1 **1. Title Page**

2 **(1) Title**

3 Ribosome profiling in *Streptococcus pneumoniae* reveals the role of methylation of 23S

4 rRNA nucleotide G748 on ribosome stalling.

5

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36 **2. Abstract**

37 **(1) Background**

38 Many nucleotides in 23S rRNA are methylated post-transcriptionally by
 39 methyltransferases and cluster around the peptidyltransferase center (PTC) and the
 40 nascent peptidyl exit tunnel (NPET) located in 50S subunit of 70S ribosome.
 41 Biochemical interactions between a nascent peptide and the tunnel may stall ribosome
 42 movement and affect expression levels of the protein. However, no studies have shown
 43 a role for NPET on ribosome stalling using an NPET mutant.

44

45 **(2) Results**

46 A ribosome profiling assay in *Streptococcus pneumoniae* demonstrates for the first time
 47 that an NPET mutant exhibits completely different ribosome occupancy compared to
 48 wild-type. We demonstrate, using RNA footprinting, that changes in ribosome
 49 occupancy correlate with changes in ribosome stalling. Further, statistical analysis
 50 shows that short peptide sequences that cause ribosome stalling are species-specific and
 51 evolutionarily selected. NPET structure is required to realize these specie-specific
 52 ribosome stalling.

53

54 **(3) Conclusions**

55 Results support the role of NPET on ribosome stalling. NPET structure is required to
56 realize the species-specific and evolutionary conserved ribosome stalling. These
57 findings clarify the role of NPET structure on the translation process.

58

59 **3. Keywords**

60 ribosome stalling

61 23S rRNA modification

62 *Streptococcus pneumoniae*

63 ribosome profiling

64 ribosomopathy

65

66 **4. Background**

67 Endogenous rRNA modifying enzymes methylate or pseudouridylate specific
68 rRNA nucleotides at functionally important regions in the ribosome, such as the
69 peptidyl transferase center (PTC) [1]. Approximately one-third of modified residues of
70 23S rRNA are clustered around the nascent peptide exit tunnel (NPET) [2]. While the
71 role of rRNA modification remains unclear, though it is generally believed that they
72 have fine-tune functions of the ribosome in translation [3], especially under the stress

73 conditions [4], via the biochemical interactions between the nascent peptide and tunnel
74 [5,6]. These interactions may stall ribosome movement and thus affect the expression
75 level of the protein [6]. However, no studies have shown the role of NPET in ribosome
76 stalling using an NPET mutant.

77 Some modifications of NPET are important for determining antibiotic
78 resistance or susceptibility [7,8]. While the role of the methylation at G748 (m¹G748) in
79 *Streptococcus pneumoniae* remains unclear, we previously showed that inactivation of
80 the methyltransferase RlmA^{II}, which methylates the N-1 position of nucleotide G748
81 located near the PTC, results in increased resistance to telithromycin (TEL) in
82 *erm*(B)-carrying *S. pneumoniae* [7].

83 We explored the role of NPET structure in translation initially by establishing
84 the ribosome profiling assay in *S. pneumoniae* and investigating ribosomal distribution
85 in both wild-type and RlmA^{II}-deficient *S. pneumoniae*. Subsequent analysis showed that
86 m¹G748 is responsible for ribosome stalling and plays a role in species specificity.

87

88 **5. Results**

89 **(1) The loss of N-1 methylation at G748 greatly changes the distribution of**
90 **ribosomes.**

91 We constructed two *S. pneumoniae* mutant strains, Sp284 and Sp379 to
92 investigate the role of NPET. Sp284 is a RlmA^{II}-disrupted mutant harboring pTKY1111
93 encoding *tlrB* of the S1 strain [7]. Sp379 is a RlmA^{II}-disrupted mutant harboring
94 pTKY1127 encoding *tlrB* of Sp44 [7]. The latter strains display no methyltransferase
95 activity because of the C23R mutation [7]. The difference between Sp284 and Sp379 is
96 only the capability to methylate G748.

97 We also performed a deep sequencing-based ribosome profiling analysis in
98 Sp284 and Sp379. Ribosome profiling captures a global snapshot of ribosome
99 positioning and density on template mRNAs with single-nucleotide resolution. The
100 *erm*(B) operon, where ribosomes stall at the *ermBL* region [9], was selected, for
101 example, to examine the distribution of ribosomes (Fig. 1A). Ribosome position and
102 density in the *erm*(B) operon in Sp379 was completely different from positions and
103 density in Sp284 (Fig.1A).

