An RNA-seq based comparative approach reveals the transcriptome-wide interplay between 3′-to-5′ exoRNases and RNase Y

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RNA degradation is an essential process that allows bacteria to control gene expression and adapt to various environmental conditions. It is usually initiated by endoribonucleases (endoRNases), which produce intermediate fragments that are subsequently degraded by exoribonucleases (exoRNases). However, global studies of the coordinated action of these enzymes are lacking. Here, we compare the targetome of endoRNase Y with the targetomes of 3′-to-5′ exoRNases from Streptococcus pyogenes, namely, PNPase, YhaM, and RNase R. We observe that RNase Y preferentially cleaves after guanosine, generating substrate RNAs for the 3′-to-5′ exoRNases. We demonstrate that RNase Y processing is followed by trimming of the newly generated 3′ ends by PNPase and YhaM. Conversely, the RNA 5′ ends produced by RNase Y are rarely further trimmed. Our strategy enables the identification of processing events that are otherwise undetectable. Importantly, this approach allows investigation of the intricate interplay between endo- and exoRNases on a genome-wide scale.
The ability to modulate gene expression enables bacteria to rapidly adapt to their environment. Ribonucleases (RNases) regulate transcript abundance, leading to RNA maturation (e.g., for tRNAs, rRNAs), stabilization or degradation. Eventually, all transcripts—even the most stable—are degraded by RNases, leading to the renewal of the nucleotide pool. As a general rule, RNA degradation starts with an endonucleolytic processing in the RNA body, leading to the generation of decay intermediates. Those are further digested by exorNases and, finally, by oligoRNase/nanoRNases. The main endoRNases that have been demonstrated to initiate RNA decay are RNase E in Gram-negative bacteria and its functional orthologue RNY in Gram-positive bacteria. However, in many Gram-positive bacteria, RNA degradation can also be initiated by the complex of RNases J1/J2, which displays both endo- and 5′-to-3′ exoribonucleolytic activities. The initial processing of a transcript is the limiting step of the RNA decay and the access of endoRNases to transcripts is usually restricted. For instance, RNase E favours 5′-monophosphorylated (5′P) transcripts and cleaves 2 nt upstream of a uridine (U) in A/U rich regions. RNY also prefers 5′P transcripts, and additional requirements have been described depending on the orthologue studied. In Staphylococcus aureus, RNY processes transcripts preferably downstream of a guanosine (G). In Streptococcus pyogenes, a G is required for the in vivo processing of the speB transcript, encoding a major virulence factor. In Bacillus subtilis and S. aureus, RNY processes relying on proximal RNA secondary structures. The decay intermediates, once generated, are cleared immediately from the cell by 3′-to-5′ exorNases. In E. coli, the decay intermediates are mainly degraded by the 3′-to-5′ exorNases II, R and PNPass. In B. subtilis and S. pyogenes, the major 3′-to-5′ exorNase is PNPass and RNase R. In addition, in B. subtilis, the 3′-to-5′ exorNases PH and YhaM participate in RNA decay, albeit with lower efficiency than the main 3′-to-5′ exorNase. S. pyogenes YhaM exhibits a very short processivity (3 nt on average) on a large number of RNA 3′ ends, the impact of which on mRNA decay is currently unknown.

With the emergence of RNA sequencing techniques allowing the global detection of RNase cleavage sites, several targetomes of endoRNases have been determined, such as those of RNY in S. aureus and B. subtilis. To date, the activity and specificity of 3′-to-5′ exorNases towards decay intermediates produced by a given endoRNase have never been studied on a global scale.

Here, we present a comparative RNA-seq based approach that allows us to dissect the complex landscape of RNA ends in S. pyogenes. We study the interplay of endoRNase Y with 3′-to-5′ exorNases in S. pyogenes, a pathogen causing a wide range of diseases in humans. We determine the first targetome of RNY in this bacterium and compare it with three 3′-to-5′ exorNase targetomes, previously characterized by our laboratory. We show on a global scale that RNY largely acts in concert with PNPass during RNA degradation. In this regard, we demonstrate a role of the RNY-PNPass interplay in the control of the differential stability of polycistronic mRNAs and the decay of 5′ regulatory elements. This strategy allows us to elucidate the interplay and dynamics of endoRNase- and exorNase-mediated RNA processing events otherwise not detectable when RNases are studied separately.

Results

In vivo RNY targetome. To identify RNY processing positions, we compared the abundance of RNA ends (5′ and 3′) in the S. pyogenes wild type (WT), RNY deletion mutant (Δrny) and complemented RNY deletion mutant (Δrny::rny) strains, as described previously for other RNases in this bacterium and complemented RNY Y deletion mutant (Δrny::rny) strains, as described previously for other RNases in this bacterium and complemented RNY Y deletion mutant (Δrny::rny) strains, as described previously for other RNases in this bacterium.

