

The 5'-NAD cap of RNAIII modulates toxin production in *Staphylococcus aureus* isolates.

1 **Hector Gabriel Morales-Filloy¹, Yaqing Zhang¹, Gabriele Nübel¹, Shilpa Elizabeth George²,**
2 **Natalya Korn², Christiane Wolz², Andres Jäschke^{1*}**

3 ¹ Institute of Pharmacy and Molecular Biotechnology (IPMB), Heidelberg University, Heidelberg,
4 Germany

5 ² Interfaculty Institute of Microbiology and Infection Medicine, University of Tübingen, Tübingen,
6 Germany

7 *** Correspondence:**

8 Prof. Dr. Andres Jäschke

9 jaeschke@uni-hd.de

10 **1 Abstract**

11 Nicotinamide adenosine dinucleotide (NAD) has been found to be covalently attached to the 5'-
12 ends of specific RNAs in many different organisms, but the physiological consequences of this
13 modification are largely unknown. Here we report the occurrence of several NAD-RNAs in the
14 opportunistic human pathogen *Staphylococcus aureus*. Most prominently, RNAIII, a central
15 quorum-sensing regulator of this bacterium's physiology, was found to be 5'-NAD-capped to a
16 significant extent. NAD incorporation efficiency into RNAIII was found to depend *in vivo* on the -
17 1 position of the P3 promoter. Reduction of RNAIII's NAD content led to a decreased expression
18 of alpha- and delta-toxins, resulting in reduced cytotoxicity of the modified strains. These effects
19 to not seem to be due to changes in RNAIII's secondary structure upon NAD attachment, as
20 indicated by largely unaltered patterns in *in vitro* chemical probing experiments. Our study
21 represents a large step towards establishing a biological function of the 5'-NAD cap, which for
22 RNAIII in *S. aureus* is to modulate the expression of virulence factors.

23 **2 Importance**

24 Numerous organisms, including bacteria, are endowed with a 5'-NAD cap in specific RNAs. While
25 the presence of the 5'-NAD cap modulates the stability of the modified RNA species, a significant
26 biological function and phenotype have not been assigned so far. Here, we show the presence of
27 a 5'-NAD cap in RNAIII from *S. aureus*, a dual-function regulatory RNA involved in quorum-sensing
28 processes and regulation of virulence factor expression. We also demonstrate that altering the
29 natural NAD modification ratio of RNAIII leads to a decrease in exotoxin production, thereby
30 modulating bacterium's virulence. Our work unveils a new layer of regulation of RNAIII and the
31 *agr* system that might be linked to the redox state of the NAD molecule in the cell.

32 **3 Introduction**

33 The discovery of the nicotinamide adenosine dinucleotide (NAD) bacterial 5'-cap in regulatory
34 RNAs in *Escherichia coli* (Cahová, Winz, Höfer, Nübel, & Jäschke, 2015) challenged a long-standing
35 dogma. To date, the 5'-NAD cap has been reported in Gram-negative and Gram-positive (*Bacillus*

36 *subtilis* (Frindert et al., 2018)) bacteria as well as in eukaryotes such as *Saccharomyces cerevisiae*
37 (Kellner et al., 2014; Walters et al., 2017), the mammalian cell line HEK293T (Jiao et al., 2017) and
38 plants from the genus *Arabidopsis* (Wang et al., 2019; Zhang et al., 2019). This novel nucleotide
39 modification in RNA appears to be present in most organisms if not ubiquitous. However, no
40 NAD-modified RNA has been reported in pathogens yet. The opportunistic pathogen
41 *Staphylococcus aureus* is a gram-positive bacterium responsible for the majority of nosocomial
42 infections (David & Daum, 2010). After the invasion of host tissue, *S. aureus* can cause
43 bacteremia, sepsis, endocarditis, and toxic shock syndrome (Lowy, 1998). The pathogenicity of *S.*
44 *aureus* is strongly related to the expression of numerous virulence factors, many of which are
45 under the control of the *agr* quorum-sensing system (Bronesky et al., 2016; Quave et al., 2016;
46 Traber et al., 2008). The *agr* locus harbours two promoters (P2 and P3) located back to back in
47 the chromosome (Janzon, Lofdahl, & Arvidson, 1989; Peng, Novick, Kreiswirth, Kornblum, &
48 Schlievert, 1988) (Figure 1A). P3 encodes a regulatory RNA (RNAIII) that additionally contains a
49 small open reading frame (ORF) for delta-toxin (Hld) (Janzon & Arvidson, 1990; Novick et al.,
50 1993), whereas P2 regulates the transcription of a polycistronic RNA (RNAII) that encodes the Agr
51 proteins Agr A/B/C/D (Figure 1A) (Novick et al., 1995). With this quorum-sensing mechanism, *S.*
52 *aureus* senses the cell density based on the extracellular concentration of the self-produced
53 autoinducer peptide (AIP) (Recsei et al., 1986). The AIP binds to the AgrC histidine kinase
54 membrane receptor and activates AgrA regulator by phosphorylation (Figure 1A). AgrA~P
55 activates transcription of the P2 and P3 promoters, producing RNAII and RNAIII, respectively
56 (Figure 1A) (Bronesky et al., 2016; Koenig, Ray, Maleki, Smeltzer, & Hurlburt, 2004). AgrA~P
57 furthermore activates transcription of phenol-soluble modulins (*psmA* and *psmB*) (Queck et al.,
58 2008), which are small peptides with surfactant activity that contribute to cell membrane
59 disruption and therefore to *S. aureus* cytotoxicity (M. Li et al., 2009; Peschel & Otto, 2013).
60 RNAIII plays a crucial bifunctional role in the switch from the adhesion phase towards invasion
61 (Bronesky et al., 2016; Koenig et al., 2004), which ultimately determines the infective behaviour
62 of the bacterium. On the one hand, it inhibits translation of *rot* mRNA, which encodes for a
63 repressor (Rot) that blocks the transcription of several toxins (Boisset et al., 2007; Geisinger,
64 Adhikari, Jin, Ross, & Novick, 2006; Said-Salim et al., 2003). Also, RNAIII represses the expression
65 of surface proteins, e.g., protein A or coagulase through an antisense mechanism (Boisset et al.,
66 2007; Bronesky et al., 2016; Huntzinger et al., 2005). On the other hand, it activates the
67 production of several hemolytic toxins, such as alpha-toxin (Hla) (Morfeldt, Taylor, von Gabain,
68 & Arvidson, 1995) and the self-encoded Hld.
69 The addition of the canonical N7-methyl guanosine (m⁷G) cap to the 5'-ends of eukaryotic mRNAs
70 proceeds co-transcriptionally once the transcript has reached a length of ~25 nucleotides. The
71 NAD cap is, however, incorporated in a different way in prokaryotes and probably in all
72 organisms: the ubiquitous redox coenzyme NAD is incorporated as the very first nucleotide (*ab*
73 *initio*) into RNA during transcription initiation by the RNA polymerase, acting as a non-canonical
74 initiating nucleotide (NCIN) that competes with ATP at A-initiating promoters (Bird et al., 2016;
75 Frindert et al., 2018). Thus, transcription appears to constitute the primary source of 5'-NAD-
76 RNAs in the cell. NAD incorporation into RNA has furthermore been demonstrated to strongly
77 depend on the promoter sequence around the transcription start site (TSS) (Frindert et al., 2018;
78 Vvedenskaya et al., 2018). In *E. coli* and *B. subtilis*, the -1 position turned out to be the most
79 important one for NAD incorporation, likely due to pseudo-base-pairing between the

80 nicotinamide moiety of NAD and the nucleotides at the -1 position of the DNA non-coding strand
81 (thymidine (T) and cytidine (C)) (Vvedenskaya et al., 2018).

82 The functions of the NAD cap are not yet fully understood, but in prokaryotes, it seems to confer
83 stability to RNA. In *E. coli*, the NAD cap protects RNA against 5'-processing by the RNA-
84 pyrophosphohydrolase (RppH) and thereby against RNaseE degradation (Cahová et al., 2015),
85 whereas in *B. subtilis*, it stabilises RNA against exoribonucleolytic attack by RNase J1 (Frindert et
86 al., 2018). Furthermore, *E. coli* possesses an NAD-decapping machinery based on the Nudix
87 phosphohydrolase NudC (Frick & Bessman, 1995), which efficiently hydrolyses the NAD cap and
88 leaves a 5'-monophosphate RNA (p-RNA) (Cahová et al., 2015). Despite Nudix hydrolase motifs
89 being present in several enzymes of *S. aureus*, no NudC ortholog has been found to date. In
90 mammalian cells, the NAD-decapping machinery works in a very different way. The DXO enzymes
91 first remove the entire NAD cap from an mRNA (releasing NAD and p-RNA) and then proceed
92 with exonucleolytic mRNA decay (Jiao et al., 2017).

93 In this study, we reveal by NAD captureSeq the presence of NAD-capped mRNAs and regulatory
94 RNAs in the opportunistic pathogen *S. aureus*. The highly expressed, multifunctional, protein-
95 coding, and regulatory RNA, RNAIII, is found to bear the NAD cap. Additionally, we study the
96 importance of the -1 and +1 positions of the P3 promoter for the incorporation of NAD into RNAIII
97 *in vivo*. Finally, we also investigate the consequences of changing RNAIII's NAD modification ratio
98 on *S. aureus* virulence. The obtained results are interpreted in the context of the current
99 knowledge of *S. aureus*'s pathobiology.

100 **4 Results**

101 **4.1 *S. aureus* possesses NAD-capped RNAs.**

102 To detect and quantify NAD-capped RNAs in *S. aureus*, Liquid Chromatography coupled with Mass
103 Spectrometry (LC-MS) analysis of total RNA samples was carried out. Two RNA samples from *S.*
104 *aureus* ATCC 25923 were washed extensively to remove contaminating free NAD before
105 enzymatic treatment with *E. coli* NudC and alkaline phosphatase. Upon this treatment, NAD-RNAs
106 will liberate N-ribosyl nicotinamide (r-NA) that can be sensitively detected by MS analysis. The
107 results revealed an average amount of 25.25 ± 1.64 fmol of NAD-RNA per microgram of RNA,
108 which corresponds to an estimated amount of 897 ± 58 NAD-RNA molecules per cell (Table S1)
109 (Chen, Kowtoniuk, Agarwal, Shen, & Liu, 2009). These results indicated the presence of NAD-
110 capped RNAs in *S. aureus*.

111 In order to identify NAD-capped RNAs, NAD captureSeq was performed (Winz et al., 2017) Total
112 RNA from *S. aureus* ATCC 25923 (isolated at the late exponential phase) was treated per duplicate
113 either with ADP-ribosyl cyclase (ADPRC), allowing selective biotinylation of NAD-RNA, or mock-
114 treated (minus ADPRC control). The captured RNAs were reverse transcribed and PCR-amplified
115 to generate a DNA library for Illumina sequencing (Figure S1A). The distribution of the obtained
116 normalised mapped reads revealed 96.87% mRNAs, 2.46% tRNAs, and 0.68% rRNAs with
117 enrichment values up to 50-fold. Enriched hits ($P < 0.05$, log₂-fold change > 2 , base mean > 10)
118 were visualised with the Integrated Genome Browser (Nicol, Helt, Blanchard, Raja, & Loraine,
119 2009) to identify reads that clustered at the 5'-ends of transcripts initiating with adenosine (+1A)
120 (Figure 1B). 67.7% of the initial enriched hits (21 out of 31) had these properties (Table S2) and

121 are indicated as green dots in the upper right sector of the scatter plot (Figure 1B). The TSS
122 analysis of the enriched hits showed T (61.9%) or A (28.6%) at position -1 of the promoter
123 predominantly. G or T usually occupy the +2 position with the same occurrence (33.3%) whereas
124 +3 position is dominated by A (57.1%). The predominant nucleobase at position -3 and -2 of the
125 promoter was A (71.4% and 47.6% respectively, Figure S1B). Most enriched RNAs turned out to
126 be mRNA 5'-fragments (Table S2). However, the most abundant and the most enriched hit was
127 the bifunctional RNAIII (*hld*/RNAIII gene, Figure 1B, green star). Notably, the other transcript from
128 the agr locus, RNAII, which has a similar promoter and is reported to also initiate with A (Novick
129 et al., 1995; Reynolds & Wigneshweraraj, 2011), was not enriched at all (Figure 1B, red star).

130 **4.2 The multifaceted regulatory RNAIII contains a 5'-NAD cap.**

131 The obtained reads for *hld*/RNAIII gene mapped clearly on its 5'-UTR (Figure 1C). As NAD
132 captureSeq does not use a fragmentation step and size-selects (at the very end, after PCR
133 amplification) amplicons that correspond to RNAs < ~170 nt, information about full-length mRNA
134 species cannot be obtained from these data. To quantitatively investigate the length distribution
135 of NAD-RNAIII, qPCR was performed on the enriched cDNA obtained directly from reverse
136 transcription, before any amplification or size fractionation using gene-specific primers (Table S3)
137 targeting different regions of RNAIII, thereby comparing the cDNA from the NAD captureSeq
138 sample with the mock-treated negative control. The qPCR data revealed the most substantial
139 enrichment for the 5'-end region, but a significant presence and enrichment of full-length RNAIII
140 (Figure 1D). Northern blot analysis (which is independent of the nature of the 5'-end) indicated
141 that overall, RNAIII in *S. aureus* is predominantly full-length (Figure S1C).
142 To confirm that the NAD is covalently linked to RNAIII's 5'-end, RNAIII (enriched) and 5SrRNA
143 (non-enriched, negative control) were specifically isolated from *S. aureus* total RNA by pull-down
144 using biotinylated complementary oligonucleotides. Afterwards, RNAs were quantified, treated
145 with NudC and alkaline phosphatase, and the small-molecule fraction analysed by LC-MS (Figure
146 1E). This analysis revealed an intensive peak corresponding to nicotinamide ($m/z = 122.81$,
147 originating from r-NA, $m/z = 254.94$) in the case of RNAIII, whereas for 5SRNA the same peak
148 could only be detected at 24-fold higher input RNA concentration (Figure 1F). The NAD
149 modification ratio, i.e., the percentage of molecules of that RNA species that carry the 5'-NAD-
150 modification, of RNAIII and 5SRNA was calculated as 36.20% and 0.25%, respectively (Figure 1F).

151 **4.3 Guanosine at position -1 of the P3 promoter increases NAD incorporation into RNAIII in** 152 **vivo.**

153 To investigate the biological consequences of 5'-NAD modification in vivo, *S. aureus* strains that
154 differ strongly in their content of 5'-NAD-RNAIII, with as little as possible differences in the
155 transcriptome, had to be generated. Toward this end, we complemented a strain devoid of the
156 *hld*/RNAIII gene (*S. aureus* HG001 Δ RNAIII) with plasmids based on the shuttle vector pCG-246
157 (Helle et al., 2011). These plasmids carried the *hld*/RNAIII gene behind different promoters: pCG-
158 P3 carried the native P3 promoter, while pCG-P3(-1G) had a mutated version of this promoter (-
159 1T to -1G). To unravel whether the lack of NAD captureSeq enrichment of RNAII was due to
160 properties of its promoter, construct pCG-P2 was generated in which the P2 promoter controlled

161 the RNAIII native sequence (Figure 2A). Unlike the previously used *S. aureus* ATCC 25923, in the
162 generated strains the *hld*/RNAIII gene is located on a plasmid.