104 The difference in the ribosome occupancy between two strains in *erm*(B)
105 operon led us to speculate a general role of m¹G748 in the translation process.
106 Ribosome density across all ORFs in Sp379 was higher than in Sp284, especially
107 around the latter region, indicating an important role of m¹G748 in the translation
108 process, with differences in ribosome position and density across all open reading

109 frames (ORFs) being assessed using a metagene analysis (Fig. 1B).

110 **(2) m¹G748 affects ribosome stalling.**

111 A high number of ribosome-protected footprints (RPFs) mapping to the

112 transcriptome (mRNA-seq) at a unique position is indicative of ribosome stalling [10].

113 Thus, observed global changes imply a role for m¹G748 in ribosome stalling.

114 We tested this hypothesis by first constructing *erm*(B) operon-overexpression *S.*

115 *pneumoniae* strains Sp380 and Sp382 from Sp284 and Sp379 respectively, to clarify

116 differences between Sp284 and Sp379 (see Tables 1 and 2 for details). We then probed

117 ribosomes of Sp380 and Sp382 before and after extracting total RNA with dimethyl

118 sulfate.

119 Fig. 2A shows the secondary mRNA structure from the *ermBL* region that was

120 previously demonstrated [9]. Consistent with this report, nucleotides in the stems were

121 protected from chemical modification when probing after extracting total RNA in both

122 Sp380 and Sp382 strains. Conversely, these regions were not protected when probing

123 before extracting total RNA in Sp380 (Fig. 2B), but these regions remained unprotected

124 in Sp382 (Fig. 2B). Stems were likely disrupted in Sp380 *in vivo* presumably due to

125 ribosome stalling in the *ermBL* region. m¹G748 affects such stalling.

126 **(3) Characterization of effects of m¹G748 on ribosome stalling**

127 Ribosome stalling does not always mean an end to translation. Translation
128 resumes in some cases [11, 12]. Such cases are termed "transient stalling" [13] and may
129 contribute to cotranslational protein and be evolutionarily preferred [14, 15]. In contrast,
130 "strong stalling" does not appear to restart and requires rescue [16, 17]. We examined
131 the type of ribosome stalling affected by m¹G748 by first defining A-site peaks (see
132 Methods for details) and counted the number of A-site peaks across all CDSs (Figs. 3A
133 and 3B). Two populations observed in Sp284 (Fig. 3A) were consistent with a previous
134 report [16]. However, surprisingly, almost no transient stalling was observed in Sp379
135 (Fig. 3B), indicating a role for m¹G748 for keeping transient stalling. A slight decrease
136 in the number of peaks of strong stalling was observed compared to Sp284 (Figs. 3A
137 and 3B).

138 Stalling peptides were identified as previously described [13] to further
139 examine the role of m¹G748 on strong stalling. Briefly, we defined strong stalling as
140 described in Methods and collected nascent peptide sequences in the exit tunnel for
141 strong stalling events. We then calculated the probability of occurrence for 8,000
142 tripeptides and defined stalling peptides as tripeptides with a probability higher than
143 0.9999. Surprisingly, few stalling peptides were common between Sp284 and Sp379
144 (Fig. 3C), suggesting that the position of "strong stalling" is different between the two

145 strains.

146 Several known stalling peptides, such as PPP, KKK and KKR, are common
147 among some organisms, including *Saccharomyces cerevisiae* and *Escherichia coli* [18,
148 19]. However, stalling peptides in Sp284 and Sp379 did not include these previously
149 reported molecules [see Additional file 1], indicating that ribosome stalling is
150 species-specific.

151 **(4) m¹G748 is required to realize evolutionarily conserved ribosome stalling**

152 Stalling peptides in *S. pneumoniae* were different in previous reports, which led
153 us to speculate on how they are distributed in the proteome. We examined relationships
154 between stalling peptides and proteome in *S. pneumoniae*, by initially identifying 360 of
155 over-represented and 382 under-represented examples for three amino acids in the *S.*
156 *pneumoniae* proteome as previously [13]. Enrichment of over- and under-represented
157 tripeptides in stalling peptides were investigated using Fisher's exact test (Fig. 4).
158 Over-represented peptides were significantly enriched in the set from Sp379, with
159 under-represented peptides being significantly enriched in the stalling peptide set of
160 Sp284, indicating that the strong stalling is not evolutionarily favored [see Additional
161 file 2]. m¹G748 is likely required to realize evolutionarily conserved ribosome stalling.