Comparison of the RNY Y and 3′-to-5′ exorNase targetomes. To investigate whether the 3′ rny ends originated from 3′-to-5′ exorribonucleolytic activity, we compared the RNY Y targetome with the targetomes of three 3′-to-5′ exorNases (PNPass, YhaM and RNY) recently characterized by our laboratory. In our previous study, we identified the processing sites of 3′-to-5′ exorNases by comparing the abundance of the RNA 3′ ends between the WT and 3′-to-5′ exorNase mutant (ΔexorNase) strains. The RNA 3′ ends more abundant in the ΔexorNase strain and the 3′ ends more abundant in the WT strain were annotated as exorNase trimming start and stop positions, respectively (Supplementary Fig. 1). The targetomes were compared using two different approaches, which are described in the next two sections, and we found that 58% of the identified 3′ rny ends corresponded to 3′-to-5′ exorNase—mainly PNPass—trimming start or stop positions. We did not match these 3′ rny ends with 3′-to-5′ exorNase start or stop positions because they were likely targeted by several 3′-to-5′ exorNases at once or by unidentified RNases. Overall, we conclude that PNPass is the main 3′-to-5′ exorNase that acts in concert with RNY Y to degrade RNAs in S. pyogenes.
RNase Y processes RNAs after a guanosine. a Representation of RNA end (5′ or 3′) profiling obtained by RNA sequencing (performed in biological triplicates). The RNA ends that were more abundant in the wild type (WT) and complemented Δmy deletion strain (Δmy::my) than in the RNase Y deletion strain (Δmy) are annotated as rny_end. "NNNNNNN" represents a sequence processed by RNase Y. b The bar plot shows the number of 5′ or 3′ ends that were more abundant in the WT than in the Δmy strain (see Methods). c RNase Y cleavage (scissors) generates two processing products. We never retrieved both the RNA fragments upstream and downstream of the cleavage site for the same processing event. d Schematic drawing of total and end (5′ or 3′) coverages from RNA sequencing, illustrating RNA 5′ “unique” (U) and 3′ “stepped” (S) end positions. e Proportion of RNA 5′ and 3′ ends classified as U and S. f Sequence and structure conservation of the identified 5′ and 3′ rny_ends. The logo was created from the alignment of all sequences 10 nt on each side of the identified ends. Error bars are automatically calculated by the WebLogo library and correspond to an approximate Bayesian 95% confidence interval. The minimum free energy (ΔG) was calculated at each nucleotide position using a sliding window of 50 nt over the entire genome. The average ΔG (kcal mol⁻¹) calculated for a window of 100 nt centred on the identified ends is depicted.

3′-to-5′ exoRNases trim RNAs generated by RNase Y processing. Left bar plot: portion of RNA 3′ ends (3′ rny_ends) corresponding to 3′-to-5′ exoRNase start positions (bottom portion), 3′-to-5′ exoRNase stop positions (middle portion) and not associated with 3′-to-5′ exoRNase (top portion). Right bar plots: number of trimming starts (top) and stops (bottom) that correspond to 3′ my_ends, which were uniquely produced by PNPase and YhaM or produced by two different 3′-to-5′ exoRNases.

Therefore, we searched for 3′-to-5′ exoRNase trimming start positions that were located downstream of the 3′ rny_ends (Fig. 3a). We retrieved 19 and 5 trimming start positions for PNPase and YhaM, respectively, which could correspond to the RNase Y initial processing positions (Fig. 3a and Supplementary Data 3). We observed enrichment of G at the 19 PNPase trimming start positions (Fig. 3b), with 9 mapped to a G and 9 located 1 or 2 nt upstream of a G (Supplementary Data 3). Considering the frequency of G around the PNPase trimming start positions, we hypothesize that the initial RNase Y processing (PNPase trimming start positions) actually occurs at G (Fig. 3a and b). The 1 or 2 nt distance from G is likely due to the known nipping activity of YhaM that we observed in S. pyogenes47. Similarly, 4 trimming start positions of YhaM were located at a G (Supplementary Data 3). One other position, located at an adenosine, corresponded to a predicted PNPase trimming stop position and was identified in the transcript encoding the putative SPy_0316 protein (Supplementary Data 3). Upon RNase Y processing, the SPy_0316 mRNA was trimmed first by PNPase and then by YhaM (Fig. 3d). We indeed identified a 3′ rny end corresponding to PNPase and YhaM stop positions (Supplementary Data 2, Fig. 3d). PNPase started trimming, 34 nt upstream of the stop position (Fig. 3d and Supplementary Data 5), at a G corresponding to the initial RNase Y processing position, followed by YhaM, which stopped at the base of a stem loop predicted in the middle of the SPy_0316 open reading frame (ORF) (Fig. 3e). The YhaM trimming start position was not detected in the absence of PNPase (Fig. 3d), which confirmed that YhaM targeted the RNA 3′ end generated by PNPase. Similarly, we observed subsequent trimming of PNPase and YhaM upon RNase Y processing in the intergenic region between Spy_sRNA73113 and rplO, encoding the 50S ribosomal

