Supplementary materials for:

RNase Y-mediated regulation of the streptococcal pyrogenic exotoxin B

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 Figure S1. ropB-speB intergenic region

 A-B. Total and 5’ end coverages (black for positive strand, grey for negative strand) are indicated between brackets.

 A. Zoom on speB promoters, P (top panel) and P1 (bottom panel). The predicted −35 and −10 motifs were mapped for P and P1. The putative RopB binding sites, consisting
of inverted repeats (dark blue boxes and arrows) located within direct repeats (light blue boxes), are annotated upstream of P and P1 [1,2]. B. Characterization of the ropB-speB intergenic region by RNA sequencing analysis. The ropB (P_{ropB}) and speB (P1) TSSs are shown with black bent arrows. P_{ropB} is located –369 nt relative to the ropB start codon (not indicated here). In the experimental conditions used in this study, SPy_2041 is not transcribed.
Figure S2. Unknown RNase(s) process the speB mRNA 5’ UTR

A. Schematic drawing of speB mRNA 5’ UTR. Processing by RNase Y (orange scissors) and unknown RNase(s) (grey scissors) are indicated (left panel). The primer used (black arrow) for primer extension (right panel) and the expected cDNA sizes (green lines) are depicted. The processed 5’ ends of speB mRNA 5’ UTR were identified using primer extension analyses (right panel) in WT, my (RNase Y) deletion mutant (Δmy), mrc (RNase III) deletion mutant (Δmrc), mrnc (Mini-III) deletion mutant (Δmrnc), rhnb (RNase HII) deletion mutant (Δrhnb), pnpA (PNPase) deletion mutant (ΔpnpA), yhAM (YhaM) deletion mutant (ΔyhAM) and mr (RNase R) deletion mutant (Δmr) at early-stationary growth phase. B. Zoom on the processing sites (grey triangles) of speB mRNA 5’ UTR at positions −77 nt and −78 nt (relative to the speB start codon) retrieved by RNA sequencing analysis. The total and the 5’ end coverages are indicated between brackets.
Figure S3. Secondary structure prediction of the speB mRNA 5’ UTR

A. Schematic drawing of speB mRNA 5’ UTR (containing SpeB Inducing Peptide (SIP), orf2, SPy_2040) and speB coding DNA sequence (CDS). The positions corresponding to the cleavage sites of RNase Y and of unidentified RNase(s) are represented with orange and grey triangles, respectively. The two Gs located upstream of the RNase Y processing sites at positions – 137 nt (G₁) and – 131 nt (G₂) are indicated. The minimal folding energy (MFE, ΔG in Kcal/mol) was calculated both 100 nt upstream and downstream of the RNase Y cleavage site (– 137 nt). The numbers indicate the
distance in nt to speB start codon. **B.** RNA folding of a portion of speB 5’ UTR (from position –153 nt to the speB start codon). The free energy of the thermodynamic ensemble is –31.48 kcal/mol. The cleavages by RNase Y and unidentified RNase(s) are indicated by orange and grey scissors, respectively (right panel). The speB ribosome binding site (RBS) and start codon are represented in purple (left panel). The same structure was colored by base-pairing probabilities (right panel). The color of the unpaired regions indicates the probability of being unpaired.
Figure S4. Isoforms of speB mRNA

Expression profile of speB locus and surrounding genes resulting from RNA sequencing analysis. The 5’ ends retrieved are depicted with black bars. The genes (arrows) with the putative promoters (P and P1) and terminators are indicated. Putative ORFs (SPy_2040 and orf-2) and the sequence encoding the SpeB Inducing Peptide (SIP) are annotated in the speB 5’ UTR. speB is co-transcribed with the SPy_2038 and prsA genes [3]. The cleavages by RNase Y and by unknown RNase(s) are depicted with orange and grey triangles, respectively. The primers used in the Northern blot
analyses (Figure 4A and 4B) are indicated below the locus. The expected transcript isoforms detectable with the primers targeting the 5' UTR (T1, T2 and T3) (Figure 4A) and the CDS (T1, T2, T4, T5) (Figure 4B) are shown as black curved lines and the sizes in nt are indicated.
Figure S5. Study of speB promoter activity

A. Schematic representation of luciferase (ffluc) fusion plasmids used in Figure 4A and S4B. The speB promoters were cloned upstream of the ffluc gene (PspeB-ffluc). The –10 and –35 motifs of P and P1 are depicted with green boxes. The putative RopB binding sites are indicated in blue. A control vector with ffluc expression under the control of a constitutive promoters (P23) was included in the analysis (P23-ffluc).