163 To detect variations in RNAIII's modification ratio, we analysed RNAIII pulled down from total
164 RNA from the RNAIII-complemented *S. aureus* strains by acryloaminophenyl boronic acid (APB)
165 gel electrophoresis that separates 5'-NAD-RNA from 5'-p and 5'-ppp-RNA, combined with
166 Northern blot (Alwine, Kemp, & Stark, 1977; Igloi & Kossel, 1985; Nübel, Sorgenfrei, & Jäschke,
167 2017). As the full-length RNAIII was too long for efficient separation and precise quantification
168 (Figure S1D), a pre-treatment with a designed DNAzyme was introduced which cleaved RNAIII to
169 yield 125 nt 5'-terminal fragments (Figure 2B). The strains containing pCG-P2 showed no NAD
170 incorporation into the RNAIII transcript at all, whereas RNAIII in pCG-P3 and pCG-P3(-1G)-
171 transformed strains showed an average NAD-modification ratio of $9.82 \pm 0.15\%$ and $24.91 \pm$
172 0.37% , respectively (Figure 2C). Indeed, *S. aureus* pCG-P(-1G) was found to have a significantly
173 higher amount of NAD-RNAIII than *S. aureus* pCG-P3 (*t*-test; $P < 0.0001$), demonstrating the
174 importance of the -1 mutation on NAD incorporation efficiency, which confirms findings in other
175 microorganisms (Frindert et al., 2018; Vvedenskaya et al., 2018). In all RNAIII samples, the slower-
176 migrating band disappeared upon treatment with *E. coli* NudC, further supporting the nature of
177 the 5'-modification as NAD (Figure 2C).

178 To test whether this different NAD modification percentage in the three RNAIII constructs (pCG-
179 P3, pCG-P2, pCG-P3(-1G)) is also reflected in NAD captureSeq enrichment, we had to modify the
180 analysis to allow quantitative comparisons between different RNA species by adding pure NAD-
181 RNA spike-in controls of five different lengths as internal standards (IS; 61, 104, 205, 302 and 400
182 nt, Table S4) to allow for normalization. The NGS results revealed that the NAD capture efficiency
183 of the IS between the samples was very similar (Figure 2D, one-way ANOVA; $P = 0.8617$). When
184 comparing the *hld*/RNAIII enrichment in the different *S. aureus* strains, pCG-P3(-1G)
185 ($\log_2\text{FoldChange} = 3.57$) showed higher enrichment than *S. aureus* pCG-P3 ($\log_2\text{FoldChange} =$
186 2.77). In the case of *S. aureus* pCG-P2, no enrichment was found for *hld*/RNAIII ($\log_2\text{FoldChange}$
187 $= 0.48$). Thus, the NGS data support the results obtained by APBgel - Northern blot analysis and
188 confirm that a -1G in the P3 promoter significantly enhances NAD incorporation into RNAIII (*t*-
189 test; $P = 0.036$, Figure 2E). Analysis of the NAD captureSeq data from the three mutant strains
190 revealed a similar pattern of NADylation as in the ATCC 25923 wild type strain (Table S5, Table
191 S6 and Table S7). For pCG-P3 we obtained 11 enriched hits (4 of them common with the wild
192 type), in pCG-P3(-1G) 12 (4 commons), and pCG-P2 6 (3 commons).

193 **4.4 The TSS of the P2 promoter is guanosine.**

194 We were puzzled by the observation that RNAIII from strain pCG-P2 seemed to contain no NAD
195 at all. Sanger sequencing data after cRT-PCR (Slomovic & Schuster, 2013), however, provided a
196 straightforward explanation: the transcription start site of the P2 promoter was a G instead of
197 the previously reported A (Novick et al., 1995; Reynolds & Wigneshweraraj, 2011), and NAD does
198 not compete with GTP in transcription initiation. The analysis of 96 sequencing reactions (96
199 single colonies from each strain) from *S. aureus* pCG-P3 and pCG-P2 yielded well-defined 3' and
200 5' ends of RNAIII, where the RNAIII's first nucleotide was +1A when transcribed from the P3
201 promoter, and +1G in the case of the P2 promoter (Figure 2F). A +1G was also found in RNAII (a

202 natural product of P2 promoter) from *S. aureus* HG001 wild type strain after the analysis of 10
203 Sanger sequencing reactions (10 single colonies, Figure 2F).

204 **4.5 The 5'-NAD cap of RNAIII affects Hla and Hld expression levels and thereby *S. aureus*'s** 205 **cytotoxicity.**

206 To study the accessibility of its NAD-5'-end, ³²P-body-labelled pure NAD-RNAIII was subjected to
207 *in vitro* cleavage with *E. coli* NudC. After 20 min of incubation at 37 °C, NudC had decapped ~65%
208 of the RNAs, and this value did not change upon further incubation (up to 1 hour, Figure 3A). An
209 additional unfolding-folding cycle, followed by the addition of fresh NudC, increased decapping
210 to ~83% (Figure 3A), suggesting that a fraction of NAD-RNAIII exists in a structure that is not
211 susceptible to NudC cleavage, e.g., with the 5'-end involved in a double-strand (Höfer, Abele,
212 Schlotthauer, & Jäschke, 2016).

213 To test the effect of the promoter mutations on the biosynthesis rate of RNAIII, a qPCR assay was
214 performed with cDNA from the different *S. aureus* strains (total RNA harvested in late
215 exponential phase and reverse transcribed). The assay revealed no significant difference in RNAIII
216 content between pCG-P3 and pCG-P3(-1G) strains (*t*-test; *P* = 0.1221, Figure 3B). On the other
217 hand, pCG-P2 showed a significantly lower amount of RNAIII than pCG-P3 (*t*-test; *P* = 0.0276,
218 Figure 3B).

219 Strains pCG-P3 and P3(-1G) differ only in one nucleotide, the -1 position of the P3 promoter,
220 which leads to the same RNAIII transcript, just with different NAD modification levels. To test the
221 influence of this single mutation on the transcriptome; RNA-Seq was performed on total RNA
222 from both strains. Their transcriptomes were found to be very similar to each other, with only 4
223 upregulated genes (*splA*, *splB*, *splC*, *splD*, all of them coding for serine proteases, Table S8) and 2
224 downregulated genes (*spa*, an uncharacterized gene with homology to *ssaA*, Table S8) in *S.*
225 *aureus* pCG-P3(-1G). The *splABCD* operon is known to be activated via RNAIII, whereas *spa* is
226 known to be inhibited by RNAIII (Queck et al., 2008). Thus, the higher NADylation in pCG-P3(-1G)
227 might enhance RNAIII's inhibitory activity. Interestingly, the prototypic RNAIII target gene, *hla*,
228 was neither upregulated nor downregulated in the RNA-Seq data. Hence, we speculated that
229 NADylation of RNAIII may influence the translation of Hla, and therefore, an analysis of Hla
230 abundance in culture supernatants was conducted. The Hla obtained in culture filtrates of *S.*
231 *aureus* pCG-P3(-1G) was significantly lower than in *S. aureus* pCG-P3 in late exponential phase
232 cultures (*t*-test; *P* = 0.0148, Figure 3C and Figure S1E). Thus, 5'-NAD capping of RNAIII might
233 modulate the interaction between the 5'-UTRs of RNAIII and *hla* mRNA as well, leading to a lower
234 translation of the latter RNA.

235 We next analysed whether NADylation impacts the translation efficiency of the RNAIII-encoded
236 Hld. According to LC-MS of culture filtrates, half the amount of Hld was found in pCG-P3(-1G)
237 (with the higher amount of 5'-NAD cap) compared to pCG-P3 (Figure 3C, Figure S1F). This finding
238 indicates that RNAIII's 5'-NAD cap might impair *hld* mRNA translation. A decreased production of
239 two major hemolytic toxins, Hla and Hld, should lead to a less cytotoxic *S. aureus* strain. Indeed,
240 an assay with *S. aureus* pCG-P3 and pCG-P3(-1G) culture supernatants in a human THP1
241 macrophage line (Figure 3D) revealed a significantly reduced cytotoxicity of pCG-P3(-1G)
242 compared to pCG-P3 (Tukey's multiple comparisons-test; *P* = 0.0004).

243 **4.6 Hld's Shine-Dalgarno sequence is accessible *in vitro* regardless of the presence of a 5'-** 244 **NAD cap.**

245 In order to analyse whether 5'-NAD capping of RNAIII affects the secondary structure of its 5'-
246 domain, we performed Selective 2'-Hydroxyl Acylation and Primer Extension (SHAPE) (Weeks &
247 Mauger, 2011; Wilkinson, Merino, & Weeks, 2006). SHAPE is an approach to probe the structure
248 of every nucleotide within an RNA simultaneously. The SHAPE reagent chemically modifies
249 (acylates) the 2'-OH position of flexible nucleotides (Wilkinson et al., 2006). A subsequent DNA
250 synthesis by reverse transcriptase stops one nucleotide before the position of a modified 2'-
251 ribose position, reporting the site of a SHAPE modification in the RNA (Weeks & Mauger, 2011;
252 Wilkinson et al., 2006). 1-methyl-7-nitro-isatoic anhydride (1M7) was chosen as SHAPE reagent
253 (Mortimer & Weeks, 2007). To determine whether RNAIII's secondary structure is modulated by
254 the nature of its 5'-end, pure 5'-NAD-RNAIII and ppp-RNAIII were required. Pure full-length (514
255 nt) NAD-RNAIII could not be prepared, as no method exists for its preparative separation from
256 full-length ppp-RNAIII. Therefore, we first probed a shorter version of RNAIII consisting of
257 nucleotides 1 to 113, designated as RNAIII leader, which could be prepared by *in vitro*
258 transcription and APB-gel purification as pure NAD-capped (NAD-RNAIII leader), or pure
259 triphosphorylated version (ppp-RNAIII leader). To exclude the possibility that the truncated
260 RNAIII leader sequences fold differently than the full-length RNAIII, we compared in a second
261 step pure full-length ppp-RNAIII with impure full-length NAD-RNA (containing NAD-RNA and ppp-
262 RNA ~ 1:1). The SHAPE data showed very similar nucleotide reactivity profiles for NAD-RNAIII
263 leader and ppp-RNAIII leader (Figure 4A, Figure 4B and Figure S1G), indicating an accessible SD
264 sequence regardless of their 5'-end modification.

265 When shaping full-length ppp-RNAIII and the ppp-/NAD-RNAIII mixture, both the overall SHAPE
266 reactivity profile and SD's accessibility were similar to that of the ppp-RNAIII leader (Figure 4C,
267 Figure 4D)

268 **5 Discussion**

269 **5.1 A short, regulatory, and protein-coding NAD-capped RNA in *S. aureus*.**

270 In this study, we have demonstrated the existence of 5'-NAD-capped RNAs in the opportunistic
271 pathogen *S. aureus*. Since the discovery of the prokaryotic NAD cap in 2015, the presence of NAD-
272 RNAs in many different organisms has been confirmed, and this modification might be
273 ubiquitous. Nevertheless, the biological function of the 5'-NAD cap in prokaryotes is still unclear.
274 In the case of *B. subtilis*, also a gram-positive bacterium, the NAD cap stabilises RNA against
275 exoribonucleolytic attack by RNase J1 *in vitro* (Frindert et al., 2018). As in other organisms
276 (Cahová et al., 2015; Frindert et al., 2018), several mRNAs were found to be enriched in the NAD
277 captureSeq of *S. aureus* ATCC 25923. Most of these mRNAs encode proteins involved in different
278 cellular processes: redox reactions, membrane transport, biosynthesis-related enzymes,
279 phosphatases, kinases, and hydrolases (Table S2). Similar classes were observed in the *S. aureus*
280 mutant strains (pCG-P3, pCG-P3(-1G) and pCG-P2), with several hits common with the wild type
281 ATCC 25923 strain, i.e., NADP reductases, synthetases, and transcription factors.

282 Interestingly, the mutant-specific hits encoded for proteins that were similar to the ones found
283 in the wild type strain, especially reductases such as the peptide methionine sulfoxide reductase

284 or redox enzymes like the superoxide dismutase (Table S5, Table S6 and Table S7). Of particular
285 interest is the enrichment of mRNAs that encode for NAD- NADP-utilizing enzymes (2-
286 dehydropantoate 2-reductase, L-lactate dehydrogenase). A similar observation has been made
287 in *B. subtilis*, where L-threonine 3-dehydrogenase (*tdh*) was enriched (Frindert et al., 2018). These
288 findings underpin the possibility that some NAD-mRNAs might act as a cofactor of their encoded
289 enzyme. Moreover, the NAD cap could act as an enzyme-binding site to its mRNA as an additional
290 regulatory mechanism (Jaschke, Hofer, Nubel, & Frindert, 2016). However, the existence of this
291 feedback mechanism still needs to be demonstrated empirically.

292 The *hld*/RNAIII gene was by far the most enriched of all hits. The results showed a higher NAD
293 modification level of RNAIII in the wild type strain (~36%) compared to pCG-P3 (~10%). This
294 phenomenon might be due to the different *S. aureus* strains used (ATCC 25923 and plasmid-
295 carrying HG001) although these values were obtained by different approaches (LC-MS vs
296 Northern blot). The variation in NAD content is supported by a different *hld*/RNAIII enrichment
297 in NAD captureSeq between both strains after, with a higher value obtained in ATCC 25923 (Table
298 S2, Table S5). Furthermore, a distinct NAD modification level on RNAIII could be the cause of
299 some differences in the infective behaviour between the strains. Nevertheless, RNAIII is still
300 strongly NAD-modified whenever transcribed by P3 promoter, and this together with its high
301 expression level, its role as a crucial intracellular effector of the quorum-sensing system, and the
302 additional presence of an embedded ORF for the *hld* gene (Figure 1A) make RNAIII a promising
303 candidate for unveiling a biological function of the NAD cap.

304 **5.2 The P3 promoter acts as a driver of NAD incorporation.**

305 The -1 position of the P3 promoter is found to modulate the incorporation of NAD into the
306 nascent RNAIII. We also tested the effect of a promoter exchange on NAD transcriptional
307 incorporation. The other promoter of the *agr* locus, P2, seems not to be prone to introduce NAD
308 into RNAII (Log2FC = -1.65 in NAD captureSeq). These two promoters share several
309 characteristics: phosphorylated AgrA activates transcription of both, they have a suboptimal
310 interspace region between the -35 and -10 boxes, and both have been predicted to initiate with
311 a +1A (Novick et al., 1995; Reynolds & Wigneshweraraj, 2011). Placing the P2 promoter upstream
312 of the RNAIII sequence led to the total abolishment of NAD incorporation into RNAIII (Figure 2C
313 and Figure 2E). The unexpected revelation of the TSS of the P2 promoter being G instead of A
314 (Figure 2E) provided us with an additional “NAD zero” control in our experiments, constituting a
315 transcript that differed only in one nucleotide from the native one but was devoid of any 5'-NAD.
316 This control further demonstrated that NAD captureSeq is not biased towards highly expressed
317 RNAs unless they bear the NAD cap. Moreover, it also confirmed the reliability of the APBgel-
318 Northern blot combination as a tool for analysing NAD-capped RNAs.