Fig. 2 3′-to-5′ exoRNases trim RNAs generated by RNase Y processing. Left bar plot: portion of RNA 3′ ends (3′ rny_ends) corresponding to 3′-to-5′ exoRNase start positions (bottom portion), 3′-to-5′ exoRNase stop positions (middle portion) and not associated with 3′-to-5′ exoRNase (top portion). Right bar plots: number of trimming starts (top) and stops (bottom) that correspond to 3′ my_ends, which were uniquely produced by PNPase and YhaM or produced by two different 3′-to-5′ exoRNases.
Fig. 3 RNase Y-generated RNAs are degraded by PNPase until secondary structures are encountered. a Upper panel: example of 3′ end coverage profiling from RNA sequencing. Middle panel: the RNA ends that were more abundant in the WT than in the Δmy strain are indicated below the coverage and depicted with purple arrowheads (3′ my ends). The RNA ends corresponding to the trimming start and stop positions of exoRNases are depicted with green and red arrowheads, respectively (see Supplementary Fig. 1). The 3′ my ends were paired to 3′-to-5′ exoRNase stop positions (Supplementary Data 2 and Fig. 2, bottom). Bottom panel: the 3′-to-5′ exoRNases (’pacman’ symbols) started trimming upon RNase Y (scissors) processing and stopped before the RNA termini. The 3′ my ends corresponding to the 3′-to-5′ exoRNase stop positions were compared with the exoRNase trimming start positions located downstream. The 3′-to-5′ exoRNase start position corresponds to the initial RNase Y processing position (Supplementary Data 3). b The logo, displaying the information (bits), was created from the alignment of all sequences surrounding the 19 identified PNPase trimming start positions. c Total coverage of SPy_0316 (encoding a putative transcriptional regulator) in WT obtained by RNA sequencing, and schematic representation of the locus. The grey rectangle indicates the region where the processing sites of RNase Y, PNPase and YhaM were identified. d 3′ end coverage of a portion of SPy_0316 in the WT, Δmy, YhaM deletion mutant (ΔyhaM) and PNPase deletion mutant (ΔpnpA) strains. The coverage scales are indicated between brackets. RNase Y processed the RNA after a G, corresponding to the detected PNPase trimming start positions. PNPase trimmed 34 nt of the SPy_0316 RNA 3′ end. This new RNA 3′ end was subsequently nibbled by YhaM. e RNA folding of the region 100 nt upstream of the 3′ my ends corresponding to YhaM trimming stop positions. YhaM started trimming after PNPase stopped, at the base of the stem loop structure, and consequently removed 2 nt from the RNA 3′ end. f Structure conservation at the 183 PNPase stop positions previously identified. The decrease in the minimum free energy (ΔG, kcal mol⁻¹) is indicative of RNA structures likely preventing PNPase from degrading the RNA.
structures, and we could not predict any structure. Here, we corresponded to the RNA 3'-end trimming start positions of PNPase and two YhaM trimming start positions corresponding to the RNA 3'-end trimming start positions of exoRNases (see Supplementary Fig. 2). The concerted action of these RNases is likely involved in the sRNA 3'-end processing17, YhaM also trims RNA 3'-ends after terminator regions and endoRNase (grey scissors) produced the 5'-ends of previously identified decay intermediates (Fig. 4b and Supplementary Fig. 3). Notably, it is known that PNPase usually releases 2- to 5-nt-long oligoribonucleotides, which are then further degraded by oligoRNase/nanoRNases25. For simplicity, in the following text, we write that PNPase degrades RNAs up to their termini.

Pairing 3' rny_ends and exoRNase trimming start positions. Six PNPase and two YhaM trimming start positions corresponded to the RNA 3'-ends produced by RNase Y (Fig. 2 in green and Fig. 4a). Therefore, the 3'-ends produced by RNase Y are targeted by these exoRNases. Their detection in this analysis suggests that a portion of the RNAs had not yet been subjected to 3'-to-5' exoRNase degradation.

PNPase trimming start positions were located at a G and probably corresponded to the RNA 3'-ends generated by RNase Y (Fig. 4b and Supplementary Fig. 3). For the rofA, Spy_sRNA482963, csrA and htrA transcripts, PNPase trimming starts corresponded to the 3'-ends of previously identified decay intermediates (Fig. 4b and Supplementary Fig. 3), which were degraded by PNPase up to the 5'-end of the decay intermediate17. The two other targets, namely, rpsU and the intergenic region between Spy_sRNA1696905 and Spy_sRNA1696905, were also likely degraded up to the RNA termini (5'-ends), as the PNPase stop positions were not detected (Supplementary Fig. 3). Notably, it is known that PNPase usually releases 2- to 5-nt-long oligoribonucleotides, which are then further degraded by oligoRNase/nanoRNases25. For simplicity, in the following text, we write that PNPase degrades RNAs up to their termini.

Pairing 5' rny_ends to exoRNase trimming start positions. We identified 190 RNA 5' rny_ends that could not be paired with 3' rny_ends, meaning that the RNA 3'-ends produced during the same processing events were not detected in the WT strain (Fig. 5a, Supplementary Data 1). In the comparisons described above, we observed that most of the 3' rny_ends were targeted by 3'-to-5' exoRNases. Therefore, 3'-to-5' exoRNases most likely also degraded the RNA fragment upstream of the RNase Y processing positions, which explains why we were not able to detect those RNAs.

To investigate this hypothesis, we paired the 5' rny_ends to 3'-to-5' exoRNase trimming start positions. In particular, we screened for trimming start positions within 10 nt upstream of the 190 RNA
5′ ends generated by RNase Y (Fig. 4a, Supplementary Data 5). We determined that 12% of the RNA 5′ ends were located in proximity of PNPase trimming start positions, indicating that the generated RNA fragment upstream of the processing site was degraded by this exoRNase (Fig. 5b; Supplementary Data 5). The observation that a majority of the PNPase trimming start positions were located up to 4 nt apart from the 5′ ends generated by RNase Y could be explained again by the activity of YhaM. The remaining 88% of the RNA 5′ ends were not associated with 3′-to-5′ exoRNase trimming start positions (Fig. 5b); therefore, the fate of the RNA fragment upstream of RNase Y processing could not be determined with our comparative analysis.

The identification of RNA 5′ and 3′ ends, the generation of which was RNase Y dependent, coupled with the comparison of exoRNase trimming start and stop positions, allowed us to provide an accurate and precise annotation of the RNase Y targetome. Overall, PNPase appears to be the major 3′-to-5′ exoRNase that degrades the RNA 3′ ends produced by RNase Y (Figs. 2 and 5). Interestingly, the PNPase-RNase Y double-deletion strain (ΔpnpAΔrny) grew slower than both the Δrny strain and the YhaM-RNase Y double-deletion strain (ΔyhaMΔrny) (Supplementary Fig. 4), which indicates that RNase Y and PNPase genetically interact and play an important role in bacterial physiology.