B. The speB promoter activity was examined by luminescence assay performed in the WT and speB deletion mutant (ΔspeB) containing the luciferase fusion plasmids (P23-ffluc and PspeB-ffluc) at mid-logarithmic (ML) and early-stationary (ES) growth phases. Values indicate luminescence intensity of the samples relative to the control plasmid (P23-ffluc), normalized to the OD_{620 nm}. Mean and standard deviations (error bars) were calculated from three independent experiments, each with technical triplicates.
Figure S6. *covR* mRNA stability is affected by RNase Y

Study of the *covR* transcript stability by rifampicin assay at mid-logarithmic phase of growth in WT and *rny* (RNase Y) deletion mutant (Δ*rny*) (lower panel). The minutes after stopping transcription upon the addition of antibiotic are indicated. 16S rRNA was used as a loading control. The primer used is indicated by a black arrow.
Figure S7. An sRNA arises from speB mRNA 5’ UTR processing

Expression profile of a small RNA (sRNA) previously identified in speB 5’ UTR by sRNA sequencing (Spy_sRNA1699993) [4]. Total, 5’ end and 3’ end coverages are indicated between brackets. Orange and grey bars pinpoint the positions of RNase Y and unidentified RNase(s) cleavage sites annotated in this study, respectively. The green bar denotes the putative sRNA 3’ end.
## Supplementary Table I. Strains, plasmids and oligos used in this study.

| Strain | Relevant characteristics | Source |
|--------|--------------------------|--------|
| **Streptococcus pyogenes** | | |
| WT | | |
| EC2224 | SF370 (M1 serotype) | ATCC 700294 |
| Δrny | EC2246 EC2224Δrny::lox72 | [5] |
| Δrnc | EC2249 EC2224Δrnc::lox72 | [5] |
| Δrnr | EC2254 EC2224Δrnr::lox72 | This study |
| ΔpnpA | EC2297 EC2224ΔpnpA::lox72 | This study |
| Δrny::rny | EC2298 EC2246Δlox72::rny-TT3-lox72 | This study |
| ΔyhaM | EC2347 EC2224ΔSPy_0267::lox71-ermAM/B-ermAM/B-lox66 | This study |
| ΔrnhB | EC2251 EC2224ΔrnhB::lox72 | This study |
| Δmrnc | EC2271 EC2224Δmrnc::lox72 | This study |
| ΔspeB | EC2356 EC2224ΔspeB::lox72 | This study |
| **Saccharomyces cerevisiae** | | |
| S228C | BY4741 (Host for cloning) | Euroscarf, Frankfurt |
| **Escherichia coli** | | |
| RDN204 | Top10 (Host for cloning) | Invitrogen |

| Plasmid | Relevant characteristics | Source |
|---------|--------------------------|--------|
| **Plasmids used for gene deletion in S. pyogenes** | | |
| pEC454 | pUC19Δlox71-ermAM/B-lox66 | Laboratory collection |
| pEC455 | pEC85QgyrA-cre | Laboratory collection |
| pEC707 | pUC19, pMB1, ampR | New England Biolabs |
Oligo | Sequence 5’-3’ * | F/R a | Usage a | Target a
--- | --- | --- | --- | ---
*rny* | | | | |
OLEC3584 | GTAACGCCAGGTTTTCCCAGTCACGACGCTCTTCAAAACGAA AAGAGG | F | Cloning | Up fragment (pEC802)
OLEC3579 | CGAGAAAAAAGGCCCACTTTTGTGGGCCTTTTTACGCAA GCACTACTTGGCATTAACCCGCCTCATT CT | R | Cloning | Iox71-ermAM/B-lox66 (pEC454)
OLEC3480 | AAGGGGGCTTTTTTCGGAATTACCGATTCGTAATGCA CATTATAGTTATCCCG | F | Cloning | Down fragment (pEC802)
OLEC3572 | TACGTTTGCATATAATGTTATGCTATACGAAAGTTATTT TTTTCTTCC | R | Cloning | Upstream *my*
OLEC2000 | ATAGCATAAGATTACGAAACGGTAAAAGAGGAATTATC CCTCTTTTATGA | F | LM-PCR | Up fragment (pEC749)
OLEC3585 | GGGGATAAATTTTCACACACGAAACACGGCTAAAAATCACAAGT GAATACCTGG | R | LM-PCR | Dw fragment (pEC749)
OLEC2785 | TGCCAAATCGTTGAAAATCAT | F | PCR, SEQ | Upstream *my*
OLEC2503 | GACAGCTCATCGGTAGTGAAG | R | PCR, SEQ | Downstream *my*

