1 Methylation of 23S rRNA G748 and the ribosomal protein L22 Lys-94 are critical
 2 factors for maintaining the association between ribosome stalling and proteome
 3 composition in *Streptococcus pneumoniae*

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9

10 **Abstract**

11 **Background:** 23S rRNA modification located at the nascent peptides exit tunnel plays
 12 an important role in both translation processes and the binding of the antibiotics.
 13 Methylation of the guanine at position 748 (m¹G748) in 23S rRNA in *Streptococcus*
 14 *pneumoniae* is involved in the ribosome stalling and the binding of the antibiotic
 15 telithromycin (TEL). The disruption of the gene encoding RlmA^{II} which methylates 23S
 16 rRNA G748 results in the increased resistance of TEL in *S. pneumoniae*. However, an
 17 isolated high-level TEL-resistant *S. pneumoniae* strain indicated that additional
 18 undescribed factors were involved in TEL resistance in *S. pneumoniae*.

19 **Results:** We successfully isolated a high-level TEL-resistant *S. pneumoniae* RlmA^{II}
 20 mutant and determined the whole-genome sequence. The lysine residue at the position
 21 94 in ribosomal protein 22 (L22 K94) was critical in binding of TEL to the ribosome. A
 22 growth competition assay showed that L22 K94 was required for the function of
 23 m¹G748. Ribosome profiling revealed that m¹G748 and L22 K94 were both essential to
 24 maintain the relationship between the ribosome stalling and proteome composition.

25 **Conclusion:** In *S. pneumoniae*, the combination of methylation status of G748 and the
 26 residue at position 94 in L22 are essential for both the distribution of ribosome stalling
 27 and the binding of TEL to ribosomes.

28

29 **Keywords:** ribosome stalling, 23S rRNA modification, *Streptococcus pneumoniae*,
30 ribosome profiling, nascent peptide exit tunnel.

31

32 **1. Background**

33 Ribosomes are the cellular location where proteins are synthesized. The
34 structure of the peptidyltransferase center (PTC) is essential for the protein translation
35 reaction. This structure consists of not only 23S rRNA but also the result of the
36 biochemical interaction between the nascent peptides and the nascent peptides exit
37 tunnel (NPET) [1,2]. Therefore, the structure of the NPET is believed to include
38 ribosomal protein and rRNA, and rRNA modification is critical for the function of the
39 ribosome in translation of mRNA to protein [3]. Previously, we demonstrated that
40 ribosome stalling is affected by the methylation of the 23S rRNA at position G748,
41 which is located at the NPET near the PTC [4].

42 Several modifications located in the NPET play important roles in determining
43 antibiotic resistance or sensitivity [5]. Previously, we showed that the inactivation of the
44 methyltransferase RlmA^{II}, which methylates the N-1 position of nucleotide G748
45 (m¹G748), or the inactivation of the methyltransferase RlmCD, which methylates the

46 N-5 position of nucleotide U747 (m⁵U747) and C-5 position of U1939, results in
 47 increased resistance to telithromycin (TEL) in *erm*(B)-carrying *Streptococcus*
 48 *pneumonia* [6,7]. The minimum inhibitory concentration (MIC) for TEL in the
 49 RlmA^{II}-disrupted or RlmCD-disrupted *S. pneumoniae* mutants is 16 to 32 or 8 µg/mL,
 50 respectively. However, Walsh et al. isolated a high-level TEL-resistant *S. pneumoniae*
 51 whose MIC for TEL was more than 512 µg/mL, [8] indicating that additional factors
 52 associated to the TEL-resistance are present other than m¹G748 and m⁵U747.

53 In this study, we sought to identify the additionally factors that are associated
 54 with the high-level TEL resistance in *S. pneumoniae*. We initially isolated a high-level
 55 TEL-resistant *S. pneumoniae* mutant from an RlmA^{II} mutant and determined the
 56 whole-genome sequence of these mutants. Subsequent analysis showed that the lysine
 57 residue at position 94 in the ribosomal protein L22 (L22 K94) is a key factor involved
 58 in high-level TEL resistance and that L22 K94 is necessary for the function of m¹G748.

