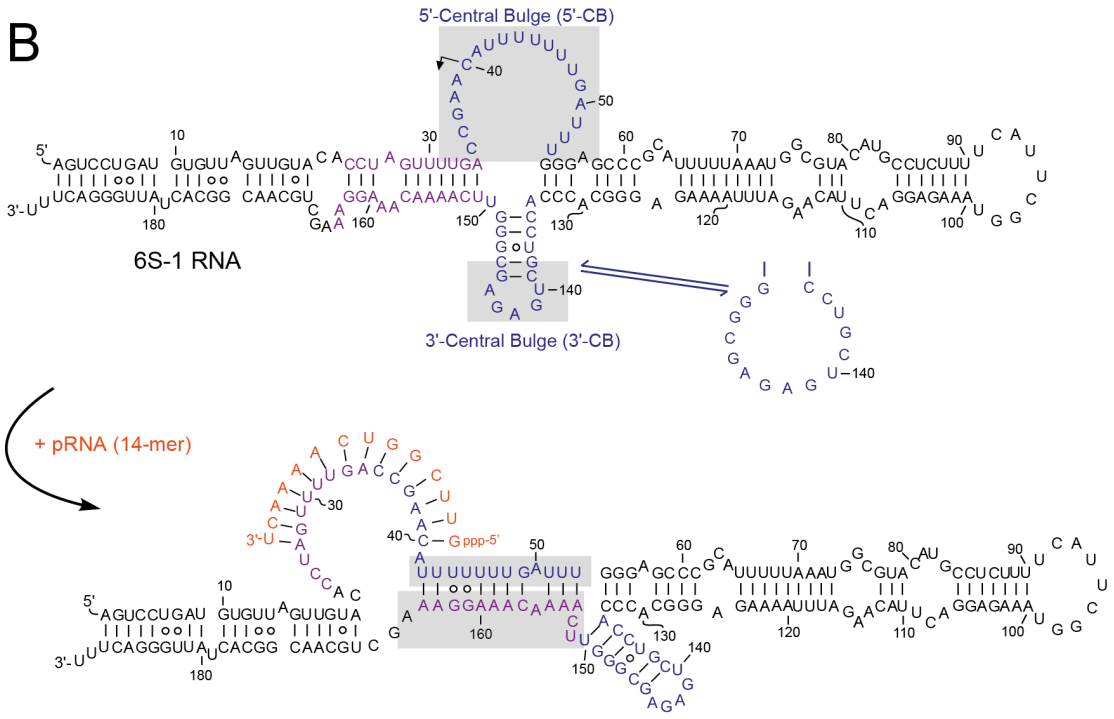
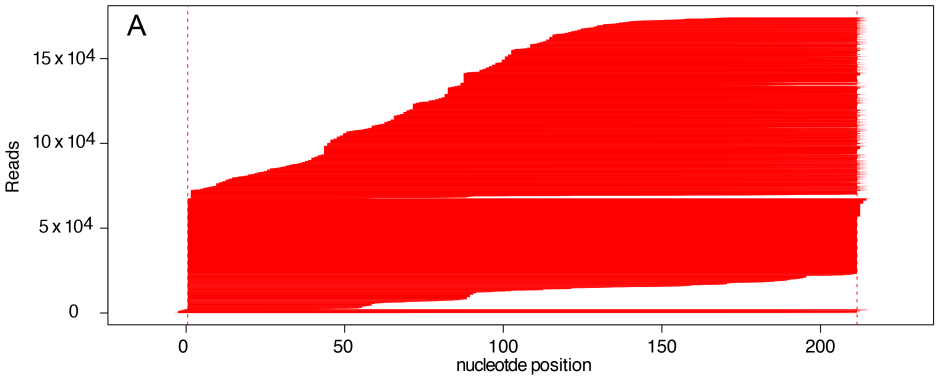
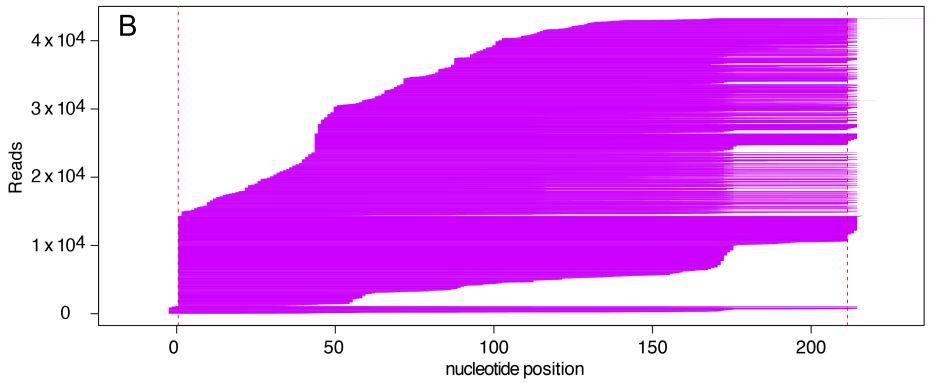