RNase Y produces short RNA fragments. To identify fragments with both ends produced by RNase Y (two cleavages in the same RNA molecule) (Fig. 6a), we calculated the distance between the 5′ rny_ends and the 3′ rny_ends (Fig. 6b). We observed that, by setting a maximum distance of 1000 nt, in a majority of the cases, the 5′ rny_ends and the 3′ rny_ends were 50–200 nt apart (Fig. 6b, Supplementary Data 6). Examples of these fragments were indeed detectable in the WT, but not in the Δrny strain, when examined by northern blot analyses (Fig. 6c and Supplementary Fig. 5). We further explored whether the 3′ rny_ends of these putative fragments were targeted by 3′-to-5′ exoRNases and noticed that 60% of them were trimmed by PNPase and/or YhaM (Supplementary Data 6, exemplified in Fig. 6d). The reason why these fragments were detectable (not degraded in the WT strain) remains unknown. We observed a decrease in the MFE at the fragment 3′ ends, indicating the presence of a stable structure (Supplementary Fig. 6a and b). Therefore, it is possible that the fragments were highly resistant to degradation because they were protected by this structure.

Among the RNA fragment 3′ ends generated by RNase Y, 23% were trimmed by YhaM. In some cases, we observed that the fragment present in the WT (Fig. 6c and Supplementary Fig. 5c–e) was not detected in the ΔyhaM strain, by neither northern blot analyses nor RNA sequencing (Supplementary Fig. 5c–e). We therefore wondered whether, in the absence of YhaM, these fragments were digested by PNPase or RNase R. However, in both the ΔpnpAΔyhaM and ΔrrnΔyhaM double-deletion strains, we did not detect the fragments by northern blot analyses (Supplementary Fig. 5c–e). It is possible that YhaM exerts a protective role by preventing further degradation of these fragments. Alternatively, the redundance between RNase R and PNPase or the involvement
of another RNA could explain the absence of the fragment in the ΔpnpAΔyhaM and ΔmrnΔyhaM strains.

RNase Y produces decay intermediates degraded by PNPase. As recently shown in *E. coli*, PNPase is actively involved in the degradation of small RNA fragments derived from transcripts targeted by sRNAs. We previously observed that PNPase rapidly degraded decay intermediates produced by endorRNases in *S. pyogenes*. The 5′ ends of these decay intermediates were identified as RNA ends that were more abundant in the ΔpnpA strain than in the WT strain (185 5′ ΔpnpA_ends) (Fig. 7a). Here, we observed a conserved G located upstream of the decay intermediate 5′ ends that was not observed at the decay intermediate 3′ ends (Fig. 7b). Based on the RNase Y cleavage signature inferred from our analysis, we propose that the decay intermediates harboring a G at the 5′ end (127 decay intermediates) were generated by RNase Y (Fig. 7a and b). Indeed, the decay intermediates, visualized by northern blot analyses, were detected in the ΔpnpA strain but not in the ΔpnpAΔrny strain, indicating that RNase Y was involved in their production (Fig. 7c and Supplementary Fig. 6). The decay intermediate 3′ ends could result from RNase Y processing—followed by exoRNase trimming, explaining the lack of G conservation—or from processing by another endorRNase (Fig. 7a and Supplementary Fig. 6).

Role of RNase Y and PNPase in the 5′ regulatory element degradation. A portion of the decay intermediates degraded by PNPase are derived from endorRNase processing of regulatory RNA 5′ UTRs (e.g., T-boxes and riboswitches). Here, we observed that some of these decay intermediates were produced by RNase Y (Fig. 8 and Supplementary Fig. 7). For example, RNase Y processing generated decay intermediates from the serS and thrS T-box RNA 5′ UTRs, providing access for PNPase to digest these RNAs further up to the 5′ end (Fig. 8a and Supplementary Fig. 7). For all the regulatory elements analysed, the decay intermediates accumulated in the ΔpnpA strain and were not present in the ΔpnpAΔrny strain, demonstrating that RNase Y is required for initiation of the decay of the premature terminated transcripts derived from the T-box and riboswitches (Fig. 8b and Supplementary Fig. 7).

Regulation of operon expression by RNase Y and PNPase. We examined the impact of RNase Y and PNPase on operon expression by studying the *rsmC-cdd-bmpA* operon, described below (Fig. 9), and the *tsf-rpsB* operon (described in Supplementary Fig. 8), which was strongly upregulated in Δrny (Supplementary Data 7).
Based on the comparative analysis, we concluded that the **rsmC-cdd-bmpA** operon was targeted by both RNase Y and PNPase (Fig. 9a). This operon encodes a 16 rRNA methyltransferase (**rsmC**), a cytidine deaminase involved in pyrimidine metabolism (**cdd**) and a lipoprotein (**bmpA**) (Fig. 9a). RNase Y processed the transcript between **cdd** and **bmpA**, and a PNPase trimming start position was located a few nucleotides upstream of the 5′ **rmy** _end_ (Fig. 9b). This observation indicates that the upstream fragment, corresponding to the **cdd** and **rsmC** ORFs, is subjected to PNPase degradation (Fig. 9b, Supplementary Data 5).

To establish the impact of RNase Y and PNPase activity, we assessed the stability of the different transcript isoforms of the **rsmC-cdd-bmpA** operon by northern blot analyses (Fig. 9d). The stability of the full-length **rsmC-cdd-bmpA** transcript (~2900 nt) was greatly increased in the Δ**rny** strain (Fig. 9d). The **rsmC-cdd** RNA isoform (~1700 nt), which was barely detectable in the WT, was stabilized in the Δ**rsmC-cdd**-bmpA strain (Fig. 9d). This result suggests that the **rsmC-cdd** isoform, arising from RNase Y processing, is rapidly degraded by PNPase. The **bmpA** isoform appeared to be more stable than the **rsmC-cdd** isoform in the WT strain, and the stability of this RNA was not affected by PNPase (Fig. 9e). In summary, the sequential activity of RNase Y and PNPase in the **cdd-bmpA** intergenic region ensures differential stability of the **rsmC-cdd** and **bmpA** RNAs.