319 **5.3 NAD-RNAIII as a modulator of *S. aureus* physiology.**

320 For validating the function of RNAIII's NAD cap, it was essential that the RNAIII expression of the
321 different *S. aureus* strains was not altered by the changes introduced in the promoter sequence.
322 qPCR analysis confirmed the lack of bias in RNAIII content between *S. aureus* pCG-P3 and pCG-
323 P3(-1G) (Figure 3B). This experiment also showed lower RNAIII levels in the pCG-P2 strain (Figure
324 3B), which suggest that the P2 promoter is less active than the P3 promoter in these experiments.

325 This finding is in agreement with the fact that *in vitro*, AgrA-mediated activation of transcription
326 is more prominent at P3 than at P2 (Reynolds & Wigneshweraraj, 2011). On the other hand, a
327 recent *in vivo* study reported AgrA~P affinity to P2 to be higher than to P3, previously assessed
328 by Koenig et al. (2004), and this differential affinity being crucial for the *agr* positive feedback
329 loop (Garcia-Betancur et al., 2017).

330 According to transcriptome sequencing, the higher NAD modification ratio of RNAIII in *S. aureus*
331 pCG-P3(-1G) (Figure 2C and Figure 2E) appears to downregulate the transcription of *spa* (protein
332 A) and *ssaA* (staphylococcal secretory antigen SsaA) (Table S6). *SsaA* mRNA has been proposed
333 (but not yet demonstrated) to base-pair with the 3'-end of RNAIII (Lioliou et al., 2016). *spa* mRNA
334 is known to base-pair with RNAIII, in particular with helix 13 (Huntzinger et al., 2005), thereby
335 blocking *spa*'s Shine-Dalgarno sequence, leading to *spa* mRNA translation repression and
336 degradation by endoribonuclease III (Bronesky et al., 2016). While within the primary sequence
337 helix 13 is far away from the NAD-5'-end, in one of the proposed secondary structures of RNAIII,
338 the 5' and 3'-ends base-pair with each other (Novick et al., 1993), thereby localising the NAD in
339 the vicinity of helix 13. Hence, it is conceivable that the 5'-NAD cap affects the folding of RNAIII
340 and its interaction with the *spa* mRNA. A stronger interaction might lead to a decreased protein
341 A production, which would ultimately favour the dissemination of *S. aureus* pCG-P3(-1G) isolates
342 (Bronesky et al., 2016). Furthermore, four genes for a protease family occurring only in *S. aureus*,
343 (*SplA/B/C/D* operon) (Reed et al., 2001) were upregulated in the strain with increased NAD-
344 RNAIII. Also, the *Spl* operon is induced by the *agr*-system with a mechanism that has not been
345 elucidated but that might involve RNAIII (Reed et al., 2001). The *Spl* proteases have been
346 implicated in tissue dissemination processes due to their ability to degrade host proteins (Paharik
347 et al., 2016). Noteworthy, a recent study reported a decrease in the amount of Hld in the spent
348 medium obtained from an *S. aureus Spl* deletion mutant, suggesting a positive correlation
349 between RNAIII and *Spl*. On the other hand, the ΔSpl strain did not show alterations in Hla levels,
350 proving that the *agr* system was not inhibited (Paharik et al., 2016).

351 At this point, one could expect that the upregulation in *Spl* proteases in pCG-P3(-1G) together
352 with the downregulation of protein A and SsaA would generate a more invasive bacterium than
353 pCG-P3. Nevertheless, evidence at protein level of these two targets would be necessary to
354 predict more accurately the phenotypic effects.

355 Transcriptome sequencing did not reveal significant changes in another major target of RNAIII,
356 namely *hla* (Log2FC = -0.14). However, as RNAIII activates translation of *hla* mRNA by base pairing
357 between the 5'-UTRs of both RNAs (Morfeldt et al., 1995), an effect on the mRNA level of this
358 gene was not expected.

359 On the protein level, the phenotypic effects were more notorious. Since pCG-P3(-1G) exhibits an
360 upregulated *Spl* operon, an increase in Hld amount in supernatant could be expected. In contrast,
361 a significant decrease of Hld in cell culture filtrates of *S. aureus* pCG-P3(-1G) was observed (Figure
362 3C).

363 One explanation would be that the 5'-NAD cap in RNAIII might impair Hld translation. In
364 eukaryotes, there are conflicting reports regarding the translatability of NAD-RNAs (Jiao et al.,
365 2017; Wang et al., 2019), and there are no comparable data on prokaryotes which are not known
366 to sense and require "cap" structures in translation. It has been suggested that the 3'-end of
367 RNAIII blocks Hld translation by base-pairing with its Shine-Dalgarno (SD) sequence, making it
368 inaccessible to ribosomes, thereby delaying the translation of Hld by 1h after RNAIII transcription

369 (Balaban & Novick, 1995). Thus, the 5'-NAD cap might favour the interaction between the 5'-end
370 and and some downstream sequences due to the pseudo-base-pairing of the nicotinamide
371 moiety of the initiating NAD, which has been proposed to base-pair with C and T of DNA in the
372 open promoter (Vvedenskaya et al., 2018). Hence, it appears plausible that the 5'-NAD cap of an
373 RNA interacts similarly with C and uridine (U) residues inside the same molecule. Nevertheless,
374 even if one assumes that NAD-RNA is not translated while ppp-RNA is, this could not explain how
375 a 15% reduction in ppp-RNAIII in pCG-P3(-1G) compared to pCG-P3 (Figure 2C) can cause a 50%
376 decrease in translation product yield. We assume that this effect may be due to yet unknown
377 factors that modulate *hld* translation.

378 In the case of Hla, the changes observed in pCG-P3(-1G) compared to pCG-P3 are slightly lower
379 than for Hld (Figure 3C), which might imply a lower influence of the 5'-NAD-cap on the RNAIII-*hla*
380 mRNA interaction than on RNAIII translation. Indeed, the fact that the H2 hairpin of RNAIII, which
381 is the one predicted to interact with *hla* mRNA, is only nine nucleotides upstream of *hld*'s SD
382 sequence suggests that both might be similarly affected by the 5'-NAD cap. Like in the case of
383 Hld translation, nicotinamide's base-pairing flexibility might increase the probability of
384 generating a 5'-end-3'-UTR duplex in RNAIII's secondary structure, sequestering both the SD
385 sequence and H2 helix, and explaining the phenotypes obtained in *S. aureus* pCG-P3(-1G) (Figure
386 3C and Figure 3D). Surprisingly, the *in vitro* analysis of RNAIII leader's secondary structure by
387 SHAPE did not reveal differences related to the 5'-NAD cap (Figure 4A). In both RNAIII leader
388 variants, the SD appeared to be accessible (Figure 4B). This structure would likely be compatible
389 with translation of the embedded Hld ORF regardless of the presence of the 5'-NAD cap. Of note,
390 the analysis was done with a shortened version of RNAIII with the aim of detecting the presence
391 of a mechanism that would block co-transcriptional translation. Furthermore, SHAPE
392 measurements with full-length RNAIII revealed an accessible SD sequence as well (Figure 4B,
393 Figure 4C, Figure 4D), an observation already reported in the chemical probing of RNAIII
394 performed by Benito et al. (2000). Our data suggest that the lack of RNAIII's 3'-domain would not
395 exert any effect on Hld and Hla translation (Figure 4B, Figure S1G). In contrast, a mutant *S. aureus*
396 strain with a truncated RNAIII devoid of its 3'-end domain (211 nt) showed an earlier Hld
397 translation compared to the wild type strain (Balaban & Novick, 1995). Likewise, deletions within
398 3'-end region of RNAIII also inhibit Hla expression (Novick et al., 1993). Thus, although the *in vitro*
399 RNAIII SHAPE experiments do not explain the obtained phenotypes with decreased Hld and Hla,
400 in a regulated cellular co-transcriptional translation environment, the 5'-NAD cap could cause
401 some differences and, induce different phenotypes.

402 While RNAIII has been studied intensively as a central regulator of bacterial physiology, a large
403 number of different targets affected by it, and the diversity of mechanisms of action make it
404 rather unlikely that only one stable secondary structure induces all these responses. Instead,
405 inside the cell likely exists an equilibrium between different folds, each of them suited for specific
406 purposes (Benito et al., 2000). The existence of such alternative folds has been hinted at by the
407 inaccessibility of a fraction of *in vitro-transcribed* NAD-RNAIII to NudC cleavage, and its
408 modulation by an unfolding / folding cycle (Figure 3A). Thus, the existence in the cell of an RNAIII
409 subpopulation that is sensitive to its 5'-modification state and is responsible for both *hld* and *hla*
410 activation remains plausible (Balaban & Novick, 1995).

411 The fact that the RNAIII sequence is highly conserved among *S. aureus* strains led us to
412 hypothesize that the incorporation of NAD into RNAIII should be strictly regulated and therefore

413 the evolution selected the -1T at P3 promoter. In such a way, *S. aureus* may ensure the right
414 (intermediate) level of NAD incorporation into RNAIII and preserves its optimal ensemble of
415 secondary structures. Assuming this, the 5'-NAD cap might serve as modulator of RNAIII's
416 secondary structure. Moreover, the NADylation of RNAIII is likely dependent on the bacterial
417 redox state and might be a new mechanism to fine-tune *agr* activity.

418 **5.4 Outlook.**

419 In this study, we have discovered another prokaryotic organism that possesses NAD-capped
420 RNAs. However, it constitutes the first evidence of this phenomenon in pathogenic bacteria to
421 date. More importantly, we have found that a crucial regulatory and protein-coding RNA (RNAIII)
422 is strongly NAD-modified and that alterations on its NAD-modification state lead to significant
423 effects that modulate *S. aureus*'s cytotoxicity that could be related to an alternative secondary
424 structure of RNAIII. However, and despite these exciting findings, the role of 5'-NAD-RNAIII
425 remains unclear. Further studies will be conducted to unravel how exactly the NAD cap changes
426 RNAIII's secondary structure and how this change contributes to the overall secondary structure
427 ensemble of RNAIII. Furthermore, *in vivo* studies applying our mutant *S. aureus* strains to animal
428 infection models are planned to study the pathogenicity of this bacterium.

429 **6 Materials and Methods**

430 **6.1 Bacterial strains.**

431 *S. aureus* ATCC 25923 was the selected strain to detect the presence of NAD covalently linked to
432 RNA by UPLC-MS analysis, NAD captureSeq, and pull-down of specific RNAs. *S. aureus* HG001 was
433 used as wild-type and for the mutational studies. The pCG-246-based (Helle et al., 2011) shuttle
434 plasmids pCG-P3, pCG-P2 and pCG-P3(-1G) were first grown in *E. coli* K-12 strain, then
435 transformed into the restriction-deficient *S. aureus* RN4220, and finally electroporated into *S.*
436 *aureus* HG001 Δ RNAIII as described before (Charpentier et al., 2004). For the cytotoxicity assay,
437 the constructs were introduced into *S. aureus* Δ RNAIII Δ psma β . Genomic DNA from *S. aureus*
438 ATCC 25923 was used to amplify the P3 promoter, P2 promoter, and RNAIII sequences (Q5 Hot
439 Start High-Fidelity DNA Polymerase, NEB). The bacterial strains used in this study are summarised
440 in Table S9.

441 **6.2 Generation of constructs.**

442 The RNAIII sequence with the native P3 promoter sequence upstream was amplified from
443 Genomic DNA from *S. aureus* ATCC 25923. The PCR amplification was carried out with the primers
444 Fwd-P3-RNAIII/Rv-P3-RNAIII; as a consequence, the restriction sites *Bam*HI and *Eco*RI were
445 introduced in the amplicon. The P2 promoter sequence was fused to RNAIII by the following
446 procedure: a PCR with the primers Fwd-P2/Rv-P2hyb was performed in order to generate a P2-
447 amplicon with a 20 bp overhang on its 3'-end which was complementary to RNAIII's first 20 nt.
448 The PCR with primers Fwd-RNAIII/Rv-RNAIII originated a second amplicon containing the whole
449 RNAIII sequence. Both amplicons were used as primers and subjected to PCR in order to generate
450 the P2-RNAIII product, which contained *Bam*HI and *Eco*RI restriction sites. Both PCR products

451 were treated with *Bam*HI and *Eco*RI and cloned into the pCG-246 shuttle vector (Helle et al., 2011)
452 yielding the constructs pCG-671 and pCG-672. The RNAIII terminator sequence was introduced
453 afterwards in both of the constructs by site-directed mutagenesis generating pCG-P3 and pCG-
454 P2 plasmids. Besides, a mutation at the -1 position (-1T to -1G) respect to the TSS of the P3
455 promoter was inserted by site-directed mutagenesis, yielding plasmid pCG-P3(-1G). All primers
456 used are summarised in Table S3.

457 **6.3 NAD captureSeq and RNA-seq Next Generation Sequencing (NGS).**

458 In order to identify those NAD-RNAs from *S. aureus*, we have made use of NAD captureSeq as
459 described previously (Winz et al., 2017) and applied to 100 µg total RNA from *S. aureus* ATCC
460 25923 per sample isolated at the late-exponential phase ($OD_{600} = 1.5$). A NAD captureSeq variant
461 was also used in order to compare the enrichment of specific RNAs between *S. aureus* strains
462 (pCG-P2, pCG-P3, and pCG-P3(-1G)). For that, 15 fmol of variable length (61, 104, 205, 302, 400
463 nt) NAD-RNAs were spiked into each sample, acting as internal standards (IS). The PCR products
464 were purified by polyacrylamide (PA) gel electrophoresis (PAGE), and the range between 150 and
465 300 bp selected (Figure S1A). This size selection helped to remove the primer dimers as well.
466 Afterwards, the quality of the samples was analysed by Bioanalyzer measurements (Bioanalyzer
467 2100, Agilent Technologies, Santa Clara, California) using the Agilent DNA 1000 Kit (Agilent). The
468 libraries were multiplexed and submitted to NGS on a NextSeq500 sequencer (Illumina, San
469 Diego, California) with the following parameters: single-end reads, 75-bp read length, read depth
470 400 million. Besides, 20% of PhiX control DNA was spiked into the samples to provide sufficient
471 read complexity. The NGS data analysis was done using an in-house pipeline. Briefly, after
472 obtaining the raw reads, the leading guanines were trimmed, and the 3' adapter was removed
473 by clipping. Once processed, the reads were mapped to the reference genome *Staphylococcus*
474 *aureus subsp. aureus* NCTC 8325 (European Nucleotide Archive, Assembly: GCA_000013425.1)
475 and to the IS RNA sequences (in the case of the quantitative NAD captureSeq, Table S4). The
476 software used for mapping was Burrows-Wheeler Aligner (BWA-MEM, version: 0.7.13) (H. Li,
477 2013). To identify the gene hits, HTSeq (version: 0.6.0) (Anders, Pyl, & Huber, 2015) was used.
478 Also, DESeq (Anders & Huber, 2010) was used for statistical analysis. Finally, the obtained hits
479 were checked and visualised with the Integrated Genome Browser (Nicol et al., 2009). RNA-seq
480 analysis was conducted with total RNA of *S. aureus* pCG-P2, pCG-P3, and pCG-P3(-1G) strains
481 (biological triplicates, 5 µg total RNA each sample) isolated at the late-exponential phase (OD_{600}
482 = 2.5). For removal of ribosomal RNA from samples, the Ribo-Zero rRNA Removal Kit (Gram-
483 Positive Bacteria, Illumina) was used. The libraries were prepared with NEBNext Ultra II
484 Directional RNA LibraryPrep Kit for Illumina (New England Biolabs (NEB), Ipswich,
485 Massachussets) together with the NEBNext Multiplex Oligos for Illumina (NEB). The removal of
486 primer excess and primer dimers was done by the Agencourt AMPure XP RNA Clean beads
487 (Beckman Coulter, Brea, California). The amount of sample was assessed by Qubit HS DNA assay
488 (Thermo Fisher Scientific, Waltham, Massachussets), whereas the sample quality was checked
489 with Agilent Tape Station D1000 for DNA. Samples were equimolarly pooled before 50 SE
490 sequencing on the Illumina HiSeq platform. NGS data analysis was performed with an in-house
491 pipeline, and the reads were mapped to the reference genome *Staphylococcus aureus subsp.*
492 *aureus* NCTC 8325 (European Nucleotide Archive, Assembly: GCA_000013425.1).