162

163 **6. Discussion**

164 We investigated the role of NPET in translation using a ribosome profiling
165 assay in *S. pneumoniae*. The finding that the loss of the methyl group at m¹G748 had a
166 notable impact on the distribution of the ribosomes and ribosome stalling (Figs. 1 and 2)
167 highlights the importance of the NPET structure and explains why rRNA modifications
168 are clustered near the PTC and NPET. The role of NPET using other NPET mutants will
169 be investigated in the future.

170 Alteration of NPET results in changes to the stalling peptide set (Fig. 3C). Thus,
171 translation depends not only on mRNA sequence but also on the structure of NPET.
172 Prediction of ribosome stalling based only on mRNA sequence would be difficult.
173 Using NPET information is crucial because the structure of the NPET is not necessarily
174 unique in a cell [21], with some studies having tried to predict ribosome occupancy or
175 stalling [19,20], and performance of developed software could be improved with
176 additional parameters for NPET structure.

177 A global change in stalling position may result in a global effect on cell
178 proteome. This effect might explain why RlmA^{II} mutants of *S. pneumoniae* are rarely
179 clinically isolated [22]. The loss of the m¹G748 methyl group inhibits binding of
180 telithromycin to the exit tunnel [7], and may also cause a decrease in fitness of *S.*

181 *pneumoniae* in a clinical setting. This concept might be useful since antibiotics that
182 compromise targets that maintain healthy ribosomes would not cause resistant bacteria.
183 TEL-resistant *S. pneumoniae* ribosomes are unhealthy.

184 The ribosomopathy encompasses diseases caused by abnormalities in the
185 structure or function of ribosomal proteins or rRNA genes or other genes whose
186 products are involved in ribosome biogenesis [23-25]. Skeletal muscle atrophy [26],
187 Diamond–Blackfan anemia [24] and Treacher Collins syndrome [24] are examples of
188 ribosomopathy. However, no reports regarding exit tunnel-induced ribosomopathy exist.
189 The present study examined *S. pneumoniae*; however, exit tunnel-derived
190 ribosomopathy might be common among organisms, including humans. For example,
191 ribosomal protein L17 (RPL17) is upregulated in parallel with stress vulnerability [27].
192 RPL17 is located near the end of the exit tunnel [21]. Therefore, RPL17 could be
193 responsible for ribosome stalling. This concept, exit-tunnel-induced ribosomopathy,
194 might explain the mechanism of the disorder in the future.

195

196 **7. Conclusions**

197 We demonstrate the role of m¹G748 on ribosome stalling in *S. pneumoniae*.
198 m¹G748 is required to realize species-specific and evolutionarily conserved stalling.

199 The loss of the methyl group at m¹G748 has a great impact on the distribution of the
200 ribosomes and ribosome stalling, and results in exit-tunnel-induced ribosomopathy. This
201 might be the reason why RlmA^{II} mutants of *S. pneumoniae* are rarely clinically isolated.
202 This study is the first to show the role of NPET using an NPET mutant. These findings
203 clarify the role of NPET structure on the translation process.

204

205

206 **8. Methods**

207 **(1) Bacterial strains, plasmids, and media**

208 Bacterial strains and plasmids are shown in Tables 1 and 2, respectively. *S. pneumoniae*
209 strain S1 with reduced TEL susceptibility (MIC, 2µg/ml) was clinically isolated in
210 Japan [7]. Pneumococci were routinely cultured at 37°C and 5% CO₂ in air in a
211 brain-heart infusion with 0.5% yeast extract (BHI-Y) broth and BHI-Y agar,
212 supplemented with 5% horse blood. *E. coli* was grown in L broth (1% bact-tryptone,
213 0.5% bact yeast extract, 0.5% sodium chloride, pH 7.4) and L agar. When necessary,
214 medium was supplemented with kanamycin (25–500 µg/mL), spectinomycin (100
215 µg/mL) and ampicillin (25 µg/mL).