Fig. S5: Recombinant RNase Y-catalyzed *in vitro* cleavage of 6S-1 RNA alone, after annealing of a synthetic pRNA analog, termed pLNA (14 nt, 5'-GTTCGGTCAAAACT, all positions are LNA) or in the presence of σ^A-RNAP. (**A**) Representative denaturing (8 M urea) PAGE analysis. For details, see Supplementary Methods above. M, 5'-^{32}P-endlabeled RNA ladder, with the nucleotide length of individual bands indicated at the left margin; control LNA (14 nt, 5'-TCCTTCTCCTTGCC, all positions are LNA), LNA oligomer not complementary to 6S-1 RNA. (**B**) 6S-1 RNA secondary structure as determined by chemical and enzymatic probing (Beckmann et al., 2012), before (top) and after annealing of a pRNA 14-mer (orange letters, bottom sketch) that rearranges the 6S-1 RNA structure. Central bubble nucleotides are shown in blue and the helical region that is disrupted upon pRNA synthesis or annealing is depicted in magenta. Gray boxes mark the regions where we observed prominent RNase Y cleavages. The stem-loop structure in the 3'-central bubble is shown to be in equilibrium with open loop conformations in the free 6S-1 RNA structure, in line with RNase Y cleavages in this region observed in the absence of Mg^{2+} (lane 3, 5 and 7 *vs.* 14, 16 and 18 in panel A) which apparently destabilizes formation of the stem-loop. Probing also suggested that the stem-loop is more structured upon pLNA association and/or is presented differently in free 6S-1 RNA versus the 6S-1:pRNA hybrid structure (Beckmann et al., 2012). This finding is consistent with reduced cleavage in the region of ~nt 140 in the presence of pLNA and absence of Mg^{2+} (panel A, lane 9 *vs.* 7).