493 **6.4 Total RNA isolation.**

494 *S. aureus* strains were grown at 37 °C with shaking at 200 rpm in LB broth (Lennox), and cells
495 were harvested at OD₆₀₀ = 2.5 (experiments conducted with *S. aureus* HG001 plasmid-
496 transformed strains) and OD₆₀₀ = 1.5 (experiments conducted with *S. aureus* ATCC 25923). The
497 RNA was extracted using TRIzol reagent (Thermo Fisher Scientific). Briefly, the pelleted cells were
498 resuspended in TE buffer (30 mM Tris-HCl, 1 mM EDTA, pH = 8.0) supplemented with lysozyme
499 20 mg/mL (Sigma-Aldrich, Saint Louis, Missouri) and 80 µg/mL lysostaphin (Sigma-Aldrich) and
500 incubated 30 min at 37 °C. The cells were incubated for 30 min at -80 °C before the addition of
501 the TRIzol reagent. Afterwards, the protocol proceeded according to the manufacturer's
502 instructions. Samples were treated with DNase I (1.5 U/mg total RNA, Roche, Basel, Switzerland)
503 at 37 °C for 1 hour to remove the genomic DNA, then they were P/C/I-chloroform-extracted and
504 precipitated. The RNA was washed twice with 80% ethanol and dissolved in Millipore water. The
505 quality of the RNA was assessed by agarose gel electrophoresis (Figure S1H). The concentration
506 and purity of the total RNA were measured by Nanodrop (Thermo Fisher Scientific), paying
507 attention to OD₂₆₀/OD₂₈₀ and OD₂₆₀/OD₂₃₀ ratios.

508 **6.5 Quantitative PCR.**

509 To confirm the enrichment of the obtained hits at the cDNA level and thereby rule out the
510 possible PCR bias, qPCR measurements were carried out as described before (Cahová et al.,
511 2015). 20 µL reactions were performed in triplicate using 3 µL cDNA (1:50 diluted) as a template.
512 When confirming the cDNA enrichment of the hits, APDRC-treated sample cDNA with cDNA of
513 the minus ADPRC control were compared by the $2^{-\Delta\Delta C_T}$ -method (Livak & Schmittgen, 2001). The
514 5SrRNA gene was used as internal control and Millipore water as a negative control. Another
515 qPCR assay was performed to check the *hld*/RNAIII levels of the different *S. aureus* strains (pCG-
516 P2, pCG-P3, and pCG-P3(-1G)). 5 µg of total RNA (harvested at late-exponential phase, biological
517 triplicates) from each strain was reverse transcribed with Superscript IV reverse transcriptase
518 (Thermo Fisher Scientific) following the manufacturer's instructions. 3 µL cDNA (1:100 diluted)
519 per 20 µL reaction were used as the template. The *gyrB* gene was used as internal standard and
520 Millipore water as a negative control. Standard curves were determined for each gene, using
521 purified chromosomal DNA at concentrations of 0.005 to 50 ng/ml. All qPCR experiments were
522 performed in a Light Cycler 480 instrument (Roche) using the Brilliant III Ultra-Fast SYBRGreen
523 qPCR Mastermix (Agilent Technologies). The data were analyzed with the Light Cycler 480
524 Software (Agilent Technologies). The primers used for qPCR analysis are listed in Table S3.

525 **6.6 Detection of Hld by HPLC/ESI-MS.**

526 The detection of Hld levels from different *S. aureus* strains was performed by high-performance
527 liquid chromatography/electrospray ionisation mass spectrometry (HPLC/ESI-MS) of overnight
528 culture filtrates as described earlier (Queck et al., 2008) with minor changes. The column (Zorbax
529 SBC8, 2.1 x 50 mm, 3.5 µ, Agilent Technologies) was run at 0.3 ml/min with a gradient of 0.1%
530 trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile. HPLC-MS experiments
531 were performed on a Bruker microTOFQ-II ESI mass spectrometer (Bruker, Billerica,
532 Massachussets) connected to an Agilent 1200 Series HPLC system equipped with a multi-

533 wavelength detector (Agilent Technologies). ESI was used with a capillary voltage of 4,500 V, and
534 the collision voltage was set to 10 eV. The drying gas temperature was 200 °C, the drying gas flow
535 rate was 6 Lmin⁻¹ and the detector was operated in positive ion mode. Mass spectra were
536 recorded on a range from 250 m/z to 3,000 m/z. The two m/z peaks in the mass spectrum for Hld
537 (formylated and deformylated version, Figure S1F) were used to calculate PSM concentration by
538 integration using ACD/Spectrus software (Advanced Chemistry Development (ACD), Toronto,
539 Canada).

540 **6.7 Western blotting of Hla.**

541 One milliliter of cell culture filtrates from each strain (harvested at OD₆₀₀ = 2.5) was filtrated and
542 concentrated (final volume 20 µL) through ultracentrifugal filters (30 kDa MWCO, Amicon, Merck,
543 Darmstadt, Germany) and analysed by 10% SDS-PAGE. After the run, SDS-PA gels were blotted
544 onto Amersham Hybond P 0.2 µm PVDF membranes (GE Healthcare, Chicago, Illinois) using an
545 EasyPhor Semi-Dry-Blotter (Biozym Scientific, Hessisch Oldendorf, Germany). Membranes were
546 blocked in TBST (Tris-buffered saline with 0.1% Tween 20) supplemented with 5% milk powder
547 (Carl Roth, Karlsruhe, Germany). Afterwards, membranes were incubated with anti-
548 staphylococcal alpha-toxin rabbit antibody (1:5000, Sigma-Aldrich) in washing buffer (TBST with
549 1% milk powder). After washing, Alexa Fluor Plus 488-conjugated goat anti-rabbit IgG (Thermo
550 Fisher Scientific) in washing buffer (1:10000) was applied over the membranes. Membranes were
551 washed with 50 mM Tris-HCl, pH 7.25, and subsequently scanned using a Typhoon FLA 9500
552 imager (GE Healthcare). Quantification was done with ImageQuant TL software (GE Healthcare).

553 **6.8 Detection of NAD covalently bound to RNA by UPLC/MS.**

554 Samples (total RNA and pull-down RNA) were prepared as described before (Frindert et al., 2018).
555 Briefly, RNA was extensively washed with decreasing urea concentrations in ultracentrifugal
556 filters (10 kDa MWCO, Amicon, Merck). RNA was recovered and subjected to NudC and alkaline
557 phosphatase (Sigma-Aldrich) treatment (1 h at 37 °C, 2 h for pull-down RNA) in the presence of
558 the MS internal standard (d4-ribose nicotinamide, Toronto Research Chemicals, Ontario,
559 Canada). Afterwards, the reaction mixtures were filtered through 10 kDa ultracentrifugal filters
560 (Merck) and dried under reduced pressure. Then, samples were analysed by UPLC-MS following
561 the previously described protocol (Frindert et al., 2018). A calibration curve was recorded for r-
562 NA for each analytical batch. M/z peaks of the mass spectra from the analyte and internal
563 standard were integrated with TargetLynx software (Waters corporation, Milford,
564 Massachusetts). The amount of injected r-NA was calculated by integration of the corresponding
565 m/z peak using the TargetLynx software and the calibration curve.

566 **6.9 Gel electrophoresis and Northern Blot analysis.**

567 RNA was separated by denaturing PAGE. For NAD captureSeq NGS library generation (Qualitative
568 and Quantitative), the cDNA amplification products were purified by native PAGE. APBgels (Nübel
569 et al., 2017) were used to separate and purify NAD-RNA and ppp/p-RNA. Northern blot analysis
570 was performed as described before (Cahová et al., 2015). RNA was separated either by PAGE or
571 PAGE-APBgels (200 V, 90 min), blotted onto a Whatman Nytran SuPerCharge nylon blotting

572 membrane (Merck) for 3 h (4 h for APBgels at 4 °C to avoid buffer evaporation) at 250 mA using
573 an EasyPhor Semi-Dry-Blotter (Biozym Scientific), and UV-crosslinked. Membranes were pre-
574 hybridized in Roti-Hybri-QuickBuffer (Carl Roth) for 2 h at 48 °C. Afterward, 5 µL RNA radiolabeled
575 probe (Table S3) was added and incubated overnight at 48 °C in a hybridization oven with
576 rotation. The templates for the probes were prepared by PCR using Taq DNA polymerase
577 (prepared laboratory stock). Probes were prepared by *in vitro* transcription (IVT) in the presence
578 of 35 µCi α -³²P-ATP and 35 µCi α -³²P-CTP (3,000 Ci/mmol, each, Hartmann Analytic,
579 Braunschweig, Germany). The IVT reactions were treated with DNase I (Roche), P/C/I-
580 chloroform-extracted, ethanol-precipitated and dissolved in 30 µL Millipore water. The blot was
581 washed for 30 min with wash solution 1 (2x SSC, 0.1% SDS), 30 min with wash solution 2 (1x SSC,
582 0.1% SDS) for 30 min with wash solution 3 (0.25x SSC, 0.1% SDS). Radioactive RNA was visualized
583 using storage phosphor screens (GE Healthcare) and a Typhoon FLA 9500 imager (GE Healthcare).

584 **6.10 Preparation of NAD-RNAIII and ppp-RNAIII markers and IS NAD-RNAs.**

585 The RNAIII markers used in the Northern blot analysis were prepared by IVT. The IVTs were
586 performed as described earlier (Huang, 2003). Each 100 µl IVT reaction contained: 1.5-2 µg DNA
587 template and 4 mM of each NTP (6 mM NAD for NAD-RNAs; ATP concentration was reduced to
588 2 mM, or without NAD for ppp-RNAs). The reactions were performed in transcription buffer (40
589 mM Tris-HCl pH = 8.1, 1 mM spermidine, 22 mM MgCl₂, 0.01% Triton X-100, 10 mM dithiothreitol
590 (DTT), 5% DMSO). Reactions were stopped upon addition of denaturing gel loading buffer (10%
591 TBE in formamide containing 0.05% bromophenol blue, 0.5% xylene cyanol blue) and purified by
592 PAGE with standard running conditions (1x TBE buffer, 600 V). RNAs were excised after the run,
593 eluted overnight in 0.3 M sodium acetate (pH = 5.5) at 19 °C, isopropanol-precipitated and
594 dissolved in Millipore water. DNA templates for the RNAIII markers were prepared by PCR
595 amplification of genomic DNA from *S. aureus* ATCC 25923 using Q5 Hot Start High-Fidelity DNA
596 Polymerase (NEB) and the primers listed on Table S3. The PCR products were purified with the
597 QIAquick PCR purification kit (Qiagen, Hilden, Germany) before the IVT reactions.

598 The 5 different IS NAD-RNAs used for NAD captureSeq were also prepared by IVT (Huang, 2003).
599 As a template, regions of *E. coli*'s K12 genome with no homology within *S. aureus* genome were
600 PCR amplified (Q5 Hot Start High-Fidelity DNA Polymerase (NEB)) with the primers summarised
601 in Table S3. The PCR products were purified with QIAquick PCR purification kit (Qiagen) before
602 IVT. IVT reactions were PAGE purified, as described above. Afterwards, the RNAs were
603 additionally purified on APBgels (5% PA, 0.4% APB) (Nübel et al., 2017), loaded with APBgels
604 loading buffer (8 M urea, 10 mM Tris-HCl pH = 8.0, 50 mM EDTA, xylene cyanol and bromophenol)
605 and run in 1x TAE (550 V). The sequences of each IS NAD-RNA are summarised in Table S4.

606 **6.11 Preparation of radiolabeled pure NAD-RNAIII and NudC cleavage assay.**

607 The radiolabeled (body labelled) pure NAD-RNAIII was prepared by IVT essentially as described
608 before (Huang, 2003) but with the presence of 100 µCi α -³²P-UTP (3,000 Ci/mmol, Hartmann
609 Analytic) per 100 µL reaction. The IVT reaction was first PAGE-purified and afterwards purified
610 on APB gels as described above to obtain 100% NAD-modified RNAIII, which was subjected to
611 NudC cleavage. The NudC cleavage assay was carried out as described before (Cahová et al.,
612 2015) but with a ratio of 2 NudC:1 RNAIII. 10 µL reactions per triplicate in 1x degradation buffer

613 (25 mM Tris-HCl pH = 7.5, 50 mM NaCl, 50 mM KCl, 10 mM MgCl₂, 10 mM DTT) were incubated
614 for 5, 10, 20, 30, 60 and 120 min at 37 °C. Reactions were stopped by addition of APBgel loading
615 buffer and analysed on APBgels (6% PA, 0.7% APB). The stability of NAD-RNAIII in 1x degradation
616 buffer was assessed by incubating it for 2 hours at 37 °C in the absence and the presence of the
617 enzyme (Figure 1, Figure 3A).

618 Additional reactions per triplicate were performed to test the influence of RNAIII's secondary
619 structure on NudC cleavage capacity. After a 60 incubation at 37 °C in the presence of NudC,
620 samples were heated up to 75 °C for 2 min and cooled down to 25 °C (NAD-RNAIII folding after
621 digestion). Afterward, fresh NudC was added, and samples underwent a second round of
622 incubation (60 min at 37 °C, Figure 1, Figure 3A).

623 Radioactive RNA was visualised using storage phosphor screens (GE Healthcare) and a Typhoon
624 FLA 9500 imager (GE Healthcare).