59

60 **2. Results**

61 **2.1. Mutation of L22 K94E produces high-level TEL resistance in *S. pneumoniae***

62 We previously isolated TEL-resistant mutants from S1, a clinically isolated *S.*
 63 *pneumoniae* strain with low TEL susceptibility (MIC, 2 µg/mL), by selection with 8 and

64 16 µg/mL of TEL to yield 10 and 6 mutants, respectively. Each of the 16 strains (Sp32
65 to Sp47) contained one unique nucleotide difference in the *tlrB* gene that encodes
66 RlmA^{II} but did not contain any shared mutations [6]. This result indicates that mutations
67 involving high-level TEL resistance would occur only after the mutation of the *tlrB*
68 gene. Thus, we sought to isolate high-level TEL resistance mutants from Sp36, one of
69 16 mutants described above, by selection with 128 µg/mL of TEL. As a result, several
70 mutants (Sp48 to Sp52) were isolated that all increased MICs (> 512 µg/mL).

71 To determine the mutations involved in high-level TEL resistance,
72 whole-genome sequencing of strains S1 and Sp52 was performed by next-generation
73 sequencing technology, and open reading frame (ORF) sequences were analyzed for the
74 two strains. As a result, one mutation was found in a gene encoding a ribosomal protein
75 L22, leading to an Lys94Glu alteration in this protein in Sp52. Furthermore, this
76 mutation was confirmed in mutants Sp48 to Sp51 but was absent in mutants Sp32 to
77 Sp47. According to the three-dimensional crystal structure of the *Escherichia coli* 50S
78 subunit of the 70S ribosome bound to TEL (Protein Data Bank: 3OAT) [9], the L22 K90
79 mutant protein, which corresponds to L22 K94 in *S. pneumoniae*, is a component of
80 NPET and is located opposite m¹G748; this residue appears to form three hydrogen
81 bonds to the oxygen atom in TEL via the methyl group at the end of side chain in K90

(Figure 1). Therefore, the K94E mutation would lose these hydrogen bonds. These results suggest that the L22 K94E mutation leads to the high level of TEL resistance in *S. pneumoniae*.

2.2. L22 K94 is necessary for the function of m¹G748

We were unable to isolate L22 K94E mutant directly from S1, which implies a genetic interaction between m¹G748 and L22 K94. The *S. pneumonia* mutant that harbors the wild-type *tlrB* and the mutated L22 K94E may exhibit a lower fitness compared with that of S1, Sp36, and Sp52. To examine this hypothesis, we introduced a plasmid encoding the wild-type *tlrB* gene to Sp52 (Sp375) and compared the growth for the following four strains: S1, Sp36, Sp52, and Sp375 (Figure 2A). As expected, Sp52 and Sp375 exhibited much lower growth compared with that of S1 and Sp36. To clarify the growth difference between Sp52 and Sp375, we performed a growth competition assay between these two strains. As a result, Sp375 rapidly disappeared during subculturing (Figure 2B). This result clearly demonstrated that L22 K94 is required for the functioning of m¹G748.

2.3. m¹G748/L22 K94 affects ribosome stalling

We previously showed that m¹G748 affects ribosome stalling. Thus, to investigate the role of L22 K94 in ribosome stalling, we performed ribosome profiling

100 assay in four strains: WT, S1ΔrlmA^{II}/pRlmA^{II}(wild-type); L22 mutant,
 101 Sp52ΔrlmA^{II}/pRlmA^{II}(wild-type); Double mutant, Sp52ΔrlmA^{II}/pRlmA^{II}(C23R);
 102 RlmA^{II} mutant, S1ΔrlmA^{II}/pRlmA^{II}(C23R); note that RlmA^{II} with a mutation of C23R
 103 exhibits no methyltransferase activity [6]. The *erm*(B) operon, where the ribosomes stall
 104 at the *ermBL* region [10], was selected as an example to observe the distribution of
 105 ribosomes. The ribosome position and density in the *erm*(B) operon were completely
 106 different between the four strains (Figure 3A).

107 The difference in the ribosome occupancy among these four strains in the
 108 *erm*(B) operon led us to speculate on the general role of m¹G748 and L22 K94 in
 109 ribosome stalling. We previously defined ribosome stalling as a A-site density (see
 110 Method section) higher than eight based on the result of the RNA footprinting assay [4].
 111 Thus, we performed metagene analysis to obtain an overview of global differences in
 112 ribosome stalling across all ORFs (Figure 3B). This showed that ribosome stalling
 113 across all ORFs in mutants was higher than that of WT especially around the 100-500
 114 residue region, indicating the important role of m¹G748 and L22 K94 in ribosome
 115 stalling.