*mnhB* | | | | |
OLEC3340 | GGGTGTGGAGTCGCGAAGTGAAAGCTAATCATGC | F | Cloning | Up fragment (pEC748)
OLEC2517 | TATAATGATATGCTATACGGAACGGTAAATACGTGCCGATCCATA TCCC | R | LM-PCR | Cloning (pEC748)
OLEC2518 | ATAGCATAAGATTACGAAACGGTAAAAGAGGAATTATC CCTCTTTTATGA | F | LM-PCR | Dw fragment (pEC748)
OLEC3341 | GGGTGTGGAGTCGCGAAGTGAAAGCTAATCATGC | R | Cloning | Cloning (pEC748)

*Note:* a uppercase letters indicate PCR primers.

Chromosomal complementation of *rny* in *S. pyogenes*

pEC802 | pRS426*omyr-rny*-TT3-lox71-PermAM/B-ermAM/B-lox66-mydw | This study

*speB* ectopic expression in *S. pyogenes*

pEC85 | repDEG-pAMB1, aphIII-Pjhl1, ColeE1 | Laboratory collection
pEC2146 | pEC85PgyrAspeB | This study
pEC2249 | pEC85PgyrA-speB(G-137A) | This study
pEC2250 | pEC85PgyrA-speB(G-131A) | This study
pEC2263 | pEC85PgyrA-speB(G-137A_G-131A) | This study
pEC2264 | pEC85PgyrA-speB(Δ-147-121) | This study
pEC2265 | pEC85PgyrA-speB(Δ-157-111) | This study

Luminescence assay in *S. pyogenes*

pEC2173 | pLZ12Km2-P23R:TA:ffluc | Addgene plasmid # 88900
pEC2248 | pEC2173PspeB | This study
| Oligo | Sequence 5'-3' | F/R | Usage | Target |
|-------|--------------|-----|-------|--------|
| OLEC2520 | TGTACAAGCAGAAAACTGATAAGACTTTAAGG | F | SEQ | Upstream mhB |
| OLEC2521 | CATAATATCTATTTTTAGGAAACTGTCATTA | R | SEQ | Downstream mhB |

**mrnc**

| OLEC2034 | GATGATGAGCTCCCTGTCAGAACTTTGAAGGTGAGG | F | Cloning | Up fragment (pEC749) |
| OLEC3353 | TAGCATACTATATCGAACCTGAATTTTATACCATCCTTTGAAATTTCATTGTAACCTGAAC | R | LM-PCR | Dw fragment (pEC749) |
| OLEC2033 | GGTGCTAGCTCAGAATAGTATTTTTCTTCATGAG | F | LM-PCR | R | Cloning |
| OLEC2006 | CCTCGTGTATTGAATTATAGCA | F | SEQ | Upstream mrnc |

**speB**

| OLEC7565 | AAAAGATCCAGTCTCAAATACGTTAGCATG | F | Cloning | Up fragment (pEC2145) |
| OLEC7566 | CATAATGATGTCAGAACAGCTGAATATTTCATTCTTCTTTGAAATTTCATTGTAACCTGAAC | R | LM-PCR | Dw fragment (pEC2145) |
| OLEC7569 | AAAAGATCGTGTGTTGTTATGAGATTCAAGCTGAAAGG | R | Cloning | |
| OLEC7563 | TGAATGCCTCATGAAATTTACGCGG | F | PCR, SEQ | Upstream speB |
| OLEC7570 | GTTGGTTGGCTCTATTGGTAAGGT | R | PCR, SEQ | Downstream speB |