625 **6.12 Pull-down of RNAIII and 5SrRNA.**

626 RNAs were specifically isolated out of 300 µg total RNA as described previously (Cahová et al.,
627 2015; Frindert et al., 2018). Briefly, 150 µL Streptavidin Sepharose High Performance beads (GE
628 Healthcare) were loaded onto Mobicol Classic columns (MoBiTec, Göttingen, Germany), washed
629 with 1x PBS and supplemented with biotinylated DNA probes (Biomers, Ulm, Germany, Table S3)
630 dissolved in 1x PBS (75 µL, 25 mM, with incubation for 10 min at 25 °C with shaking at 1000 rpm).
631 After washing and equilibration of the beads (with 1x PBS and pull-down buffer (10 mM Tris-HCl
632 pH 7.8, 0.9 M tetramethylammoniumchloride, 0.1 M EDTA pH 8.0), total RNA dissolved in pull-
633 down buffer was added. RNA was then incubated for 10 min at 65 °C and then for 25 min at room
634 temperature with rotation (Tube Rotator, VWR, Radnor, Pennsylvania). Beads were washed with
635 Millipore water, and RNA was eluted upon addition of 2mM EDTA (pre-heated to 75 °C). The
636 eluted RNA was precipitated with 0.5 M ammonium acetate and isopropanol in a reaction tube
637 and dissolved in Millipore water. Northern blot analysis with the pull-down eluates and probes
638 vs RNAIII and 5SrRNA was performed (Figure S1I). Afterwards, RNA was further purified by 8%
639 PAGE and the bands corresponding to RNAIII and 5SrRNA excised (Figure S1J). After RNA
640 precipitation and washing, RNA concentration was measured with Qubit RNA BR Assay Kit
641 (Thermo Fisher Scientific). The purified RNA was used for: Northern bot analysis (Figure S1K) and
642 NudC treatment with subsequent UPLC-MS analysis as described above. For DNAzyme treatment,
643 the precipitated RNA was used without further PAGE purification (Figure 1E).

644 **6.13 Generation of RNA-cleaving DNA enzymes.**

645 Four different RNA-cleaving DNAzymes were generated following the instructions by Joyce
646 (Joyce, 2001) to generate a shorter RNAIII. DNAzymes were tested on RNAIII *in vitro*, and the
647 most efficient one was selected for further experiments (DNAzyme II, Figure S1L). The cleavage
648 of RNAIII by DNAzyme II (37 nt, Table S3) generated a 125 nt product containing RNAIII's 5'-
649 terminus. For the *in vitro* cleavage assays, 10 µL reactions were set up in 1x DNAzyme buffer (10
650 mM Tris-HCl pH = 8.0, 5 mM NaCl) supplemented with 1 µM NAD/ppp-RNAIII, 0.2 mM DTT, 25
651 mM MgCl₂, and 1 µM DNAzyme II. First, RNAIII was folded in the presence of 0.2 mM DTT by
652 incubation for 2 min at 75 °C, followed by a cool down to 25 °C. Afterwards, the remaining
653 reagents were added (MgCl₂, DNAzyme II) and reactions were incubated 1 h at 37 °C. Reactions

654 were stopped by addition of denaturing gel loading buffer and analysed by classical PAGE and
655 APBgels followed by Northern blot as described above (Figure S1L). The same procedure was
656 followed with RNAIII obtained after pull-down (biological triplicates, 2 μ M DNAzyme II) but
657 including an additional NudC treatment (1.5 μ M NudC, 1h 37 °C) right after RNA cleavage as a
658 negative control (to deplete NAD-RNAIII). NAD-RNAIII and ppp-RNAIII treated with DNAzyme II
659 and I were used as reference markers in the Northern Blot assay of RNAIII pull down samples.

660 **6.14 Analysis of 5'-ends by circular RT-PCR and Sanger sequencing.**

661 To analyse RNAIII's TSS on the different *S. aureus* mutant strains (pCG-P3, pCG-P2, pCG-P3(-1G)),
662 circular RT-PCR was carried out as described before (Slomovic & Schuster, 2013) with small
663 differences. In essence, 10 μ g of total RNA of each strain was treated with RNA 5'-
664 Polyphosphatase (Epicentre, Madison, Wisconsin). The dephosphorylated RNAs were self-ligated
665 by T4 RNA ligase (Thermo Fisher Scientific) treatment, and the predicted 3'-end-5'-end ligated
666 region of RNAIII was amplified by nested PCR with the primers listed in Table S3. The purified
667 amplicons were then phosphorylated by T4-Polynucleotide Kinase (Thermo Fisher Scientific)
668 treatment before cloning them into the plasmid pDisplay-AP-CFP-TM (Addgene plasmid #20861
669 (pDisplay-AP-CFP-TM was a gift from Alice Ting (Howarth, Takao, Hayashi, & Ting, 2005)),
670 previously digested with EcoRV (NEB) and dephosphorylated by alkaline phosphatase (Thermo
671 Fisher Scientific)). The blunt-end ligation was carried out by T4-DNA ligase (Thermo Fisher
672 Scientific). The ligation mixture was transformed into *E. coli* DH5 α competent cells. The
673 transformed cells were streaked on Petri dishes containing LB-agar supplemented with ampicillin
674 (100 μ g/mL, Carl Roth) and incubated overnight at 37 °C. Single colonies were picked, the
675 plasmids isolated and subjected to high throughput Sanger sequencing (Microsynth SeqLab,
676 Göttingen, Germany). All the enzymatic treatments were performed according to the
677 manufacturer's instructions.

678 **6.15 Cytotoxicity assay.**

679 Overnight cultures were sub-cultured to OD₆₀₀ = 0.05 in LB-broth (Lennox) and then grown for ~
680 22 h. Bacterial supernatants were harvested and filtered through a 0.45 μ m filter (Merck) and
681 analysed for its cytotoxic potential. The cytotoxicity assay was performed essentially as described
682 (Munzenmayer et al., 2016) with the exception that 1x10⁵ THP1 cells were seeded in 96-well cell
683 8culture plates in a final volume of 100 μ L. Differentiated THP1 macrophages were treated with
684 200 μ L of each bacterial supernatant. THP1 cells with culture medium were used as negative
685 control and THP1 cells treated with Phosphate Buffered Saline (PBS) containing 1% TritonX-100
686 were used as a positive control. The cytotoxic potential of the samples was determined from
687 THP1-cell supernatants after 3 hours by using the Cytotoxicity Detection Kit (Roche) according to
688 the manufacturer's instructions.

689 **6.16 Structural analysis of RNAIII variants by SHAPE**

690 1M7 was synthesized as described (Mortimer & Weeks, 2007). A shorter version of RNAIII
691 comprising nucleotides 1 to 113 (RNAIII leader) and full-length RNAIII with different 5'-ends (5'-
692 NAD cap or triphosphate) were prepared by *in vitro* transcription as described above. In the case

693 of full-length RNAIII, a mixture containing ~ 1:1 NAD-RNAIII and ppp-RNAIII was prepared
694 whereas the obtained NAD-RNAIII leader was 100% NAD-modified. The IVT template was
695 generated by PCR with the primers RNAIII-leader-FW and RNAIII-leader-RV. Genomic DNA from
696 *S. aureus* ATCC 25923 was used for the PCR. The NAD-supplemented IVT reaction was double-
697 purified, first by denaturing PAGE and afterwards by APB gels, yielding NAD-RNAIII leader and
698 ppp-RNAIII leader (Figure S1M).

699 SHAPE was performed as described with minor modifications (Wilkinson et al., 2006). Briefly, 8
700 pmol of RNA dissolved in 47.8 μ L Millipore water were incubated for 2 min at 75 $^{\circ}$ C and then
701 cooled down to 60 $^{\circ}$ C with a ramp of 0.1 $^{\circ}$ C/s. Afterwards, 24.2 μ L of Dulbecco's Phosphate
702 Buffered Saline (DPBS, Sigma-Aldrich) supplemented with 3 mM MgCl₂ were added to the
703 samples, and they were incubated 2 min at 60 $^{\circ}$ C. Samples were then cooled down to 30 $^{\circ}$ C (0.1
704 $^{\circ}$ C/s) and kept 2 min at this temperature. During this step, each sample was equally divided into
705 two vials (sample vial and control vial). The vials were heated up to 37 $^{\circ}$ C, and then 4 μ L of 100
706 mM 1M7 (10 mM final concentration) was added to sample vial. 1M7 was dissolved in dry DMSO
707 (Sigma-Aldrich), so the reaction control was supplemented with the same volume of dry DMSO
708 as in the sample (4 μ L). The reaction mixtures were incubated for 20 min at 37 $^{\circ}$ C. Finally,
709 reactions were stopped by adding 1 reaction volume of Millipore water, and the RNA was
710 precipitated by ethanol in the presence of 0.3 M sodium acetate. The precipitated RNA was
711 washed twice with 80% ethanol and dissolved in Millipore water to a concentration of 0.5
712 pmol/mL.

713 For primer extension, 100 pmol of SHAPE-RT1 primer (Table S3) were radioactively labeled on its
714 5'-end with 3 μ L of γ -³²P-ATP (3,000 Ci/mmol, Hartmann Analytic) per 20 μ L reaction by using T4
715 polynucleotide kinase (Thermo Fisher Scientific), and further purified with the QIAquick
716 Nucleotide Removal Kit (Qiagen). Both steps were performed following the manufacturer's
717 instructions.

718 The primer extension assay was applied to the RNA samples by using Superscript IV reverse
719 transcriptase (Thermo Fisher Scientific). The reaction volume was set to 10 μ L. 1 pmol of treated
720 RNA per reaction was used as a template for the reaction together with 5 μ L of 5'-end
721 radiolabeled DNA primer 1 μ M. A sequencing ladder was generated by reverse transcription of 1
722 pmol non-treated *in vitro*-transcribed ppp-RNAIII leader supplemented with 0.5 mM
723 deoxynucleotide triphosphate mix each (Sigma-Aldrich), and 1 mM of the corresponding
724 dideoxynucleotide triphosphate (Jena Bioscience, Jena, Germany). Reactions were performed
725 according to the manufacturer's instructions. The removal of residual RNA was done by addition
726 of 2 μ L 1 M NaOH to the samples followed by a 5 min incubation at 90 $^{\circ}$ C. Samples were then
727 neutralised by the addition of 2 μ L 1 M HCL and stored at -20 $^{\circ}$ C in denaturing gel loading buffer
728 before being analysed by PAGE.

729 Samples processed in triplicate were analysed by 15% PA sequencing gels with standard running
730 conditions (TBE buffer, 2000 V, 4h 30 min run time, Figure S1L). Radioactive cDNA was visualised
731 with storage phosphor screens (GE Healthcare) and a Typhoon FLA 9500 imager (GE Healthcare).
732 Quantification of PA sequencing gels was done with the software SAFA (Laederach et al., 2008).
733 The intensities of the bands were normalised, and the control lanes (DMSO treated) were
734 subtracted from the sample lanes (1M7 treated) to obtain the SHAPE values for each nucleotide.
735 The SHAPE values for each nucleotide were afterwards used in the RNAstructure software (Reuter
736 & Mathews, 2010) to create a structure prediction for the RNA sequence.

737 **6.17 Statistical analysis**

738 Except for NGS data analysis, where DESeq statistics was used (Anders & Huber, 2010), all
739 statistics were analysed using the software Prism 6 version 6.01 (GraphPad, San Diego,
740 California). Error bars depict standard deviations in all experiments. For specific data sets,
741 identification of outliers was performed by Grubbs' method (Alpha = 0.2). When comparing two
742 groups, the parametric unpaired two-tailed Student's t-test was selected. For the analysis of
743 experiments involving three or more groups, the parametric one-way ANOVA test was done. The
744 applied *Post hoc* analysis was Tukey's multiple comparisons-test. Differences were considered
745 significant when $P < 0.05$.

746 **7 Author contributions**

747
748 H.M. and A.J. designed the study. H.M., N.K., Y.Z., and G.N. performed the experiments. H.M.,
749 Y.Z., G.N., S.E.G., C.W., and A.J. analyzed the data. H.M. and A.J. wrote the initial draft of the
750 manuscript, and H.M., A.J., C.W and S.E.G. edited the manuscript.

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752
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759 **9 Competing interests**

760
761 The authors declare no competing interests.

762 **10 Figure legends**

763
764 **Figure 1:**
765 **A:** Schematic representation of the cell-population-sensor Agr locus in *S. aureus*. AgrD is a
766 precursor peptide that is converted into an autoinducing peptide (AIP) upon proteolysis by AgrB
767 (transmembrane protein) and secreted to the exterior. The AIP then binds to the AgrC receptor,
768 triggering autophosphorylation of its intracellular histidine kinase domain. AgrA is activated by
769 the transfer of the phosphate group of AgrC and enhances transcription of P2 and P3 promoters,
770 leading to RNAII and RNAPIII production. RNAII encodes all the Agr proteins (AgrB/D/C/A) whereas
771 RNAPIII is a regulatory RNA also containing an ORF encoding for delta-toxin (Hld) (See text for more
772 details).
773 **B:** Scatter plot of NGS data after NAD captureSeq. Y-axis represents the enrichment of RNAs (Log2
774 Fold change) in the fully treated samples against minus ADPRC (control). The X-axis shows the
775 average of normalized counts. The dots confined in the upper right region of the plot represent
776 the RNAs significantly enriched (NAD-capped RNAs). The light green dots depict the hits

777 clustering at the 5'-UTR and with +1A. A light green star represents the *hld*/RNAIII gene, which is
778 the most enriched by far, whereas RNAII (depicted by a red star) is located in the non-enriched
779 area of the graph.

780 **C:** Distribution of normalized reads on the *hld*/RNAIII gene (grey bar) visualized with the
781 Integrated Genome Browser (Nicol et al., 2009). The sample reads (shown in blue) are the
782 normalized reads of the ADPRC-treated sample group whereas the red-labeled control reads are
783 the ADPRC-negative. The orientation of the gene is indicated in the black bar above and
784 represents the coding strand (+). RPM: Reads per million mapped reads.

785 **D:** Bar chart representing the enrichment at the cDNA level of three targeted regions of the
786 *hld*/RNAIII gene (5'-UTR, middle region, and 3'-region). The Y-axis represents the Cp fold change
787 (sample vs. negative control group) obtained by qPCR.

788 **E:** Schematic view of workflow followed for detection of NAD-RNAs in total RNA samples and
789 pulled-down RNAs.

790 **F:** Extracted ion chromatogram representing the pull-down LC-MS outcome. The Y-axis
791 represents the NAD (r-NA) intensity of RNAIII (green, from 40 ng RNA) and 5SrRNA (blue, negative
792 control, from 960 ng RNA). The X-axis indicates the retention time in minutes. The table beneath
793 shows the concentration of r-NA (NAD) per ng RNA and also the NAD modification percentage
794 for each RNA.

795
796

797 **Figure 2:**

798 **A:** Promoter sequences of the different pCG-246-derived constructs used in the study (pCG-P3,
799 pCG-P3(-1G), and pCG-P2). Highlighted are the -35 (-35, purple for P3 and light gray for P2), -10
800 (-10, brown for P3 and orange for P2) and -1 (-1, light blue for native P3, pink for mutated P3 and
801 black for native P2) regions. The first four nucleotides of RNAIII are highlighted in green.