116 **2.4. m¹G748/L22 K94 has a major impact on the interaction between the NPET**
 117 **and nascent peptides**

118 One of the reasons for ribosome stalling is the biochemical interaction between
119 nascent peptides and the NPET. To investigate the role of m¹G748 and L22 K94 on the
120 biochemical interaction in NPET, the “stalling peptides” were identified as previously
121 described [11]. Briefly, we first collected the nascent peptide sequences in the exit
122 tunnel for all of ribosome stallings and calculated the probability of the occurrence for
123 each of 8,000 tripeptides. Then, we defined the stalling peptides as tripeptides with a
124 probability higher than 0.9999 and compared the stalling peptides among the four
125 strains. Surprisingly, there were very few stalling peptides that were commonly found
126 among these strains (Figure 4A), suggesting that the biochemical interaction in the
127 NPET differs among the four strains.

128 The length of the NPET is approximately 31 amino acids [11]. To test whether
129 m¹G748 and L22 K94 directly or indirectly interact with the NPET, the frequency of the
130 occurrence of the stalling peptides at each of the 29 positions in the NPET was
131 calculated. The stalling peptides tend to occur near the PTC in all four strains (Figure
132 4B), indicating a direct biochemical interaction between m¹G748/L22 K94 and NPET.

133 **2.5. m¹G748/L22 K94 is a general factor which affects the association between** 134 **ribosome stalling and proteome composition**

135 Stalling peptides cause ribosome stalling, which essentially ends the translation

136 process [12, 13]. Therefore, the sequences of the stalling peptides are expected to be
 137 evolutionary underrepresented. To examine this hypothesis, we first identified 360
 138 overrepresented and 382 underrepresented tripeptides in the proteome of *S. pneumoniae*.
 139 Then, the enrichment of over- and underrepresented tripeptides in the stalling peptides
 140 was calculated using the Fisher's exact test (Figure 5A). While both over- and
 141 underrepresented peptides were significantly enriched in the stalling peptides set of WT,
 142 neither of these sets were enriched in the other three strains. This indicated that the
 143 stalling peptides are evolutionarily conserved and that m¹G748 and L22 K94 are
 144 important for maintaining the relationship between ribosome stalling and the proteome
 145 composition.

146 To further examine the role of m¹G748 and L22 K94 in the proteome
 147 composition, we calculated the 8,000-dimension vector representing the frequency of
 148 the occurrence of all 8,000 tripeptides in the proteome for 12 gram-positive bacterial
 149 species in which the methylation of G748 and the sequence of L22 have been revealed
 150 [14]. Then, we drew a phylogenetic tree based on this frequency vector. Surprisingly,
 151 the proteome composition segregated into two groups in a manner largely consistent
 152 with the combination of the mutated residue at position 94 in L22 and the status of
 153 G748 methylation (Figure 5B), suggesting that m¹G748/L22 K94 are the general factors

154 for controlling the ribosome stalling against the proteome composition.

155

156 **3. Discussion**

157 In this study, we successfully identified L22 K94 as the key factor involved in
158 the high-level TEL resistance in *S. pneumoniae*. We revealed that L22 K94 is required
159 for the function of m¹G748, which is important for ribosome stalling at the appropriate
160 position to maintain proteome composition. The reason TEL exhibits a strong effect
161 specifically on *S. pneumoniae*, and why TEL-resistant mutants of *S. pneumoniae* are
162 rarely clinically isolated [15] may be because the regions where TEL binds are
163 indispensable for *S. pneumoniae* to control ribosome stalling at the correct position.
164 Here, the growth of both the Double mutant and the L22 mutant were much lower than
165 that of the others (Figure 2).

166 L22 K94 is located on the opposite side of m¹G748 in the NPET [9]. Several
167 rRNA modifications are clustered around m¹G748 and L22 K94 [16]. These
168 modifications, together with m¹G748 and L22 K94, compose a narrow gate in the NPET
169 called the “discriminating gate” [1]. Thus, the discriminating gate may function to
170 control ribosome stalling at the appropriate position to maintain proteome composition.
171 This implies the structure of the discriminating gate might be different among species

172 since the discriminating gate is responsible for the proteome composition which is
 173 different among species . Therefore, antibiotics that target the discriminating gate would
 174 be species-specific and would not induce resistance in non-targeted bacteria. This is a
 175 new concept for designing antibiotics, and therefore, discriminating gate-targeting
 176 antibiotics may form a new class of antibiotics that should be investigated in future
 177 studies.