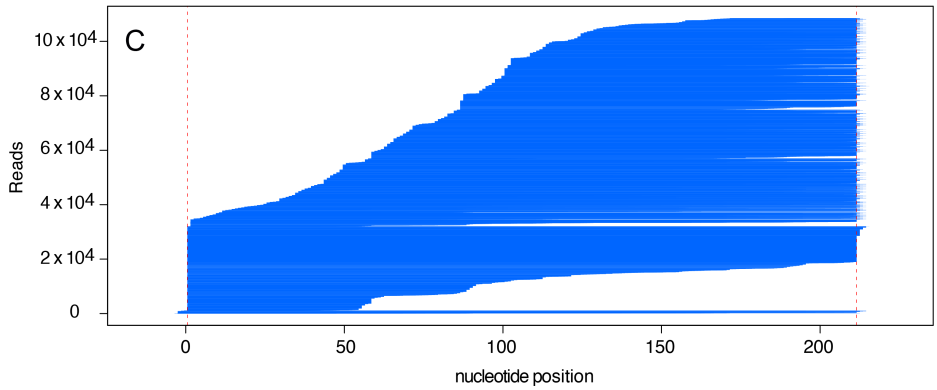
6S-2 RNA, wt, replicate 2



6S-2 RNA, Δ*(rph rnr yhaM pnp)*, replicate 2



6S-2 RNA, Δ*rny*, stat2, replicate 2.1



6S-2 RNA, Δ*rnjA*, replicate 2

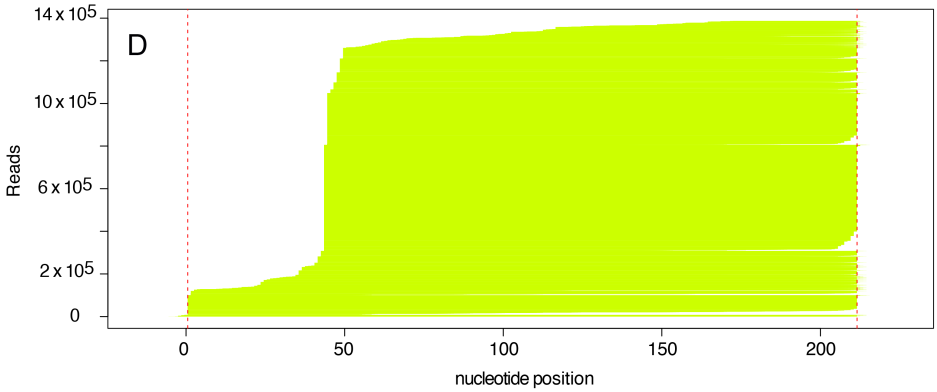


Fig. S6: Illustration of RNA-seq profiles for 6S-2 RNA in (**A**) the wt strain, (**B**) the Δ*4exo* (Δ[*rph_rnr_yhaM_pnp*]) strain, (**C**) in Δ*rny* stat2 cells, and (**D**) in Δ*rnjA* bacteria. The profiles depict read coverage (number of reads, y-axis) along the 6S-2 RNA sequence (see Fig. 9 of the main text).

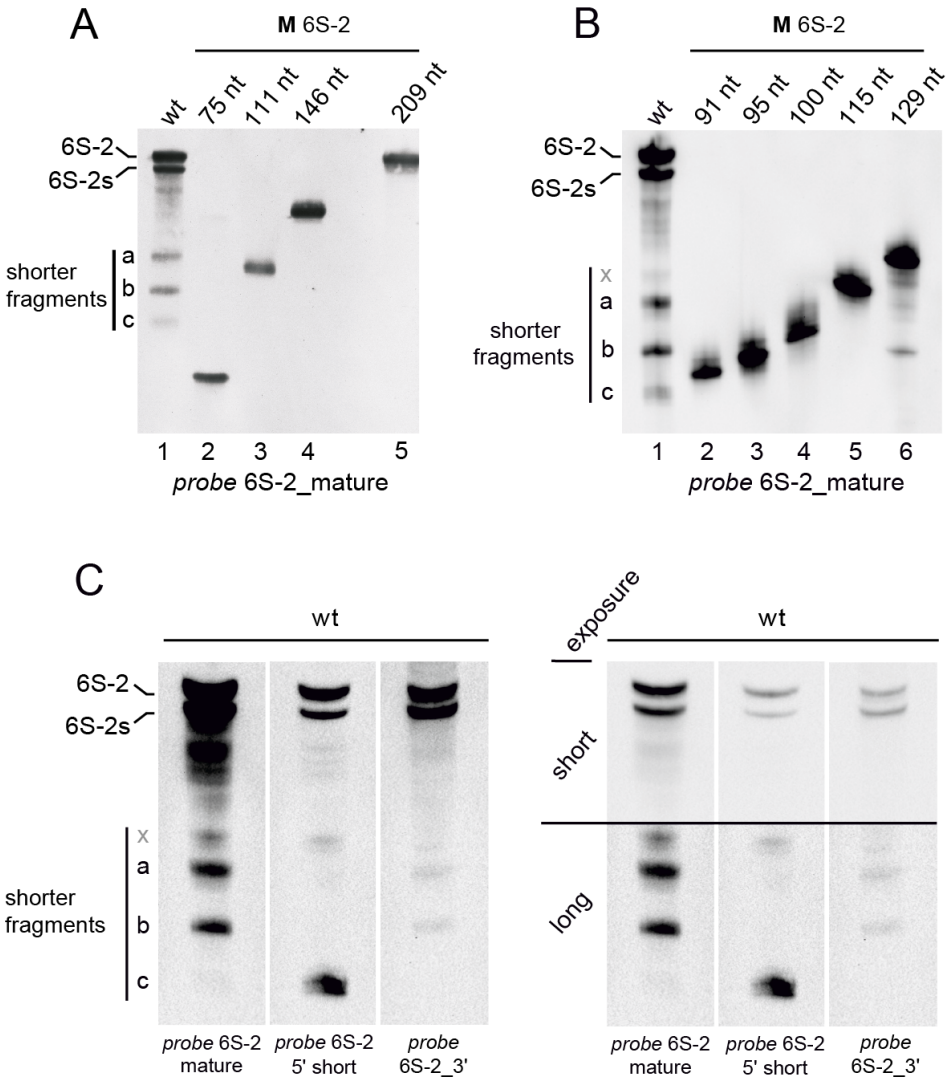


Fig. S7: Northern blot analysis of 6S-2 RNA cleavage products in total RNA isolated from stationary wt cells. (**A**) Length estimation of shorter fragments (12% denaturing PAGE) based on co-electrophoresis with RNA/DNA size markers. The 75-nt size marker in lane 2 is a DNA oligonucleotide isosequential to nt 1-75 of 6S-2 RNA; for size markers in lanes 3 to 5 (T7 transcripts), see Supplementary Methods above. (**B**) As panel A, but using different RNA size markers (see Supplementary Methods). Signal "X" has remained unassigned. (**C**) Northern blots using the three indicated probes; probe 6S_2_5' short verified that fragment "c" is a 5'-terminal fragment of 6S-2 RNA, and probe 6S-2_3' identified fragments "a" and "b" as 3'-proximal fragments (see Fig. 12 of the main text). In the illustration on the right, shorter

exposures of the membrane parts representing longer fragments up to full-length 6S-2 RNA were assembled with the (longer) membrane exposures for the shorter fragments to visualize 6S-2 and 6S-2s RNAs as sharp bands.