802 **B:** The figure shows the workflow (steps 1 to 6) followed for the pull-down of specific RNA targets
803 and the subsequent RNA cleavage by a DNAzyme (see text for more information).

804 **C:** Analysis of pulled-down RNAIII by Northern blot on APBgels after DNAzyme treatment of the
805 different *S. aureus* strains (pCG-P3, pCG-P3(-1G), and pCG-P2, each of them by triplicate). The
806 arrows indicate the bands belonging to NAD-RNAIII and ppp-RNAIII 5'-termini. As a marker, 10 ng
807 of *in vitro*-transcribed (IVT) ppp-RNAIII treated with DNAzyme were loaded in the first lane from
808 the left. Control samples underwent a NudC treatment before analysis on APBgels. The marker
809 used was mixed NAD/ppp-RNAIII instead of ppp-RNAIII. The bar chart beneath depicts the
810 quantification of NAD-modification ratio of RNAIII of *S. aureus* pCG-P3 (black) and pCG-P3(-1G)
811 (blue), based on the Northern blot without NudC treatment. Statistical significance determined
812 by *t*-test, $n = 5$.

813 **D:** The chart represents the enrichment (capture efficiency) of each of the IS NAD-RNAs in the
814 three NAD captureSeq experiments conducted: *S. aureus* pCG-P3 (black dot and line), pCG-P3(-
815 1G) (blue square and line) and pCG-P2 (red triangle and line).

816 **E:** Scatter plot of NGS data after NAD captureSeq. Y-axis represents the enrichment of *hld*/RNAIII
817 gene (log2fold change) in the fully treated samples against minus ADPRC (control). The X-axis
818 shows the average of normalised counts. Each strain is represented with markers: a black dot for
819 pCG-P3, blue triangle for pCG-P3(-1G), and red square for pCG-P2. The bar chart on the right side
820 shows the RNAIII enrichment in terms of fold change (sample vs negative control) of pCG-P3
821 (black bar) and pCG-P3(-1G) (blue bar) *S. aureus* strains. Statistical significance determined by *t*-
822 test, $n = 6$.

823 **F:** The figure shows the ligated 3'-UTR and 5'-UTR of circularised RNAs (cRNA). Sequences were
824 obtained by Sanger sequencing after cRT-PCR (see Methods).

825 $P < 0.05$; *, $P < 0.0001$; ****. Horizontal bars represent mean. Error bars depict standard
826 deviation.

827
828

829 **Figure 3:**

830 **A:** Cleavage of NAD-RNAIII by NudC. The reactions were analysed on APBgels. The bands
831 represent radiolabeled (body labelled) NAD-RNAIII and p-RNAIII. Incubation controls without
832 enzyme (NC) were included. #1, #2, and #3 depict the three replicates. Highlighted in red and
833 green are the percentages of NAD-RNAIII and pRNA after incubation. RNA folding plus the
834 addition of fresh NudC afterwards was done to equilibrate the RNAIII secondary structure pool
835 again.

836 **B:** RNAIII levels in the three different strains measured by qPCR. Y-axis represents the relative
837 expression of *hld* divided by the relative expression of the housekeeping gene *gyrB*. Statistical
838 significance determined by *t*-test, $n = 6$.

839 **C:** The two scatter plots show the total amount of Hld and Hla in the supernatant of *S. aureus*
840 pCG-P3 and pCG-P3(-1G) strains. The Hld intensity was calculated by integration of the two
841 highest MS peaks: Hld $m/z = 993.5$ and its formylated version $m/z = 1002.9$. Alpha-toxin was
842 detected by Western blotting and quantified by densitometry. Statistical significance determined
843 by *t*-test, $n = 6$.

844 **D:** Bacterial supernatants were analysed for cytotoxic potential against THP1 macrophages.
845 Percent cytotoxicity shown was normalised to the Triton control. Statistical significance
846 determined by one-way ANOVA with Tukey's post-test, $n = 21$.

847 * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, ns: non-significant. WT: wild type *S. aureus*
848 HG001. Horizontal bars represent mean.

849

850

851 **Figure 4:** Analysis of the secondary structure of RNAIII 5'-end variants by SHAPE.
852 **A:** The bar chart represents the differential SHAPE reactivity (Y-axis) throughout the nucleotides
853 of the 5'-UTR of RNAIII (X-axis). The blue bars represent NAD-RNAIII leader whereas the light grey
854 bars represent ppp-RNAIII leader (113 nt long). The region containing the SD sequence and the
855 H2 loop residues are indicated as a horizontal black lines.
856 **B:** Bar chart showing a detailed view of the SHAPE reactivity (accessibility) of the SD sequence
857 from ppp-RNAIII leader (light grey bar) and NAD-RNAIII (blue bar). Full-length (514 nt) ppp-RNAIII
858 is represented with dark grey bars. A: adenosine, G: guanosine.
859 **C:** Same as panel A but the chart compares full-length ppp-RNAIII (grey bar) vs NAD/ppp-RNAIII
860 (green bar) SHAPE experiments.
861 **D:** Same as panel B but comparing the SD sequence accessibility of full-length ppp-RNAIII (grey
862 bar) vs NAD/ppp-RNAIII (green bar).
863 Horizontal bars represent mean. Error bars depict standard deviation.
864
865

866 **Figure S1:**

867 **A:** Size selection step during purification of PCR-amplified libraries with NGS primers on a 10%
868 non-denaturing PA gel stained with SYBR Gold (Thermo Fisher Scientific). The dashed lines
869 indicate the excised area (between 150 and 300 bp). The numbers indicate three replicates of
870 minus ADPRC controls. M: Ultra Low Range DNA Ladder (Thermo Fisher Scientific).

871 **B:** TSS analysis of the 21 hits enriched in NAD captureSeq in *S. aureus* ATCC 25923. Note that
872 position +1 is not shown because all hits were selected to start with adenosine. A: adenosine, T:
873 thymidine, B: guanosine, C: cytidine.

874 **C:** Northern blot with probes against RNAIII and 5SrRNA. Ten micrograms of total RNA from *S.*
875 *aureus* ATCC 25923 were loaded on each of the probe-hybridised lanes. Radioactively labeled
876 RNA Century Marker (Thermo Fisher Scientific) was loaded on the first lane by the left.

877 **D:** APBgels-Northern blot conducted in triplicate with total RNA of *S. aureus* pCG-P3 and pCG-P2
878 strains and probe against RNAIII. IVT Full-length mixed ppp/NAD-RNAIII was loaded as a control
879 in the flanking lanes. Note that this experiment shows full-length RNAIII with no DNAzyme
880 treatment. #1, #2 and #3 represent biological replicates.

881 **E:** Membrane used for the detection of alpha-toxin (Hla) by Western blotting in culture filtrates
882 from two strains (pCG-P3 and pCG-P3(-1G)) in triplicate (#1, #2 and #3). The marker used was the
883 PageRuler Prestained Protein Ladder (Thermo Fisher Scientific). Two arrows indicate the bands
884 corresponding to Hla (target) and protein A (unspecific binding of the antibody).

885 **F:** Stacked extracted ion chromatograms from two different strains in triplicates (blue line pCG-
886 P3(-1G), black line pCG-P3). The first peak corresponds to delta-toxin (Hld) whereas the second
887 belongs to its formylated version.

888 **G:** Analysis of SHAPE-treated RNAIII leader (*in vitro*). G, U, A and C represent the RNA nucleobases,
889 which are equivalent to their cognate dideoxynucleotide used for the ladder generation. The
890 bases of the SD sequence are highlighted in red. N: NAD-RNAIII leader treated with 1M7 (+) or
891 with DMSO (-), P: ppp-RNAIII leader treated with 1M7 (+) or with DMSO (-), FL: Full length reverse
892 transcription product (113 nt).

893 **H:** Agarose gel electrophoresis analysis of total RNA samples from the different *S. aureus* strains.
894 Total RNA was isolated and treated with DNaseI as described in the methods section. M: 1 Kb
895 Plus DNA Ladder (Thermo Fisher Scientific). Numbers indicate three replicates of each strain.

896 **I:** Northern Blot of RNA pull-down eluates before PAGE purification. Samples were hybridised
897 with RNAIII and 5SrRNA radiolabeled probes.

898 **J:** The figure shows an 8% denaturing polyacrylamide gel with the pull-down products of RNAIII
899 (PD RNAIII) and 5SrRNA (PD 5S). As a size marker ppp-RNAII, I was loaded, together with Riboruler
900 Low Range RNA Ladder (Thermo Fisher Scientific). The red dashed areas indicate the excised
901 region of the gels, corresponding to RNAIII and 5SrRNA.

902 **K:** LC/MS-ready (after PAGE purification and band excision) RNAIII and 5SrRNA were subjected to
903 Northern Blot detection. RNAIII concentration was too low to be detected.

904 **L:** Screening of different DNAzymes (I, II, and III) on mixed ppp/NAD-RNAIII. The figure shows a
905 Northern blot targeting RNAIII (probe complementary to first 86 nt of RNAIII), after
906 electrophoresis on 8% polyacrylamide gel (left side) and APBgel (right side). The same ppp/NAD-
907 RNAIII was loaded without treatment in both gels as a marker. The APB gel shows the separation
908 between NAD and ppp RNAIII 5'- fragments. DNAzyme II is the most active of the three, acting
909 quantitatively.

910 **M:** Analysis of purified NAD / ppp-RNAIII leader on an APBgel. The ladder used was Riboruler Low
911 Range RNA Ladder (Thermo Fisher Scientific).

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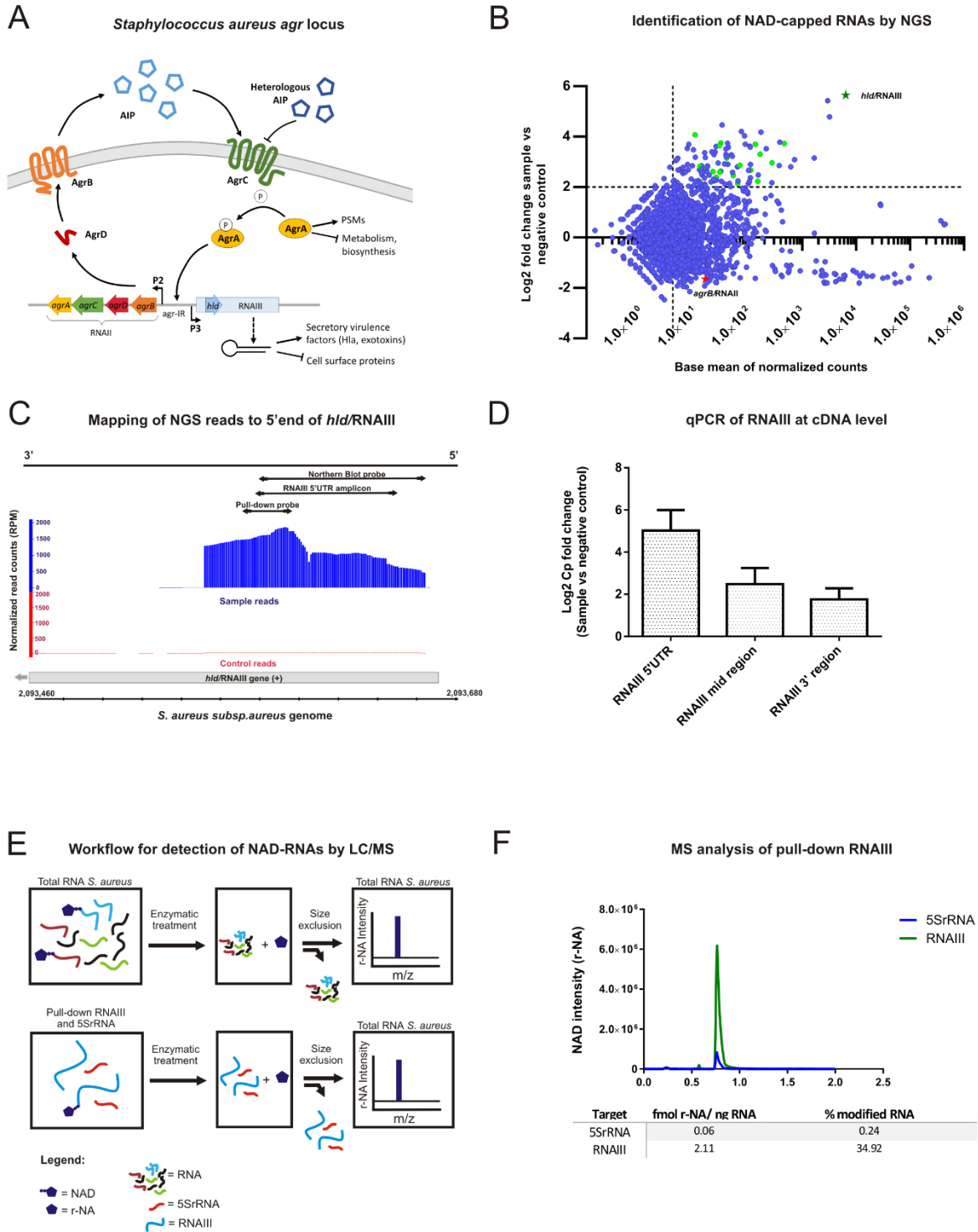
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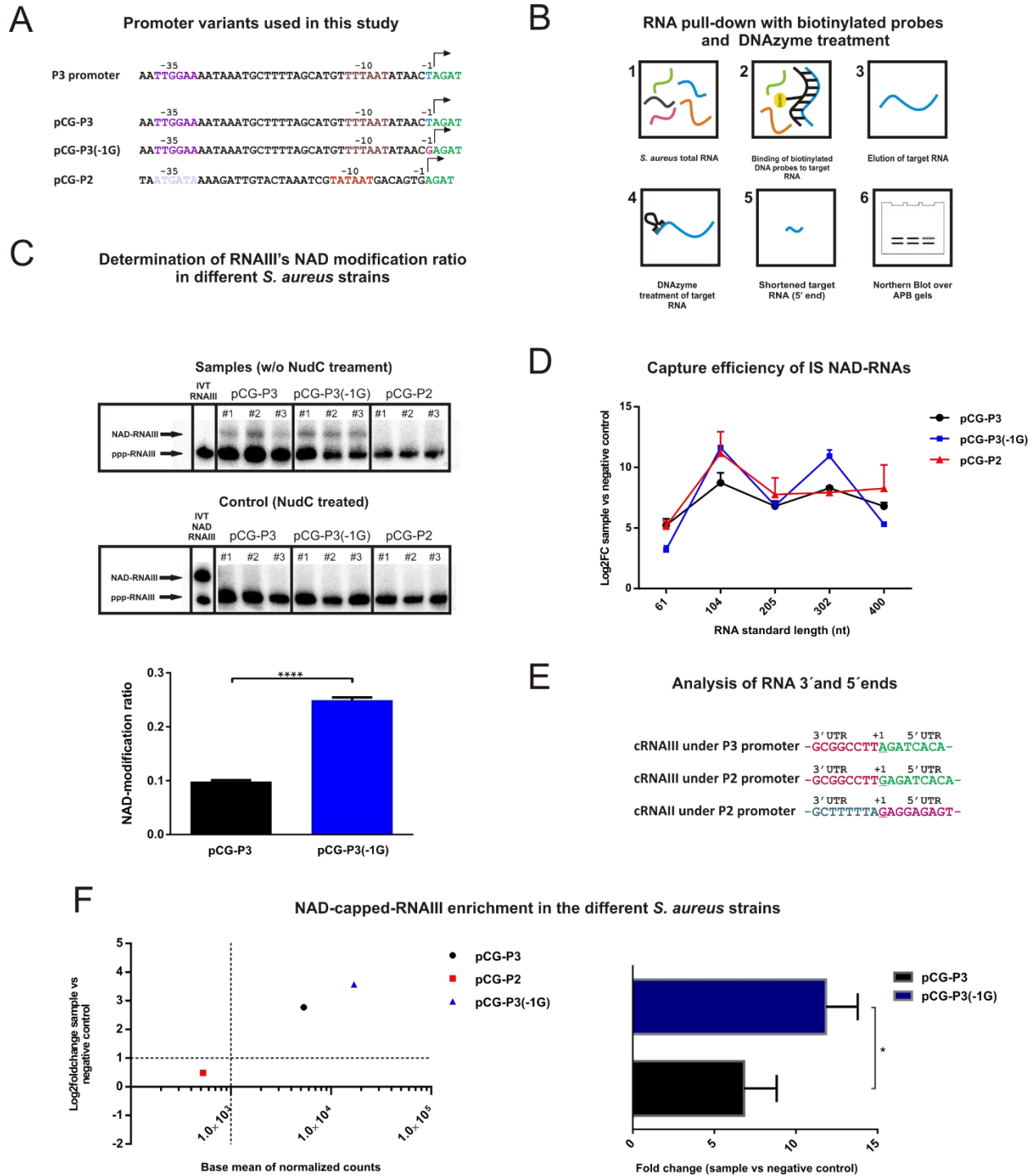
1108 **12 Figures**