216 **(2) Transformation**

217 Synthetic competence-stimulating peptide (CSP) 1 and the method of Iannelli and Pozzi
218 [28] were used to transform *S. pneumoniae* S1 into a transformation-competent state.

219 **(3) RNA-Seq**

220 *S. pneumoniae* cultures were grown to log-phase; 2.8 mL of cultures were added to 2.8
221 mL of 100°C preheated RNA lysis buffer (1% SDS, 0.1 M NaCl and 8 mM EDTA) and
222 vortexed for 2 min. The resulting lysates were added to 5.6 mL of 100°C preheated acid
223 phenol (Sigma-Aldrich) and vortexed for 5 min. After centrifuging, RNA was extracted
224 from the aqueous phase using DirectZol (Zymo Research). rRNA was removed from
225 total RNA using MICROBExpress (Ambion). Resulting total mRNA (400 ng as an
226 input) was used for constructing the DNA library, using KAPA Stranded RNA-Seq
227 Library Preparation Kit Illumina platforms (KK8400). DNA libraries were sequenced
228 using the Illumina HiSeq 1500 system and single-end reads. Illumina libraries were
229 preprocessed by clipping the Illumina adapter sequence using Trimmomatic v.0.39 [29],
230 then aligned to the S1 genome sequence [7] using HISAT2 v.2.2.1 [30].

231 **(4) Ribo-Seq**

232 Libraries were prepared as previously described with some modifications (see below)
233 [10].

234 **Cell growth and harvest**

235 *S. pneumoniae* cultures (2.4 L) were grown to log-phase. Cells were pretreated for 2
236 min with ~ 100 $\mu\text{g/mL}$ chloramphenicol. Immediately after chloramphenicol
237 pretreatment, cultures were placed on ice. Cells were pelleted by centrifugation at 8,000
238 $\times g$ for 15 min at 4°C. After decanting the supernatant, cell pellets were resuspended in
239 2.5 mL of prechilled resuspension buffer [10 mM MgCl_2 , 100 mM NH_4Cl , 20 mM Tris
240 (pH 8.0), and 1 mM chloramphenicol].

241 **Lysate preparation and Nuclease digestion**

242 Cells were sonicated on ice and centrifuged at 12,000 $\times g$ for 10 min at 4°C. Aliquots of
243 lysate, containing 25 Abs_{260} ribosome units (1 $\text{A}_{260} = 12$ $\mu\text{g}/\mu\text{L}$) [5] were digested with
244 60 U of MNase (Roche) and 60 U of SUPERase[•] In (Ambion) with the addition of
245 chloramphenicol to a final concentration of 1 mM to remove unprotected mRNA and
246 generate footprint fragments. Digestion reactions were incubated for 1 h at 25°C and
247 quenched with the addition of EGTA to a final concentration of 6 mM.

248 **Sucrose fractionation**

249 Linear sucrose gradients [5–40% (wt/vol)] were prepared with 7.6 mL of buffer A and
250 buffer B [10 mM MgCl_2 , 100 mM NH_4Cl , 2 mM DTT, 20 mM, 0.2 mM
251 chloramphenicol, Tris pH 7.8 and 5% or 40% Sucrose, respectively], by loading Buffer
252 B on Buffer A in 16PA tube and placed vertically for 12 hr at 4°C the placed

253 horizontally for 2 hr at 4°C.

254 Digested samples were carefully loaded onto prepared gradients and centrifuged at
255 $124,700 \times g$ for 8 hr at 4°C in a P28S2 rotor. Sucrose gradients were fractionated
256 manually into 200 μ L portions and A_{260} was recorded for each fraction. Absorbance
257 values were graphed in Microsoft Excel to determine ribosome footprint associated
258 fractions (RPF fractions). RPF were consequently pooled.

259 **Ribosome footprint preparation**

260 RNA was purified from RPF using the SDS/hot acid phenol. Three mL of samples were
261 first denatured with SDS to a final concentration of 1% (wt/vol) and 2.7 mL of
262 preheated acid phenol (65°C) (Sigma-Aldrich). Mixtures were vortexed for 5 min at
263 65°C. After centrifuging, aqueous phases were mixed with one volume of acid phenol,
264 and 0.9 volumes of chloroform/isoamyl alcohol (24:1). RNA was precipitated with
265 ethanol and resuspended in 10 μ L of 10 mM Tris (pH 8.0).