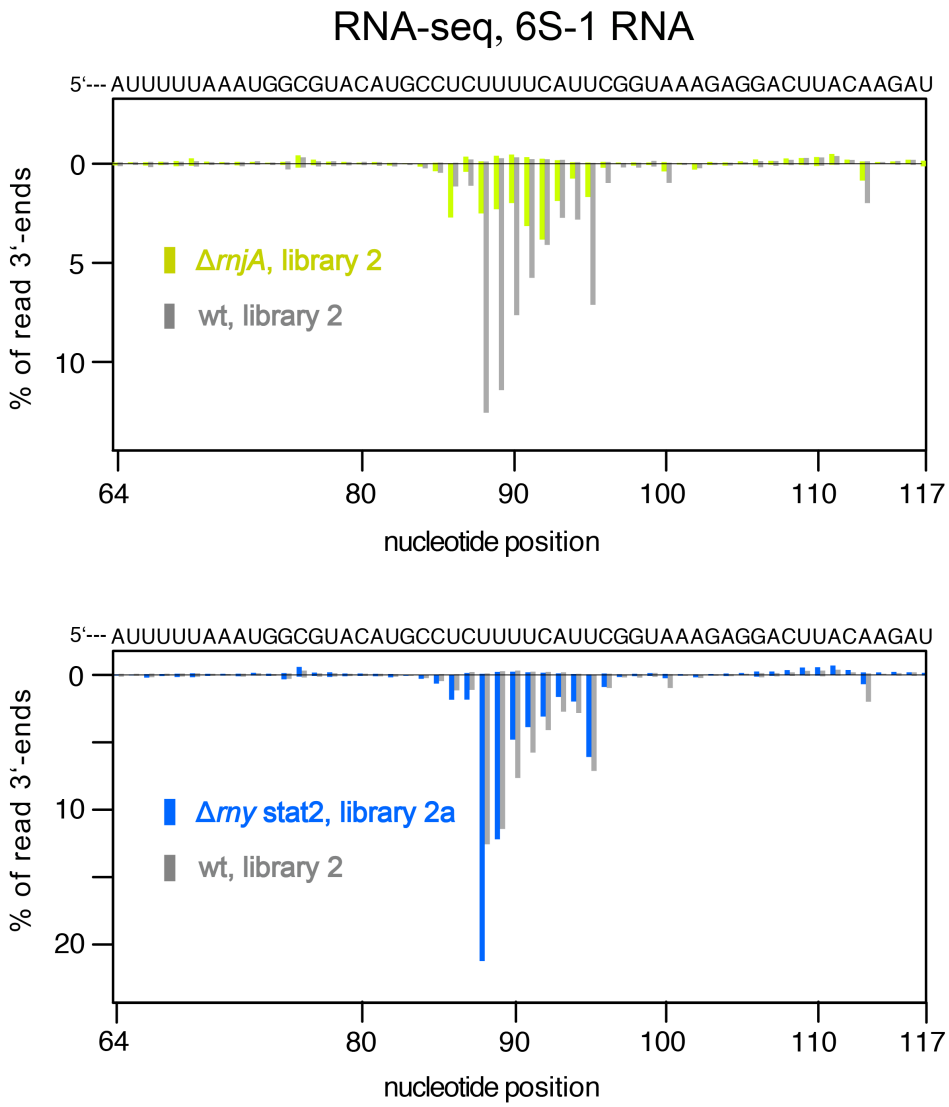


Fig. S8: Representative illustration of RNA-seq 3'-end frequencies (% of all mapped 3'-ends) at positions 86-96 (apical loop region) of 6S-1 RNA in total RNA isolated from stationary phase Δ*rnjA* and Δ*rny* stat2 cells. Gray bars indicate the values for the respective wt strain in library 2. For the designation of libraries (replicates), see Table 2 of the main text. Nucleotide sequences are given above each graph (see Fig. 1 of the main text for the complete sequence of 6S-1 RNA).