**Fig. 9 The concerted action of RNase Y and PNPase is responsible for the differential RNA stability of the rsmC-cdd-bmpA operon.** a Schematic representation of the **rsmC-cdd-bmpA** operon; the location of the promoter, terminator and probes used in the northern blot analyses and predicted RNA sizes are shown. b 5′ and 3′ end RNA sequencing coverages in the WT and Δ**rmy** strains (for the 5′ end) and in the WT and Δ**pnpA** strains (for the 3′ end) of a region comprising portions of the **cdd** and **bmpA** ORFs and the intergenic region between the two genes. The coverage scale is indicated between brackets. The 5′ **rmy** _end_ and the PNPase trimming start position identified in the **cdd-bmpA** intergenic region are depicted with purple and green arrowheads, respectively. c RNA 5′_end_ in the **cdd-bmpA** intergenic region in the WT and Δ**rmy** strains, generated by RNase Y (scissors) and identified by primer extension analysis. The primer used is depicted with an arrow and binds upstream of the RNase Y processing and PNPase start positions (‘pacman’ symbol). The size of the expected cDNA product is indicated. Shown are the results of one representative primer extension experiment (n = 3). d, e The stability of **rsmC-bmpA**_cdd_, **cdd** and **bmpA** RNAs was determined by northern blot analyses up to 45 or 8 min after the addition of rifampicin in the WT and Δ**rny** strains or in the WT and Δ**pnpA** strains. Shown are the results of one representative northern blot analysis (n = 3). The 16 rRNA was used as a loading control. Source data are provided as a Source Data file.
Discussion

We have investigated the targetome of RNase Y in the human bacterial pathogen *S. pyogenes*, using a method based on sequencing analysis of RNA 5’ and 3’ ends. We observed that the identified RNA 5’ and 3’ ends harboured distinct features in terms of sequence and structure conservation. Therefore, to further explore the origin of the RNase Y-dependent RNA ends, we developed an RNA-seq based comparative approach allowing us to juxtapose those data with 3’-to-5’ exoRNAse targetomes. This method enabled us to determine that the detected RNA 5’ ends generated by RNase Y were usually not further trimmed. The 3’ ends, depending on RNase Y, resulted mostly from PNPase trimming and YhaM nibbling following RNase Y processing.

The analysis of the RNA 5’ ends generated by RNase Y revealed the presence of a G located just upstream of the processing sites for 87.4% of the targeted RNAs (Fig. 1f). The preference of RNase Y for this nucleotide at the processing site was first described in *S. aureus*, in which 58% of the processing sites were identified to be located upstream of a G10. A recent study from our laboratory demonstrated that RNase Y also requires a G to process *speB* mRNA, encoding a major virulence factor in *S. pyogenes*11. In light of the RNase Y cleavage signature identified in this study, it is likely that the G is required for the processing of substrates other than *speB* mRNA. Interestingly, in *B. subtilis*, a preferred sequence for RNase Y cleavage was not reported. Instead, this enzyme was shown to depend on the presence of RNA secondary structures around the processing site, as exemplified by the processing of several riboswitches9, but this observation was never validated genome-wide. Similarly, *S. aureus* RNase Y processes the *saePQRS* transcript only when a secondary structure is located 6 nt downstream of the cleavage site12. In our study, the analysis of the MFE did not reveal a secondary structure in proximity of the 190 RNA 5’ ends (Fig. 1f). However, we noticed at these positions an increase in the MFE, which is consistent with the fact that RNase Y cleaves in single-stranded regions.

Although we showed that RNase Y is involved in RNA decay, we believe that, due to the limited number of direct targets identified, RNase Y might not be the major initiator of mRNA decay in *S. pyogenes*. We report 320 processing positions (identified by 5’ and 3’ end sequencing), which is consistent with previous reports for the *S. aureus* and *B. subtilis* RNase Y proteins, describing ~100 processing positions (identified by 5’ end sequencing)10,18. In *S. aureus*, the limited impact of RNase Y on global transcript stability is consistent with the low number of detected direct targets16. In contrast, for RNase E, the major endoRNase initiating RNA decay in Gram-negative bacteria, ~22,000 processing positions were identified in *Salmonella enterica*9. A possible explanation for the high number of RNase E processing events detected in this bacterium is the absence of RNase J1, which is found mainly in Gram-positive bacteria2,27 and performs degradation from the 5’ end of the RNAs. In the present study, it is likely that we underestimated the number of RNase Y processing sites. First, because the method used relies on the detection of at least one RNA end (5’ or 3’), we did not identify RNase Y processing events when both generated ends were subsequently degraded by exoRNases (Fig. 10a). Second, the parameters used were stringent. However, the small RNase Y targetome found here is consistent with the fact that RNase Y is not essential under standard growth conditions (Supplementary Fig. 4). Comparison of the RNase Y and 3’-to-5’ exoRNAse targetomes revealed 127 additional RNase Y processing sites that could be identified only in the absence of PNPase (due to the detection of decay intermediates), thereby increasing the total number of RNase Y processing sites identified in this study to 447 (Fig. 7 and Supplementary Fig. 6). It is possible that additional RNase Y processing positions were not detected in the Δ*npA*