266 Ribosome footprint samples were resolved on denaturing polyacrylamide gels for size
267 selection of footprint fragments. RNA samples were prepared for electrophoresis by
268 adding 2x Novex TBE-Urea Sample Buffer (Invitrogen). Ladder standards used a 0.05
269 μ g/ μ L 10-bp DNA Ladder (Invitrogen), prepared in 2x Novex TBE-Urea Sample Buffer
270 and 10 mM Tris (pH 8.0). Samples were resolved on a 15% TBE-Urea gel in $1 \times$ TBE

271 buffer for 65 min at 200V. Gels were stained for 3 min with SYBR Gold Nucleic Acid
272 Gel Stain (diluted from 10,000x in 1x TE; Invitrogen) and visualized by UV
273 transillumination. A band between 20 and 45 was excised using the 10-bp DNA ladder
274 to identify footprint fragments. RNA was recovered using the ZR small-RNA PAGE
275 Recovery Kit (Zymo Research) following the manufacturer's protocol, except that RNA
276 was eluted from the final spin column with 15 μ L of 10 mM Tris (pH 8.0). Collected
277 RNA was quantified and characterized by using a small-RNA chip on the Agilent
278 BioAnalyzer (Agilent Technologies).

279 **Dephosphorylation**

280 Three hundred pmol of footprint were denatured for 2 min at 80°C and placed on ice.
281 The 3' ends were dephosphorylated with T4 polynucleotide kinase (T4 PNK; NEB) in
282 the following reaction mix: 1 $\times$ T4 PNK reaction buffer (without ATP), 20 U
283 SUPERase[•] In, and 10 U T4 PNK. Reactions were incubated at 37°C for 1 hr. The
284 enzyme was then heat-inactivated for 10 min at 75°C. RNA was precipitated with
285 isopropanol and resuspended in 10 μ L of 10 mM Tris (pH 8.0).

286 **Linker-1 Ligation**

287 Twenty pmol of dephosphorylated RNA was prepared by diluting with 10 mM Tris (pH
288 8.0). One μ g of Linker-1 (5'-App CTGTAGGCACCATCAAT ddC-d') was added to

289 RNA samples. Mixtures were denatured for 90 s at 80°C, then cooled to room
290 temperature for 15 min. Ligation of RNA to Linker-1 used the following reaction
291 components: 20% (wt/col) PEG, 10% DMSO, 1 × T4 Ligase reaction buffer, 20 U
292 SUPERase[•] In, and 10 U T4 Ligase 2, truncated (NEB). Reaction mixtures were
293 incubated at 37°C for 1 h. 2 × TBE-Urea sample buffer was added to reaction mixtures.
294 Samples were resolved on a 10% TBE-Urea gel in 1 × TBE buffer at 200V. Gels were
295 stained for 3 min in SYBR Gold Nucleic Acid Gel Stain and visualized by UV
296 transillumination. A band between 30 and 70 nt was excised using the 10-bp DNA
297 ladder to mark ligated product size. Ligated RNA was recovered using the ZR
298 small-RNA PAGE Recovery kit. Ligated products were eluted from the final spin
299 column with 6 µL of 10 mM Tris (pH 8.0).

300 **Phosphorylation**

301 The collected 3' ligated samples were incubated for 2 min at 80°C and placed on ice.
302 The 5' ends were phosphorylated by T4 PNK in the following reaction mix: 1 × T4 PNK
303 Reaction Buffer, 20 U SUPERase[•] In, 10 U T4 PNK and 1 mM ATP. Reactions were
304 incubated at 37°C for 1 hr. The enzyme was heat-inactivated for 10 min at 75°C. RNA
305 was precipitated with isopropanol and resuspended in 6 µL of 10 mM Tris (pH 8.0).