**mr**

| OLEC2897 | AAAAGATCCAGTGATTGGCTGAAATTTTCATTCTTCTTTGAAATTTCATTGTAACCTGAAC | F | Cloning | Up fragment (pEC545) |
| OLEC2535 | TATAATGATGTCAGAACAGCTGAATATTTCATTCTTCTTTGAAATTTCATTGTAACCTGAAC | R | LM-PCR | Dw fragment (pEC545) |
| OLEC2536 | ATAGCATACATATATCGAACCTGAATTTTATACCATCCTTTGAAATTTCATTGTAACCTGAAC | F | LM-PCR | R | Cloning |
| OLEC2898 | AAAAGATCCAGTGATTGGCTGAAATTTTCATTCTTCTTTGAAATTTCATTGTAACCTGAAC | R | PCR, SEQ | Upstream mr |
| OLEC2538 | CTGAGTTGGCATTGAGATTGGCAGGTAAGG | R | PCR, SEQ | Downstream mr |
| OLEC2539 | TATGGCGATAGAGAATAACCATCCTCACAT | R | PCR, SEQ | |

**pnpA**

| OLEC3350 | GCTAGGGATCCGATCCCTTTCTGTGCTCCGGCAATTTCATTCTTCTTTGAAATTTCATTGTAACCTGAAC | F | Cloning | Up fragment (pEC750) |
| OLEC2541 | TATAATGATGTCAGAACAGCTGAATATTTCATTCTTCTTTGAAATTTCATTGTAACCTGAAC | R | LM-PCR | Dw fragment (pEC750) |
| OLEC2542 | ATAGCATACATATATCGAACCTGAATTTTATACCATCCTTTGAAATTTCATTGTAACCTGAAC | F | LM-PCR | R | Cloning |
| OLEC3351 | GCTAGGGATCCGATCCCTTTCTGTGCTCCGGCAATTTCATTCTTCTTTGAAATTTCATTGTAACCTGAAC | R | PCR, SEQ | Upstream pnpA |
| OLEC2544 | CTAGGAGTTGGCATTGAGATTGGCAGGTAAGG | R | PCR, SEQ | Downstream pnpA |
| OLEC2545 | ATAGAGACTCCAGGAGCGATTTTG | R | PCR, SEQ | |

**syhA** (SpEc0267)

| OLEC3361 | GAAGCTAGGCCCTTTCTCTTTCTTGCTCCCG | F | Cloning | Up fragment (pEC822) |
| OLEC2529 | TATAATGATGTCAGAACAGCTGAATATTTCATTCTTCTTTGAAATTTCATTGTAACCTGAAC | R | LM-PCR | Dw fragment (pEC822) |
| OLEC2530 | ATAGCATACATATATCGAACCTGAATTTTATACCATCCTTTGAAATTTCATTGTAACCTGAAC | F | LM-PCR | R | Cloning |
| OLEC3362 | GAAGCTAGGCCCTTTCTCTTTCTTGCTCCCG | R | PCR, SEQ | Upstream SyhA | SpEc0267 |
| OLEC2532 | GAGCCTGCTGAAACCGCTTA | R | PCR, SEQ | Downstream SyhA | SpEc0267 |
| OLEC2533 | GTCTATTGGCCTGAGGCGTTGTA | R | PCR, SEQ | |