strand due to functional redundancy between PNPase and RNase R17. Previously, a global RNA stability study in *S. pyogenes*, performed under conditions mimicking infection, revealed that deletion of *rny* causes the stabilization of 98% of the transcripts28. It would be interesting to characterize the RNase Y targetome under these conditions and to evaluate whether the increase in transcript stability correlates with RNase Y activity. Overall, RNA degradation in *S. pyogenes* must rely on another endoRNase(s) in addition to RNase Y. For example, the RNase J1/J2 complex could play an important role in RNA decay in *S. pyogenes*, as both enzymes are essential in this bacterium3.
We demonstrated that RNase Y acts principally in concert with PNPase to degrade RNAs (Fig. 2; Supplementary Data 2 and 4). These two enzymes were shown to interact with each other in B. subtilis, although this interaction was not required for the degradation of all the studied targets\(^\text{59}\). When PNPase targeted the RNA fragments generated by RNase Y processing, we detected more PNPase trimming stop positions than start positions (Fig. 2). This result supports the observation that RNA 3′ ends produced by endoRNases are generally immediately degraded by PNPase and do not accumulate in the WT strain\(^\text{18,30}\). In our analysis, we could not detect RNase Y products entirely degraded by PNPase. Therefore, we suggest that the interplay between these two enzymes likely plays a broader role in RNA decay than that observed. By examining the PNPase targetome, we observed that some decay intermediates produced by RNase Y accumulated only in ΔpnpA (Fig. 7; Supplementary Fig. 6)\(^\text{17}\). Thus, the comparison of the ΔpnpA strain in the presence or absence of RNase Y led to the identification of additional RNase Y processing positions and a more representative picture of the interplay between these two enzymes.

Interestingly, we observed that the interplay of RNase Y and PNPase performs different functions in bacteria, such as decay of regulatory elements (e.g., riboswitches and T-boxes) and maturation of polycistronic mRNA. A role of RNase Y in the turnover of regulatory elements was previously observed in both B. subtilis and S. aureus\(^\text{6,10,13}\). Efficient removal of these regulatory elements from the bacteria might be important for the recycling of the ligand. In addition, RNase Y was previously shown to play an important role in the maturation of polycistronic transcripts by uncoupling the expression of genes encoded in the same operon\(^\text{16,32}\). Here, we show that the coordinated action of RNase Y processing in intergenic regions and subsequent degradation of one of the RNA products by PNPase results in differential decay of genes encoded within the same polycistronic mRNA, as exemplified for the rsncC-cdd-bmpA operon (Fig. 9).

As demonstrated previously, YhaM trims an average of 3 nt from most of the RNA 3′ ends generated by transcriptional terminators or by endoRNases\(^\text{17}\). Therefore, it was expected that YhaM would nibble the RNA 3′ ends produced by RNase Y. This activity complicated the identification of the original processing positions of RNase Y, as the G characterizing RNase Y activity was removed from the RNA 3′ end by YhaM (Supplementary Data 2 and 5). The example of SPy_0316 mRNA degradation illustrates that YhaM also targets RNAs already trimmed by other 3′-to-5′ exoRNases (Fig. 3d and Supplementary Fig. 2). A previous study in B. subtilis suggested that YhaM could shorten the single-stranded RNA tail necessary for the binding of PNPase and RNase R to their targets, thereby protecting the RNAs from degradation by these two enzymes\(^\text{16}\). The observation that the three RNA fragments analysed in this study (Supplementary Fig. 5e) were not present in the ΔyhaM strain suggests that this hypothesis could also be valid, at least in a few cases, in S. pyogenes.

The number of RNase R trimming positions detected in S. pyogenes was limited during exponential growth in rich medium\(^\text{17}\); therefore, it was expected that the interplay between RNase Y and RNase R would also be restricted under these conditions. It is possible that RNase R and RNase Y might act in concert in different conditions than the ones tested. Here, we focused on the fate of the RNA 3′ ends generated by RNase Y, and highlighted that these ends were in most cases further trimmed by the 3′-to-5′ exoRNases (Fig. 10b). In contrast to the detected RNA 3′ ends, the fate of the corresponding transcript 5′ ends is unknown. RNase J1, which degrades the RNAs in the 5′ to 3′ direction\(^\text{6}\), might be a possible candidate enzyme for the degradation of these RNAs. Alternatively, these undetected RNAs could be degraded by the 3′-to-5′ exoRNases up to the 5′ end produced by RNase Y, as exemplified by PNPase trimming of decay intermediates (Fig. 7 and Supplementary Fig. 6). In contrast to what we observed for the detected 3′ ends, 87.4% of the RNA 5′ ends corresponded to the original RNase Y processing positions (Fig. 10c). The remaining 12.6% of the RNA 5′ ends produced by RNase Y that were not located downstream of a G might correspond to RNase J1 trimming stop positions or might be generated by an endoRNase affected by RNase Y (Fig. 10c). The conditions leading to RNA 5′ end protection (detected 5′ ends) or degradation (undetected 5′ ends) have yet to be investigated. We believe that our method might facilitate the investigation of the concerted action of RNase Y and RNase J1, for instance, in Streptococcus mutans, in which none of these enzymes are essential.

To conclude, we have developed an RNA-seq based comparative approach that allows the genome-wide characterization of the specific RNase interplay and RNA degradation in vivo. We anticipate that this methodology will enable to elucidate diverse, parallel and interconnected, regulatory processes at the RNA level.

**Methods**

**Bacterial culture.** S. pyogenes SF370 (M1 GAS and isogenic gene deletion strains (Supplementary Table 1) were grown in THY medium (Todd Hewitt Broth (THB, Becton, Dickenson) supplemented with 0.2% yeast extract (Serva bacter)) at 37 °C with 5% CO\(_2\) without shaking\(^\text{19}\). TSA (trypticase soy agar, BD Difco) supplemented with 3% sheep blood was used as a solid medium.