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Figure 1

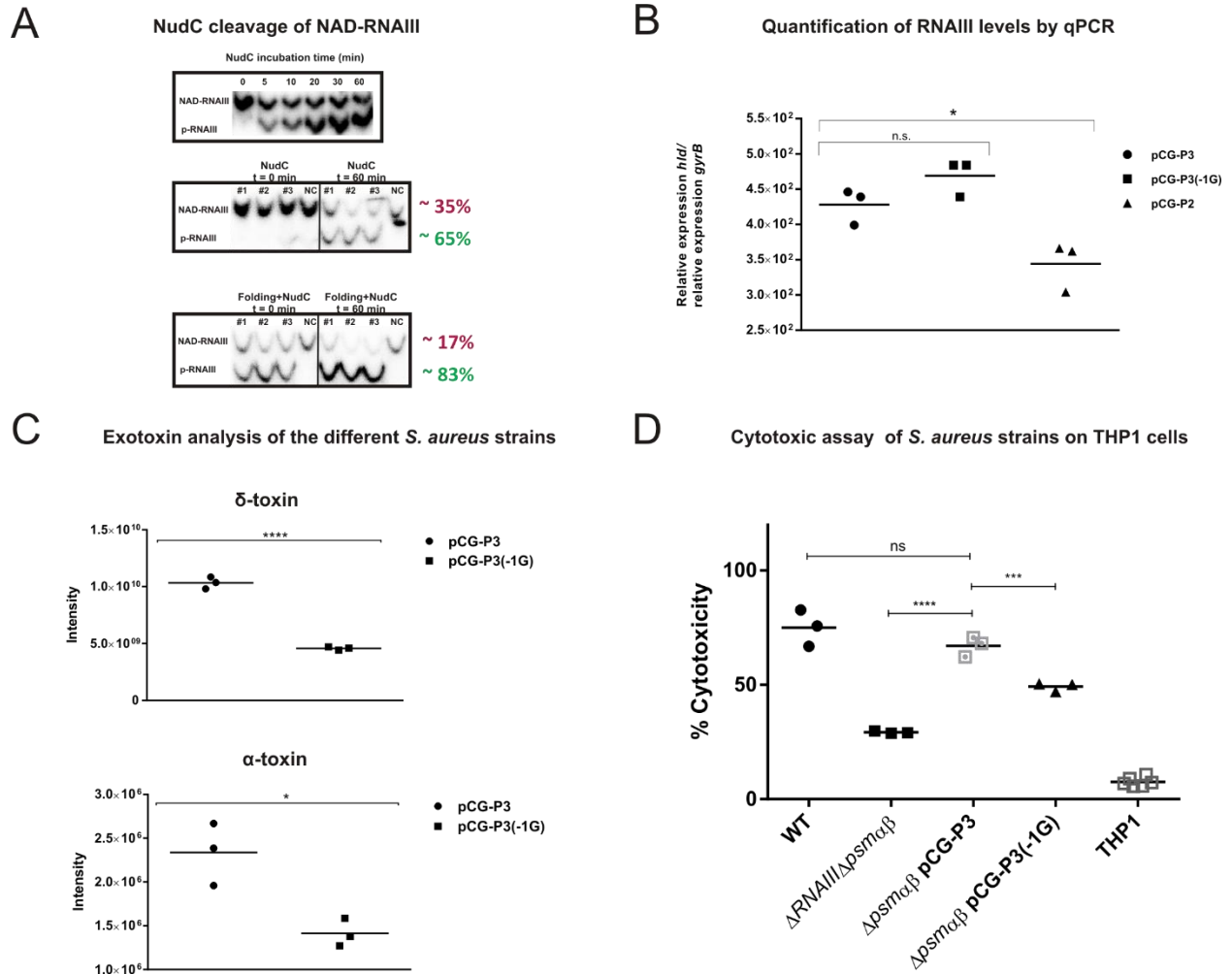
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Figure 2

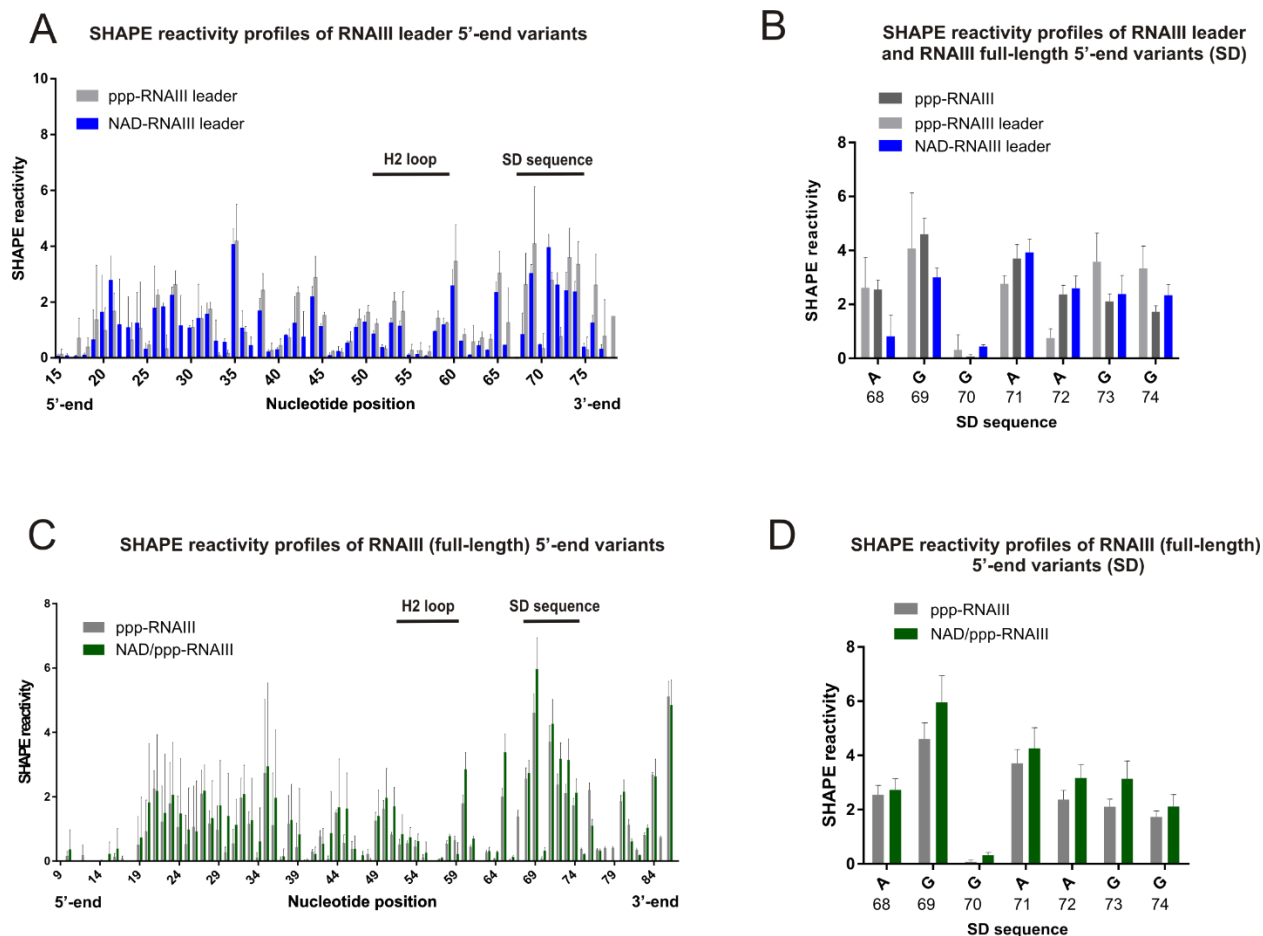
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Figure 3



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1118 **Figure 4**
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1120 **13 Supplementary tables**

1121 **Table S1:** Detection of NAD by UPLC/MS/MS in total RNA samples from *S. aureus*. I and II:
1122 reactions by duplicate performed with NudC. Column 2 shows the NudC-treated samples minus
1123 the negative control (non-treated). The third column shows the estimated NAD-RNA molecules
1124 per cell.
1125

	Sample (fmol NAD/ μ g RNA)	Sample - negative control (fmol NAD/ μ g RNA)	Estimated NAD-RNA molecules per cell
<i>S. aureus</i> I	31.52	23.61	839
<i>S. aureus</i> II	34.81	26.89	955

1126

1127 **Table S2:** Enriched hits after NAD captureSeq in *S. aureus* ATCC 25923 strain. Base mean:
 1128 normalised mapped reads, Log2FC: Log2 Fold change (sample vs negative control).

Gene annotation number	Gene product	Base mean	Log2FC	P-value
SAOUHSC_00070	HTH-type transcriptional regulator SarS	63.88	2.15	0.016
SAOUHSC_00086	3-ketoacyl-acyl carrier protein reductase putative	166.37	3.30	0.000
SAOUHSC_00204	Globin domain protein	111.18	2.67	0.002
SAOUHSC_00615	Haloacid dehalogenase-like hydrolase putative	39.83	3.15	0.002
SAOUHSC_00781	HPr kinase/phosphorylase	25.40	2.39	0.022
SAOUHSC_00834	Thioredoxin putative	12.54	2.82	0.011
SAOUHSC_00861	Lipoyl synthase	52.53	2.47	0.010
SAOUHSC_01162	Lipoprotein signal peptidase	10.36	4.07	0.000
SAOUHSC_01176	Guanylate kinase	25.63	2.28	0.032
SAOUHSC_01406	Acylphosphatase	14.93	2.70	0.008
SAOUHSC_01617	Arginine repressor	31.13	3.74	0.000
SAOUHSC_01685	Heat-inducible transcription repressor HrcA	464.91	3.73	0.000
SAOUHSC_02118	Glutamyl-tRNA(Gln) amidotransferase subunit	29.51	3.65	0.000
SAOUHSC_02260	Delta-hemolysin precursor/RNAIII	6404.64	5.64	0.000
SAOUHSC_02270	Ammonium transporter	12.55	2.22	0.047
SAOUHSC_02577	Putative 2-hydroxyacid dehydrogenase	276.48	2.96	0.001
SAOUHSC_02696	FmhA protein putative	70.17	2.86	0.002
SAOUHSC_02920	2-dehydropantoate 2-reductase	135.44	3.70	0.000
SAOUHSC_02922	L-lactate dehydrogenase	149.88	2.23	0.022
SAOUHSC_02942	Anaerobic ribonucleoside-triphosphate reductase putative	22.16	2.61	0.008
SAOUHSC_03045	Cold shock protein putative	29.67	2.60	0.009

1129

1130 **Table S3:** Oligonucleotides used in this study

Primer name	Sequence (5'-3')	Application
Fwd-P3-RNAIII	CGCGCGGATCCATAAAAAAATTTACAGTTAAGA ATAAAAAACG	pCG-671 cloning
Rv-P3-RNAIII	GCGCGAATTCAAGGCCGCGAGCTTG	pCG-671 cloning
Fwd-P2	GCGCGCGGATCCACAAATTACATTTAACAGTTAA G	pCG-672 cloning
Rv-P2hyb	CCATCACATCTCTGTGATCTCACTGTCATTATACG ATTTAG	pCG-672 cloning
Fwd-RNAIII	AGATCACAGAGATGTGATGG	pCG-672 cloning
Rv-RNAIII	GCGCGAATTCAAGGCCGCGAGC	pCG-672 cloning
Mut FW	GTTATATTTAAACATGCT	pCG-673 cloning
Mut -1G RV	GAGATCACAGAGATG	pCG-673 cloning
RNAIII term insertion FW	TAATGAGGCGCGCCTATTC	pCG-692, pCG-693 and pCG-694 cloning, introduction native RNAIII terminator
RNAIII term insertion RV	CAAAAAAGGCCGCGAGCTT	pCG-692, pCG-693 and pCG-694 cloning, introduction native RNAIII terminator
RNAIII 5'UTR FW	GTGATGGAAAATAGTTGATGAGT	qPCR
RNAIII 5'UTR RV	GCCATTGAAATCACTCCTTCC	qPCR
RNAIII mid FW	GAGTTAGTTTCCTTGGACTCAG	qPCR
RNAIII mid RV	GTTGTTTACGATAGCTTACATGC	qPCR
RNAIII 3' FW	GCATGTAAGCTATCGTAAACAAC	qPCR
RNAIII 3' RV	AGGGGCTCACGACCATACTT	qPCR
5S rRNA FW	TATAGCAAGGAGGTCACACCT	qPCR
5S rRNA RV	CTACCATCGACGCTAAGGAG	qPCR
gyrB FW	CAAATGATCACAGCATTGGTACAG	qPCR
gyrB RV	CGGCATCAGTCATAATGACGAT	qPCR
RNAIII NB probe FW	TAATACGACTCACTATAATTGAAATCACTCCTTCC TTAATTAAGATAAAAAATTCTTAAAA	Template for Northern Blot probe
RNAIII NB probe RV	AGATCACAGAGATGTGATGGAAAATAGTTGATG AGTTGTTTAAATTTAAGAATTTTATC	Template for Northern Blot probe
5SrRNA NB probe FW	TAATACGACTCACTATATAAGTTTCGACTACCATCG ACGCTAAGGAGCTTAACTTCTGTGT	Template for Northern Blot probe
5SrRNA NB probe RV	TCTGGTGACTATAGCAAGGAGGTCACACCTGTTC CCATGCCGAACACAGAAGTTAAGCTC	Template for Northern Blot probe
RNAIII PD probe	CTTGTGCCATTGAAATCACTCCTTCTT-biotin	Pull-down probe
5SrRNA PD probe	GGTGTGACCTCCTTGCTATAGTCACCAGA-biotin	Pull-down probe
RNAIII template T7 FW	TAATACGACTCACTATAAGATCACAGAGATGTGA TGG	Template for <i>in vitro</i> transcription
RNAIII template RV	AAGGCCGCGAGCTTGG	Template for <i>in vitro</i> transcription