306 **Linker-2 ligation**

307 1 μ L of 100 μ M Linker-2
 308 (5'-GAGTCTGCGTGTGATTCGGGTTAGGTGTTGGGTTGGGCCA-3') was added to
 309 phosphorylated RNA samples. Mixtures were denatured for 15 min at 65°C and placed
 310 on ice. Ligation of RNA to Linker-2 used the following reaction mix: 17.5% (wt/vol)
 311 PEG, 1 $\times$ T4 ligase reaction buffer, 20 U SUPERase[•] In, and 10 U T4 RNA Ligase1
 312 (NEB). Reaction mixtures were incubated at 37°C for 2.5 h. Two $\times$ TBE-Urea sample
 313 buffer was added and ligated RNA resolved on 10% TBE-Urea gels in 1 $\times$ TBE buffer at
 314 200V. Gels were stained for 3 min in SYBR Gold Nucleic Acid Gel Stain and visualized
 315 by UV transillumination. A band between 90 and 120 nt was excised using the 10-bp
 316 DNA ladder to identify ligated products. Ligated RNA was recovered using the ZR
 317 small-RNA PAGE Recovery kit. Products were eluted from the final spin column with 6
 318 μ L of 10 mM Tris (pH 8.0).

319 **Reverse transcription**

320 Four point five μ L of ligated samples were mixed with 1 μ L of 0.1 μ M Linker-1-RT
 321 (5'-ATTGATGGTGCCTACAG-3') and 1 μ L of 0.5 mM dNTP. The resulting mixtures
 322 were denatured for 2 min at 80°C then quickly cooled on ice. Samples were incubated at
 323 room temperature for 10 min reverse transcription using SuperScript III Reverse
 324 Transcriptase (Invitrogen) with the following reaction mix: 1 $\times$ first strand buffer, 5 mM

325 DTT, 20 U SUPERase[•] In and 200 U SuperScript III Reverse Transcriptase. Reaction
326 mixtures (10 µL) were incubated for 1 hr at 47°C. RNA products were hydrolyzed by
327 adding 1 mM NaOH to a final concentration of 0.1 mM and incubated for 15 min at
328 95°C. cDNA products were resolved from the unextended primer on a 10% TBE-Urea
329 gel in 1 × TBE buffer at 200V. Samples were prepared for electrophoresis by adding 2 ×
330 TBE-Urea sample buffer and denaturing for 5 min at 95°C. Gels were stained for 3 min
331 in SYBR Gold Nucleic Acid Gel Stain and visualized by UV transillumination. A band
332 between 90 and 120 nt was excised using the 10-bp DNA ladder to identify reverse
333 transcription products. DNA was recovered using the ZR small-RNA PAGE Recovery
334 Kit. cDNA products were eluted from the final spin column with 6 µL of 10 mM Tris
335 (pH 8.0).

336 **2nd strand DNA synthesis**

337 cDNA (100 pg) was amplified with Q5 High Fidelity Polymerase (NEB) using
338 Linker-2-partial (5'-TTAGGTGTTGGGTTGGGCCA-3') and Linker-1 as primers.
339 Amplified PCR products were purified using AMPure Bead (Beckman Coulter).
340 Double-stranded DNA was eluted from beads with 10 µL of 10 mM Tris (pH 8.0).

341 **Library preparation and sequencing**

342 KAPA Hyper Prep Kits from Illumina (KK8500) were used to construct the library with

343 a slight modification to the manufacturer's protocol. The manufacturer's protocol
344 followed the EndRepair and A-tailing steps, where, briefly, 5 μ L of KAPA frag buffer
345 was added to 45 μ L of purified double-stranded DNA. The resulting DNA library was
346 quantified and characterized using the high-sensitivity DNA chip on an Agilent
347 BioAnalyzer (Agilent Technologies). Libraries were sequenced using an Illumina HiSeq
348 1500 system and single-end reads after adding PhiX (Illumina) to a final concentration
349 of 30% (vol/vol) to improve sequencing quality.

350 **Sequence analysis**

351 Illumina libraries were preprocessed by clipping adapter sequence (Linker-1 and
352 Linker-2) using TrimmomaticPE v.0.39 [29] for the Illumina adapters and CutAdapt
353 v.2.10 [31] for linker sequences. Sequencing reads were aligned to the S1 genome
354 sequence [7] using HISAT2 v.2.2.1 [30]. The S1 gene feature file [7] was used to
355 identify the CDS region. Sequencing data were deposited in the DDBJ database with
356 accession number DRA011224.

357 **(5) Definition of the ribosome density**

358 The ribosome density (RD) was calculated as previously described [32], except that
359 ribosome footprints between 24 and 30 nt were selected for calculations. This range was
360 chosen to assess as many footprints as possible to improve statistical power and to

361 exclude fragments suspected not to represent footprints (see Additional file 3 for the
362 detail).