**Growth curves.** Bacterial cultures were grown overnight (37 °C, 5% CO\(_2\)), diluted to an OD\(_{600\text{nm}}\) of 0.02 in 5 ml of medium and centrifuged at 3200 × g for 5 min. The resuspended pellet was used to inoculate flasks containing 25 ml of THY supplemented with 0.2% yeast extract. The growth was monitored by measuring the OD\(_{600\text{nm}}\) using a microplate reader (Biotek PowerWave XS2). Growth curve experiments were performed in triplicate and the standard error of the mean was calculated.

**RNA isolation.** The overnight S. pyogenes cultures were diluted 1:200 into 300 ml of fresh THY medium and grown to an OD\(_{600\text{nm}}\) of 0.25 corresponding to the mid-logarithmic growth phase. For the RNA stability assays, 250 µg/ml of rifampicin (Sigma-Aldrich) was added when the bacteria reached the mid-logarithmic phase of growth, and samples were taken either after 0, 5, 10, 20, 30 and 45 min or after 0, 1, 2, 4 and 8 min. The cells were rapidly harvested by mixing the 25 ml of cultures with 25 ml of 1:1 ice-cold acetone/ethanol solution and by centrifugation (3500 x g for 10 min at 4 °C). The pellets were thoroughly resuspended in 5 ml of TE buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, pH 8, 50 mM NaCl, 25% sucrose). The cells were lysed by adding 100 µl of lysis buffer (20 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 20% sucrose) supplemented with 2.5 mg/ml of lysozyme and 0.5 µg/ml of mutanolysin and incubated for 5 min on ice. The samples were mixed with the lysis executor buffer (2% sodium dodecyl sulfate (SDS), 1 mg/ml Proteusine K) incubated at 95 °C for 1.5 min. Seven hundred and fifty microlitres of TRIzol reagent (Life Technologies) were added to the samples, which were subsequently inverted three times. After incubation for 5 min at room temperature, 200 µl of chloroform were added and the samples were mixed by vortexing. The samples were then incubated at room temperature for 10 min prior centrifugation at 11,300 x g for 10 min at 4 °C. The upper aqueous phase (~700 µl) was gently collected and the RNAs were precipitated with ice-cold 100% isopropanol at a 1:1 ratio at −20 °C for at least 1 h. After centrifugation at 11,300 x g for 10 min at 4 °C, the RNA pellets were washed with 1 ml of 70% ethanol, air dried for 10 min and dissolved in autoclaved Milli-Q H\(_2\)O. RNA integrity was assessed on 1% agarose gels.

**RNA sequencing and analysis.** The RNA sequencing was performed in biological triplicates using the workflow previously published by our laboratory\(^\text{17}\). After treatment with TURBO DNase (Ambion), the RNA quality was assessed using a bioanalyzer system (Agilent 2100). Subsequently, 4.5 µg of RNA was depleted of rRNAs (Ribo-Zero rRNA Removal Kit (Bacteria)) and treated with 10 U of RppH (New England Biolabs) at 37 °C for 1 h 30 min to convert the 5′ monophosphate RNAs in 5′ monophosphate RNAs. The RNAs were purified using standard extraction with phenol/chloroform/isoamylalcohol (25:24:1, Roth) and precipitated using ice-cold ethanol. The obtained RNAs were treated with T4 polynucleotide kinase (Thermo Scientific) according to the manufacturer’s instructions to allow the subsequent ligation of the sequencing adapters. After a purification step using the RNA Clean & Concentratior kit (Zymo Research), the RNAs were fragmented (Covaris M220) in a microTUBE AFA Fiber Pre-Slit Snap-Cap tubes for 140 s.
cDNA libraries were prepared using the NEXTflex Small RNA Sequencing Kit v3 (Bioo Scientific) according to the manufacturer’s instructions until step 6. The purification step was performed using Agencourt AMPure XP beads (Beckman Coulter). The cDNA libraries were sequenced on a HiSeq3000 (paired-end mode, 2 × 151 bp) at the Max Planck-Genome-centre Cologne. The data were deposited in the European Nucleotide Archive (ENA) under the accession number PRJEB29486 (Supplementary Table 1) for 30 min at 65°C and subsequently incubated on ice for 1 min and the RNAs were reverse transcribed using 1U of SuperScript III Reverse Transcriptase (Invitrogen) in the presence of 1X first strand buffer (Invitrogen), 5 mM dithiothreitol (DTT) and 40 U of RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen), for 1 h at 55°C. After inhibition of the SuperScript III enzyme by incubation at 70°C for 15 min, the cDNAs were precipitated with ice-cold 100% ethanol at -20°C for 1 h and centrifuged at 20,000 × g for 4°C for 10 min. The pellet was washed with ice-cold 70% ethanol at 20,000 × g for 4°C for 10 min and resuspended in 5 µl of 2X RNA loading dye. The cDNA products were resolved on 10% polyacrylamide/8 M urea/TBE gels and the size of the products was estimated using the AFLP 30–300 bp ladder labelled according to the manufacturer’s instructions.

RNase Y processing sites. RNase cleavage positions were identified following the previously published procedure17. In brief, the genome coverage data was pre- filtered with a count per million (rpm) value ≥ 0.05 and only the RNA ends displaying a cpm ≥ 25 were further analysed. We carried out differential expression analysis of normalized 5‘ and 3‘ RNA reads by comparing the following datasets (triplicates): WT vs. Δrny and Δrny vs. Δrny:rnpy. RNase Y ends were identified using edger (v3.20.6)36,37 with absolute log2 fold-change (log2 FC) ≥ 1 and false discovery rate (FDR) < 0.05.