RNAIII DNA enzyme I	GTTCACTGTGGGCTAGCTACAACGACGATAATCCAT	RNAIII cleavage by DNA enzyme
RNAIII DNA enzyme II	CGATAATCCAGGCTAGCTACAACGATTTACTAAGTCA	RNAIII cleavage by DNA enzyme
RNAIII DNA enzyme III	CGATTGTTGAAAGGCTAGCTACAACGAGATATCTTGTG	RNAIII cleavage by DNA enzyme
RNAIII R1	TGTTCACTGTGTCGATAATCC	cRT-PCR RNAIII
RNAIII F1	TCCTTGGAAGTCAAGTCTATG	cRT-PCR RNAIII
RNAIII R2	CACCGATTGTTGAAATGATATC	cRT-PCR RNAIII
RNAIII F2	GCTATCGTAAACAACATCGA	cRT-PCR RNAIII
RNAII F1	GTGGCAGTAATTCAGTGTATG	cRT-PCR RNAII
RNAII R1	GCATCCCTAATCGTACTTGC	cRT-PCR RNAII
RNAII R2	GATACGTGGCAAAGTGGTC	cRT-PCR RNAII
IS1 T7 FW	TAATACGACTCACTATAATATGCTTTGTATGTTCCG	Template for RNA internal standard
IS1 RV	CATTCTTTGCCTGTGACAGAC	Template for RNA internal standard
IS2 T7 FW	TAATACGACTCACTATAAGCCACGTCACCTGTCCGG	Template for RNA internal standard
IS2 RV	TAATACGACTCACTATAAGCCACGTCACCTGTCCGG	Template for RNA internal standard
IS3 T7 FW	TAATACGACTCACTATAAGCGCCGTCGGGCGTTGGT	Template for RNA internal standard
IS3 RV	GGCATATCCCTGGCGGGTGG	Template for RNA internal standard
IS4 T7 FW	TAATACGACTCACTATAACTATCTGAACCAAATATATC	Template for RNA internal standard
IS4 RV	GGCATTAAAGCAAATTTATC	Template for RNA internal standard
RNAIII-leader-FW	TAATACGACTCACTATAAGATCACAGAGATGTGATG	Template for <i>in vitro</i> transcription
RNAIII-leader-RV	CCGATTGTTGAAATGATATC	Template for <i>in vitro</i> transcription
SHAPE-RT1	CTAAGTCACCGATTGTTGAAATG	Reverse transcription for RNAIII SHAPE

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1132

1133 **Table S4:** IS NAD-RNAs used for NAD captureSeq with their sequence.

IS-RNA	RNA sequence
IS 1	AUAUGCUUUGUAUGUUCACGACGACCCGGGUCACGCACGCGGGUCUGACAGGCAAAGAAUG
IS 2	AGCCACGUCACCUUGUCCGUAUCCAUCAGACUUUGCGCCAUCGGGCCGUUGUCGACGGAAUAAACCCAC AUGUUGCGCUUCACCAGUUGGUAGGUCCAGCCGCC
IS 3	AGUCAGAGCGUAGUCAGCAUGCUAACAAGCGGCUGGCACGAUUGCUGAUUGCCUGGAAGCUGGAGCAA CAGCAACAGGAAAAUAGCGCGGCUGAAAUCGCAGCGGCGAAUGUCCAUCACCAGAUUGAACGUGGC AACCCGCGACGGACAUUACAGGGGAUGGCUUUUAUCGAAGGAUAAUGAAGGAUGAAACUGCCGGAAGG
IS 4	AGCGCCGUCGGGCGUUUGGUCGCCCCGUCGAUUUUUGUUUCUACCGUGGUCUGAACGUCAGUGAAGCU UCUGUACUGGUUACGCGCGCUUCCGAUCACAAUCCGCUACUCGUUGAAUUCAGUCCCGGCAAGCCUGAU AAUAAGGUAUGUCAGGUCUGCCACAGGGCAGACCAACGUUUGGCGUCGCGAAAACGUGAGCGCGGU GGUGGCGUAUGACUUAUCUGCCCACAUGCUGGAUGUCGUGGCACAAGCUGCCGAAGCCCGGCAACUGAA AAUAUCACCACCCGCCAGGGAUUGCC
IS 5	ACUAUCUGAACCAAAUUAUAUCACAUUUCAGCAGGGUUAUUAUUUCUAUAAACCGUUACAUAUAUC GACCUGGUCAAAAUUAUCCUUCUAAACCGAAGGUGAAGGUUGUGGUUGAGUGAAAAUUGAUCAGUAA GGCCAUAGUGCGGUGUAAUUAUAGACAGCUAAUUAAGCUCGUUGCCUCUUGUACUAUUGUUAUUAU UUUGUUUGCUAUAUUGUUUGAAAGUUUUGACAGGAUUGCCAUUAGUAGCAUGAACAAUAGUAAUAA UCUGGAUUAUUUCACUCUCUAUAUCAUUAUUUCCAUUGCAUUUAUGCUGAUCACCCUCCUGGUCAUCC UUAUUGCAAACCCAGUACCGGGCUGGGAGAAGUGCUUGUGACGAUAAAUUUGCUUAAUGCC

1134

1135 **Table S5:** Enriched hits after NAD captureSeq in *S. aureus* pCG-P3 strain. Base mean: normalised
1136 mapped reads, Log2FC: Log2 Fold change (sample vs negative control). In bold are highlighted
1137 the common hits to *S. aureus* ATCC 25923 wild type strain (Table S2) and in regular font the non-
1138 common hits.

Gene annotation number	Gene product	Base mean	Log2FC	P-value
SAOUHSC_00086	3-ketoacyl-acyl carrier protein reductase putative	60.65	2.49	0.000
SAOUHSC_00861	Lipoyl synthase	32.35	3.85	0.000
SAOUHSC_01685	Heat-inducible transcription repressor HrcA	127.01	2.84	0.000
SAOUHSC_02260	Delta-hemolysin precursor/RNAIII	5338.08	2.77	0.000
SAOUHSC_00467	Pur operon repressor	24.92	2.12	0.000
SAOUHSC_01191	50S ribosomal protein L28	18.24	2.28	0.000
SAOUHSC_01432	Peptide methionine sulfoxide reductase	13.50	3.53	0.000
SAOUHSC_01493	30S ribosomal protein S1 putative	36.32	2.05	0.000
SAOUHSC_01997	Peroxide-responsive repressor PerR	28.14	2.07	0.000
SAOUHSC_02019	Probable autolysin LytO	203.90	2.52	0.000
SAOUHSC_02409	Arginase	13.34	5.09	0.000

1139

1140 **Table S6:** Enriched hits after NAD captureSeq in *S. aureus* pCG-P3(-1G) strain. Base mean:
1141 normalised mapped reads, Log2FC: Log2 Fold change (sample vs. negative control). In bold are
1142 highlighted the common hits to *S. aureus* ATCC 25923 wild type strain (Table S2) and in regular
1143 font the non-common hits.

Gene annotation number	Gene product	Base mean	Log2FC	P-value
SAOUHSC_00086	3-ketoacyl-acyl carrier protein reductase putative	300.15	3.06	0.000
SAOUHSC_00781	HPr kinase/phosphorylase	31.15	3.96	0.000
SAOUHSC_01176	Guanylate kinase	12.58	2.53	0.000
SAOUHSC_01617	Arginine repressor	22.34	2.28	0.000
SAOUHSC_01685	Heat-inducible transcription repressor HrcA	184.56	2.50	0.000
SAOUHSC_02260	Delta-hemolysin precursor	16912.85	3.57	0.000
SAOUHSC_02577	Putative 2-hydroxyacid dehydrogenase	66.55	2.07	0.000
SAOUHSC_01191	50S ribosomal protein L28	67.43	2.27	0.000
SAOUHSC_01653	Superoxide dismutase 1	63.09	3.30	0.000
SAOUHSC_01981	Sensor histidine kinase putative	16.93	5.07	0.000
SAOUHSC_02019	Probable autolysin LytO	3110.21	3.23	0.000
SAOUHSC_02409	Arginase	26.68	3.04	0.000

1144

1145 **Table S7:** Enriched hits after NAD captureSeq in *S. aureus* pCG-P2 strain. Base mean: normalised
1146 mapped reads, Log2FC: Log2 Fold change (sample vs negative control). In blue are highlighted
1147 the common hits to *S. aureus* ATCC 25923 wild type strain (Table S2) and in green the non-
1148 common hits. Note that *hld*/RNAIII gene is not enriched in this table because due to its +1G
1149 condition.

Gene annotation number	Gene product	Base mean	Log2FC	P-value
SAOUHSC_00086	3-ketoacyl-acyl carrier protein reductase putative	47.53	2.68	0.000
SAOUHSC_00861	Lipoyl synthase	16.34	3.30	0.000
SAOUHSC_01685	Heat-inducible transcription repressor HrcA	71.22	2.81	0.000
SAOUHSC_01191	50S ribosomal protein L28	17.10	2.11	0.000
SAOUHSC_01997	Peroxide-responsive repressor PerR	18.60	2.19	0.000
SAOUHSC_02019	Probable autolysin LytO	125.51	3.02	0.000

1150

1151 **Table S8:** pCG-P3 vs pCG-P3(-1G) transcriptomic analysis. Base mean: normalised mapped reads,
1152 Log2FC: Log2 Fold change (sample vs negative control). Negative Log2 Fold change values
1153 indicate downregulation, whereas positive values account for upregulated genes.

Gene annotation number	Gene product	Base mean	Log2 Fold change	P-value
SAOUHSC_00069	Protein A	94209.43	-1.41	0.000
SAOUHSC_01938	Serine protease SplD	502.55	1.06	0.000
SAOUHSC_01939	Serine protease SplC	547.92	1.15	0.000
SAOUHSC_01941	Serine protease SplB	584.74	1.19	0.000
SAOUHSC_01942	Serine protease SplA	497.76	1.12	0.000
SAOUHSC_02576	Secretory antigen precursor SsaA putative	560.25	-1.28	0.000

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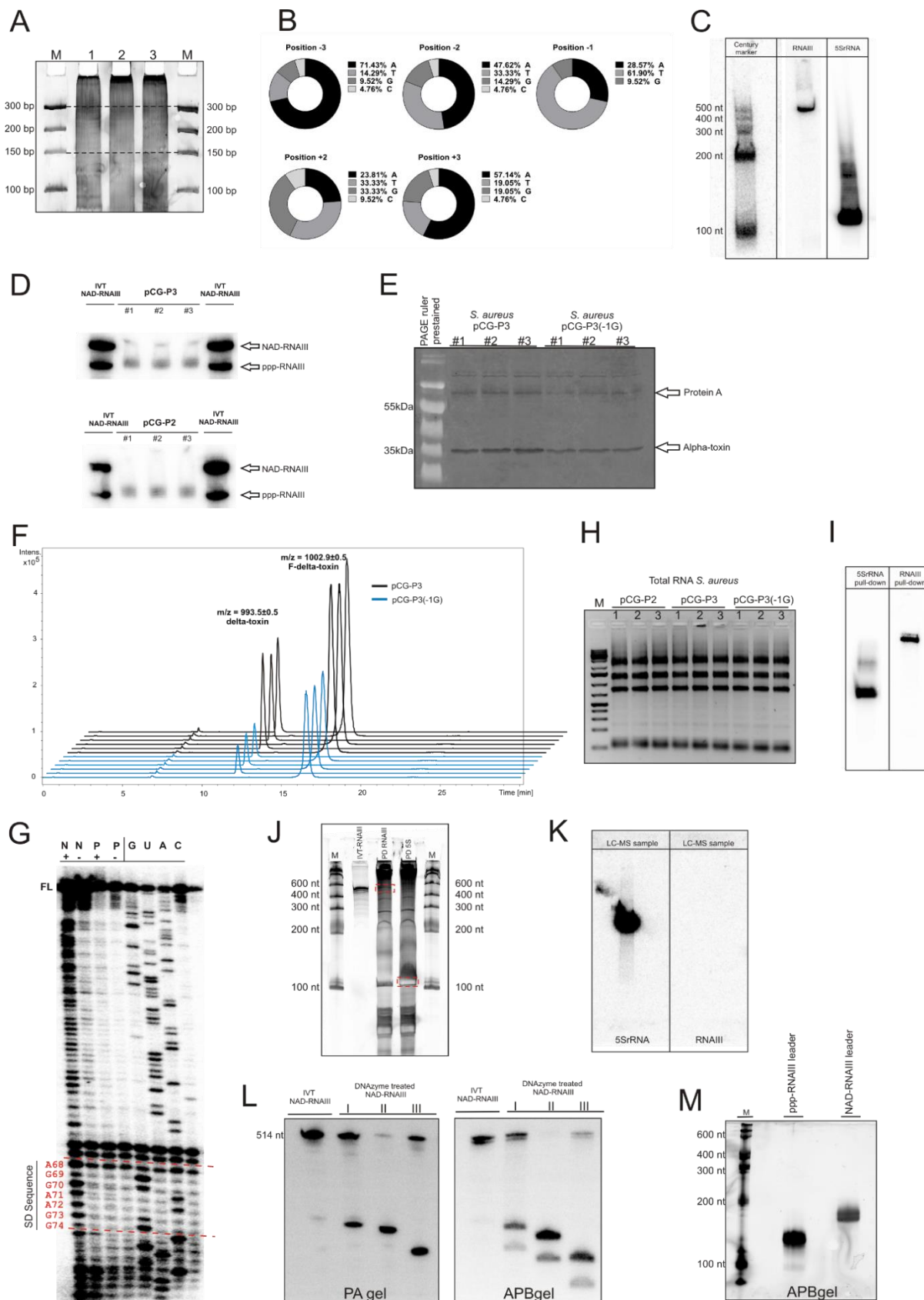
1155 **Table S9: Bacterial strains used in this study**

Bacterial strain	Source
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> ATCC 25923	American Type Culture Collection (ATCC)
<i>Escherichia coli</i> K-12	Lab strain collection
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> RN4220	Charpentier <i>et al.</i> , 2004
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> HG001	Herbert <i>et al.</i> , 2010
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> HG001 Δ RNAIII	This study
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> HG001 Δ RNAIII, Δ psm $\alpha\beta$	This study
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> HG001 Δ RNAIII pCG-P3	This study
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> HG001 Δ RNAIII pCG-P2	This study
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> HG001 Δ RNAIII pCG-P3(-1G)	This study

1156

1157 **14 Supplementary figures**

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Figure S1

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