**pEC2146**

| OLEC7968 | CTCTTCTCTCTCTCTGATA | F | Cloning | PgyR (pEC455) |
| OLEC7969 | CATAATGATGTCAGAACAGCTGAATATTTCATTCTTCTTTGAAATTTCATTGTAACCTGAAC | R | LM-PCR | speB |
| OLEC7970 | ATCATAGGCTATCATTAATGCTCGTAATTCTTTCTTTGAAATTTCATTGTAACCTGAAC | F | LM-PCR | |
| OLEC7971 | CCCAGATTCTAAGGCTTTGTAGCCTCCTCAACAGACCAC | R | LM-PCR | |
| Oligo | Sequence 5’-3’ a | F/R b | Usage c | Target d |
|-------|-----------------|-------|---------|----------|
| **pEC2249** | | | | |
| OLEC8388 | GTCAACTAACCCTATTATTTGCTATTACCAT | F | TS-PCR | speB 5’ UTR (pEC2146) |
| OLEC8389 | GTCAACTAACCCTATTATTTGCTATTACCAT | R | TS-PCR | |
| **pEC2250** | | | | |
| OLEC8390 | GTCAACTAACCCTATTATTTGCTATTACCAT | F | TS-PCR | speB 5’ UTR (pEC2146) |
| OLEC8391 | ATGGTAATAGATAAAATACACGTTTAGGTGAC | R | TS-PCR | |
| **pEC2263** | | | | |
| OLEC8392 | GTCAACTAACCCTATTATTTGCTATTACCAT | F | TS-PCR | speB 5’ UTR (pEC2146) |
| OLEC8393 | ATGGTAATAGATAAAATACACGTTTAGGTGAC | R | TS-PCR | |
| **pEC2264** | | | | |
| OLEC8394 | GTTGGGTTGTCAGTGTACATGATCAGCGACAT | F | TS-PCR | speB 5’ UTR (pEC2146) |
| OLEC8395 | ATGTCGCTGATACCATGATGACACTGACAACCCAACAC | R | TS-PCR | |
| **pEC2265** | | | | |
| OLEC8396 | GAATAATTGGGTTGGTATTACCAT | F | TS-PCR | pEC2146 |
| OLEC8397 | TTATCATACGATGCTACTGCTAACCCCAATTTATTC | R | TS-PCR | |
| **pEC2248** | | | | |
| OLEC8386 | GGAACGAAAACTCACGTTAA | F | Cloning | speB 5’ UTR |
| OLEC8387 | TACCGCGGGTGCTATTATCATAGCTGCTTATTTTGCAATTTGCT | R | Cloning | |
| **Sequencing** | | | | |
| OIRC228 | GGAACGAAAACCTACGTTAA | F | SEQ | pEC85 |
| OLEC87 | TGTGTTTACGTTGTTTTTAAAC | R | SEQ | pEC707 |
| OLEC3224 | TGTAAGGCAACGCCGCGT | F | SEQ | pEC2173 |
| OLEC3225 | CAGAGAAAGCTAGTACCC | R | SEQ | |
| OLEC3600 | CCAGGTTTCTCCAGTCACGAC | F | SEQ | |
| OLEC3590 | AGCCGATGACGATACCATACAGGA | R | SEQ | |
| OLEC1938 | TCATCGAGATGCAATCGTACTGCTATTTAAGAAAAACACACAGC | F | SEQ | |
| OLEC1937 | TTGCTGTTCATTTTATATGTGATGTC | R | SEQ | |
| OLEC5336 | GGGGGATGTCGTGAGGGG | F | SEQ | pEC802 |
| OLEC5337 | TCCGGCTCCTATGTCGTGTTT | R | SEQ | |
| **Primer extension analyses** | | | | |
| OLEC2406 | ACTACCTATTGGCAAAAGGAAC | R | PE | speB 5’ UTR |
| OLEC3903 | TACCGGGCTACCTGGAACACAACCCTCC | R | PE | |
| OLEC3904 | TATACCTCTTTTCAATATTGATATTGC | R | PE | |
| OLEC3970 | TGGTATTGACAGAACAAATTCC | R | PE | speB CDS |
| **Northern blot analyses** | | | | |
| OLEC5802 | AACCCATAGTAGGCGCCCTC | R | NB | speB 5’ UTR |
| OLEC7431 | GCACACATGCTGACACG | R | NB | speB CDS |
| OLEC1542 | CATGACGGATTTCTCATATAGTC | R | NB | covR CDS |
| OIRC243 | CGTGTACCAACCATTGACG | R | NB | 16S rRNA |

a italic: sequence annealing to the template; underlined: restriction site.
b F: forward primer; R: reverse primer.
c LM-PCR: ligation-mediated PCR; TS-PCR: two-stage PCR; SEQ: sequencing; PE: primer extension; NB: Northern blot;
d 5’ UTR: 5’ untranslated region; CDS: coding DNA sequence
### Supplementary Table II. speB regulators potentially affected by RNase Y.

| speB regulators | Function | References |
|-----------------|----------|------------|
| **Direct transcriptional regulators** | | |
| ropB            | Activator | [1,6–8] |
| covRS           | Repressor | [9–11] |
| ccpA            | Activator | [11–13] |
| **Indirect transcriptional regulators via RopB** | | |
| LacD.1          | Repressor | [14] |
| vfr             | Repressor | [15,16] |
| SIP             | Activator | [2,17] |

Except for vfr abundance [18] and ropB stability [19], which were shown to be affected by RNase Y, the effect of RNase Y on the other regulators is to be confirmed [20]. SpeB Inducing Peptide (SIP) is encoded by the speB transcript, and therefore its expression is downregulated in the my deletion strain.
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