363 **(6) Metagene analysis**

364 Each normalized RD profile was aligned by its start codon and averaged across each
365 position, with RD profiles first being scaled by their own mean density to obtain
366 metagene profiles (Fig. 1B).

367 **(7) DMS modification of ribosomes**

368 Dimethyl sulfate (DMS) modification was based on a previously published protocol [7].

369 *S. pneumoniae* cultures (2.4 L) were grown to log-phase. Cells were pelleted by
370 centrifugation at $8,000 \times g$ for 15 min at 4°C and washed and resuspended in 250 µL of

371 DMS buffer [10 mM MgCl₂, 50 mM sodium cacodylate pH 7.0]. For the modification

372 *in vivo*, DMS solution was added to a final concentration of 50 mM and incubated for 1

373 hr at 20°C. The reaction was quenched by the addition of 62.5 µL ice-cold stop buffer [1

374 M β-mercaptoethanol and 1.5 M sodium acetate, pH 7.0]. RNA was extracted using

375 SDS/hot acid phenol method. Preheated (100°C) acid phenol (0.9 volumes) was added

376 to the quenched mixture and vortexed for 5 min. After centrifugation, RNA was purified

377 from the aqueous phase using DirectZol (Zymo Research). For the modification *in vitro*,

378 RNA was first extracted from the cell suspension using the same method as for *in vivo*.

379 After purifying the total RNA, DMS solution was added to a final concentration of 50
380 mM in DMS buffer and incubated for 1 hr at 20°C then quenched. RNA was
381 precipitated with ethanol and resuspended in 20 µL of 10 mM Tris (pH 8.0).

382 **(8) Primer extension**

383 The degree of methylation of each RNA was assayed by primer extension initially
384 described by Morgan et al. [33]. Briefly, 210 µg of DMS-probed total RNA, 400 nM
385 concentration of an oligonucleotide primer containing 5' - linked fluorescein
386 isothiocyanate (FITC)
387 (5'-[FITC]GAATTAATCTAACGTATTTATTTATCTGCGTAATCA -3'), and 500 µM
388 dNTP were mixed, heated to 95°C for 1 min, and cooled to allow annealing at 53°C. A
389 mixture containing SuperScript III Reverse Transcriptase was added, and the reaction
390 was continued at 53°C for an additional 20 min. Reactions were terminated by the
391 addition of 2 × TBE-Urea sample buffer after incubation at 70°C for 15 min. Extension
392 products were resolved on a 12.5% TBE-urea gel and visualized using a
393 TyphoonFLA9000 photoimager (GE Healthcare).

394 **(9) Definition of A-site peaks**

395 We first determined the A-site corresponding to each read by an offset of $[(15/27) \times (L)]$
396 from the 5' end of the read, where L is the length of each read. Then we used normalized

397 A-site count (ribo/mRNA) as A-site peaks.

398 **(10) Definition of strong stalling**

399 Strong stalling is defined as A-site peaks with a height of higher than eight because the
400 number of A-site peaks in the *ermBL* region was around eight. This region is where
401 ribosome stalling was confirmed by RNA footprinting. The results, however, remain
402 robust also for other cutoff values of A-site density.

403 **(11) Enrichment of over-, under-represented peptide sequence**

404 Over- and under-represented peptide sequences were identified as previously described
405 [13], except that *p*-value cutoff is 0.0001 for under-represented peptides and 0.9999 for
406 over-represented peptides.

407

408 **10. Supplementary Information**

409 Additional file 1: Supplementary Tables S1

410 Additional file 2: Supplementary Tables S2

411 Additional file 3: Supplementary Fig. S1

412

413 **11. Declarations**

414 **(1) Ethics approval and consent to participate**

415 Not applicable.

416 **(2) Consent for publication**

417 Not applicable.

418 **(3) Availability of data and materials**

419 All data generated in this study have been deposited to the DDBJ depository
420 (DRA011224).

421 **(4) Competing interests**

422 The authors declare that they have no competing interests.

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426 **(6) Author contributions**

427 ST contributed to the conception and overall design of the work, all of experiments,
428 bioinformatics analysis and interpretation of Ribo-Seq data and drafting of the
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430 Ribo-Seq experiments. TH contributed to bioinformatics analysis, interpretation of
431 Ribo-Seq data, drafting of the manuscript and substantial revision of the manuscript. YT
432 contributed to the drafting of the manuscript. KH contributed to the conception and

433 overall design of the work, interpretation of data, and drafting and revision the
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437

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529

530 **13. Figure Legends**

531 **Fig. 1 Differences in ribosome density (RD) profiles between Sp284 and Sp379.**

532 (A) RD in *erm(B)* in Sp284 or Sp379. (B) Metagene profile of RD in Sp284 or Sp379.