Comparison of the RNase Y and 3‘ to 5‘ exorNase targetomes. The RNase Y targetome (i.e., 5‘ and 3‘ rny:rnpy) was compared to the PNase, YhaM and RNase R targetomes (i.e., 3‘ to 5‘ exorNase trimming start and stop positions), which were previously identified (SRP149866)17 (see Supplementary Fig. 1). Different approaches were used to perform the comparison. When at least two consecutive positions were identified as 3‘ rny:rnpy ends in a window of 5 nt, the position with the highest ratio of proportion of ends between the WT strain and the Δrny strain was selected for further analysis17. First, the 3‘ rny:rnpy ends were compared with the PNase, YhaM and RNase R trimming stop positions that were located 5 nt upstream or 5 nt downstream of the 3‘ rny:rnpy ends (+/−5 nt shift) (Supplementary Data 2). Second, the 3‘ rny:rnpy ends corresponding to 3‘ to 5‘ exorNase trimming stop positions (in Supplementary Data 2) were compared with the trimming start positions located downstream (Supplementary Data 3). The maximum distance between these trimming stop and start positions was set at 200 nt for PNase and RNase R, and 10 nt for YhaM. For RNase R, by setting a maximum distance of 200 nt, we did not identify any trimming start position downstream of the 3‘ rny:rnpy ends that matched the RNase R stop positions. Third, the 3‘ rny:rnpy ends were compared to the PNase, YhaM and RNase R trimming start positions, allowing a +/-5 nt shift (Supplementary Data 4). Finally, the 3‘ rny:rnpy ends were paired to the PNase, YhaM and RNase R trimming start positions located 10 nt upstream (Supplementary Data 5). In addition, the 3‘ rny:rnpy and 5‘ rny:rnpy ends were compared by setting minimum and maximum distances of 40 and 1000 nt, respectively, between the ends (Supplementary Data 6). This comparison allowed the identification of RNA fragments produced by RNase Y. Python (v3.6.3) was used to perform all the comparisons described.

Sequence logo and folding. RNAfold (v2.4.3)38 was used to calculate the MFE (ΔG in kcal/mol) using a sliding window of 50 nt sequences, with 100 or 200 nt centred on the position of interest. The average MFE at each nucleotide was then calculated. WebLogolib (v3.5.0) was used to generate the sequence logos39, with sequences of 20 nt centred on the processing site with a GC-content of 38.5%. The plots were generated using Python (v3.6.3) and matplotlib (v2.0.1).

Northern blotting assays. For the short RNAs, 10 µg of RNA was separated on 8% or 10% polyacrylamide/8 M urea gels (Figs. 5, 6 and 7; Supplementary Figs. 5, 6, and 8b). The RNAs were transferred onto nylon membranes (HybondTM N+) and subsequently hybridized with iodine-stained DNA probes. The membranes were prehybridized in the Rapid-hyb buffer (GE Healthcare) for 1 h at 42°C. The membranes were washed and then with 1X SSC-0.1% SDS buffer for 15 min at 42°C, respectively. The membranes were hybridized at 42°C. The membranes were washed first with 5X SSC-0.1% SDS buffer and then with 1X SSC-0.1% SDS buffer for 15 min at 42°C, respectively. The RNA sizes were estimated using the RNA LadderDECMARKer (Ambion) or the PhiX174 DNA/Hinfl Marker (Fermentas).

For the long RNAs (Fig. 8), 20 µg of RNA was separated on a 1% agarose gel (1X MOPS (20 mM MOPS free acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0), 6.6% formaldehyde) in 1X MOPS buffer with 0.7% formaldehyde for 2 h at 80 V. The RNA bands were visualized using the Integrative Genomics Viewer (IGV)33,34. Differentially expressed genes were identified using featureCounts (v3.2.5)40 and edgeR (v3.20.6)41 with absolute log2 fold-change (log2 FC) ≥ 1 and false discovery rate (FDR) < 0.05.

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NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-020-15387-6 | www.nature.com/naturecommunications

ARTICLE

Received: 13 August 2019; Accepted: 29 February 2020; Published online: 27 March 2020

NATURE COMMUNICATIONS | (2020) 11:1587 | https://doi.org/10.1038/s41467-020-15387-6 | www.nature.com/naturecommunications
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Acknowledgements
We acknowledge Richard Reinhardt and Bruno Huettel from the Max Planck-Genom-Centre Cologne (MP-GC) for RNA sequencing of the cDNA libraries. We thank Johan Reimégard and Estelle Proux-Wera from SciLifeLab (Science for Life Laboratory, Sweden) and Knut Finstermeier and Davide Chiarugi from the Charpentier group for RNA sequencing data analysis support. We acknowledge Ciarán Condon and Petra Dersch for helpful discussions. The authors are grateful to the members of the Charpentier group for constructive discussions and critical reading of the paper. This work was supported by the Max Planck Society (E.C.), the Max Planck Foundation (E.C.), the Goran Gustafsson Foundation (Goran Gustafsson Prize to E.C.), the Alexander von Humboldt Foundation [AvH research fellowship to T.T.R.], the Kempe Foundation [E.C.], Umeå University [Dnr: 223-2386-10] and the Swedish Research Council [K2013-57X-21436-04-3] [E.C.]

Author contributions
L.B., A.-L.L., A.L.R. and E.C. designed the study; L.B., A.-L.L., A.L.R., T.T.R. and K.H. performed the experiments; L.B., A.-L.L., A.L.R., T.T.R. and R.A.-B. performed the data analysis; L.B., A.-L.L. and A.L.R. interpreted the data; A.L.R. and E.C. oversaw the project; L.B., A.-L.L., A.L.R. and E.C. wrote the paper. All the authors read, edited and approved the paper.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-15387-6.

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Peer review information Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

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