533 RD across all ORFs was aligned relative to the start position.

534

535 **Fig. 2 RNA footprinting assay for the *ermBL* region.**

536 (A) The secondary structure model of *ermBL* region in *erm(B)* mRNA suggested in [9].

537 Amino acids in at the A-site of stalled ribosomes in the *ermBL* region are boxed. (B)

538 RNA footprinting of the *ermBL* region indicates a difference in ribosome stalling

539 between Sp380 and Sp382. DMS was added to bacterial cultures (*in vivo*) or the total

540 RNAs (*in vitro*). Reactions were quenched, and total RNA was purified and used in

541 primer extension assays to detect base modifications. Unmodified RNA was used as a

542 control (DMS (-)). The gel shows primer extension products from bases 117 to 152. The

543 sequence around the calculated P-site and encoded amino acids are indicated on the side

544 of the gel.

545

546 **Fig. 3 The effect of m1G748 on transient stalling and strong stalling. (A, B)**

547 Histograms showing the distribution of A-site density in Sp284 and Sp379, respectively.

548 (C) Venn diagram for stalling peptide sequences in Sp284 and Sp379. The number of

549 stalling peptide sequences is shown in parentheses. The number in the intersection

550 region reflects common stalling peptides.

551

552 **Fig. 4 Enrichment of over- and under-represented stalling peptides**

553 The number of the stalling peptides are represented at the center of the donut. The
554 p -value represents statistical significance of the over- and under-represented fractions.

555

556 **14. Supplementary Figure Legends**

557 **Fig. S1 Histograms showing the distribution of length of RFPs in Sp284 and Sp379,**
558 **respectively.**

559

560 **15. Supplementary Table Legends**

561 **Table S1 The list of stalling peptides.**

562 The first and the second columns list stalling peptides in Sp284 and Sp379 respectively.

563 The third column shows the list of stalling peptides that are common between strains.

564

565 **Table S2 The list of over- and under-represented peptides.**

566 The first and the second columns list over-represented peptides and under-represented
567 peptides in *S. pneumoniae* proteome composition.

568

569 **16. Tables**

570

571 **Table 1** **Bacterial strains**

Strain	Relevant characteristics	Reference of source
<i>Streptococcus pneumoniae</i>		
S1	TEL resistance clinical isolate	[7]
Sp36	TEL-resistant mutants of S1 isolated from 8 μg/mL TEL-containing BHI-Y agar plates	[7]
Sp274	$\Delta tlrB::aad(9)$ in S1	[7]
Sp284	Sp274 harboring pTKY1111	This study
Sp379	Sp274 harboring pTKY1127	This study
Sp380	S1 harboring pTKY1041	This study
Sp382	Sp274 harboring pTKY1041	This study

572

573 **Table 2** **Plasmids**

Plasmid	Relevant characteristics	Reference of source
pUC18	Cloning vector	Lab. collection
pLZ12-Km2	Shuttle vector	[34]
pTKY862	pLZ12-Km2 with Sp resistant cassette <i>aad(9)</i>	[35]

pTKY1041	pLZ12-Km2 with 1401 bp <i>erm</i> (B) fragment [7]
	from S1
pTKY1109	pUC18 with 328 bp <i>tlrB</i> fragment [7]
pTKY1110	pUC18 with disrupted <i>tlrB</i> fragment by [7]
	insertion of an Sp resistant cassette <i>aad</i> (9)
pTKY1111	pLZ12-Km2 with 1065 bp <i>tlrB</i> fragment from [7]
	S1
pTKY1127	pLZ12-Km2 with 1065 bp <i>tlrB</i> fragment from [7]
	Sp44

574

(A)

(B)

Figure 1 Shoji et al.

Figure 2 Shoji et al.

Figure 3 Shoji et al.

Figure 4 Shoji et al.