178 The combination of the residue at the position 94 in L22 and the status of G748
 179 methylation correlated to the composition of the proteome (Figure 5B). This means the
 180 distribution of the ribosome stalling could explain the differences among species. Since
 181 the distribution of the ribosome stalling is summarized as the probability of the
 182 occurrence for each 8,000 tripeptide, or more generally each 20^n n-length peptide, each
 183 species could be represented mathematically by using the 20^n dimension vector. We
 184 suggest that this 20^n vector “stalling vector” would be very useful as a mathematical
 185 representation of the species. For example, the distance between two species is easily
 186 calculated based on the definition of the distance in mathematics without defining the
 187 distance between nucleotides, in which the rational for the definition is unclear.
 188 Furthermore, since the distribution of the ribosome stalling depends on the cell
 189 condition or experiment condition [10], the stalling vector could be used as the

190 biological definition for the cell or experiment condition.

191

192 **4. Conclusions**

193 We identified that L22 K94 was a key factor involved in the high-level TEL
194 resistance in *S. pneumoniae* and demonstrated that the residue at position 94 in L22 and
195 the status of the methylation of G748 are essential to maintain the appropriate ribosome
196 stalling for the proteome composition. Therefore, this region in the NPET may be a new
197 target for a novel class of antibiotics.

198

199 **5. Methods**

200 **5.1. Bacterial strains, plasmids, and media**

201 Bacterial strains and plasmids are shown in Supplementary Table S1 and S2,
202 respectively. *S. pneumoniae* strain S1 with reduced TEL susceptibility (MIC, 2 µg/mL)
203 was clinically isolated in Japan [6]. Pneumococci were routinely cultured at 37°C and
204 5% CO₂ in brain-heart infusion media with 0.5% yeast extract (BHI-Y) broth and
205 BHI-Y agar, supplemented with 5% horse blood. *E. coli* was grown in L broth (1%
206 Bact-trytone, 0.5% Bact yeast extract, and 0.5% sodium chloride, pH 7.4) and L agar.
207 When necessary, the medium was supplemented with kanamycin (25–600 µg/mL),

208 spectinomycin (100 µg/mL), and ampicillin (25 µg/mL).

209 **5.2. Transformation**

210 Synthetic competence-stimulating peptide (CSP) 1 and the method of Iannelli and Pozzi
211 [17] were used to produced transformation-competent *S. pneumoniae* S1.

212 **5.3. Antimicrobial susceptibility testing**

213 Susceptibility to antibiotics was determined by the serial two-fold dilution method,
214 using Mueller-Hinton agar plates supplemented with 5% lysed horse blood.
215 Susceptibility or resistance of pneumococci to TEL was assessed in accordance with the
216 recommendations of the Clinical and Laboratory Standards Institute [18].

217 **5.4. Next-generation sequencing and data analysis**

218 Relative mutations in the bacterial genome were estimated by sequencing the genomic
219 DNA samples extracted from each strain. Total DNA were extracted using the method
220 of Blue and Mitchell [19]. For preparation of library DNA, 500 ng of total DNA was
221 sheared to 800 bp by Covaris (M&S). The sheared DNA products were purified using a
222 MiniElute PCR purification kit (Qiagen) and were then used as templates for
223 pyrosequencing with a GS Junior platform (Roche). The data were treated by a data
224 analysis pipeline. Reads from both S1 and Sp52 were assembled into contigs by a de
225 novo assembler. The nucleotide sequence polymorphisms among these strains were

226 listed by mapping sequence reads on the assembled contigs with a reference mapper
227 program. We collected nucleotide substitutions meeting the following two conditions:
228 (i) the substitution was supported by five sequencing reads, and (ii) 90% of the reads
229 supported the substitution.

230 **5.5. RNA-Seq**

231 *S. pneumoniae* cultures were grown to log-phase, and 2.8 mL of cultures were then
232 added to 2.8 mL of RNA Lysis Buffer (1% SDS, 0.1 M NaCl, and 8 mM EDTA)
233 pre-heated to 100°C and vortexed for 2 min. The resulting lysates were added to the 5.6
234 mL of 100°C-preheated acid phenol (Sigma-Aldrich) and vortexed for 5 min. After
235 centrifuging, RNA was extracted from the aqueous phase using DirectZol (Zymo
236 Research). rRNA was isolated from the total RNA using MICROBExpress (Ambion).
237 The resulting total mRNA (400 ng as an input) was used for constructing the DNA
238 library using a KAPA Stranded RNA-Seq Library Preparation Kit Illumina platforms
239 (KK8400). DNA libraries were sequenced using the Illumina HiSeq 1500 system with
240 single-end reads. The Illumina libraries were preprocessed by clipping the Illumina
241 adapter sequence using Trimmomatic v.0.39 [20] and then aligned to the S1 genome
242 sequence⁶ using HASAT2 v.2.2.1 [21].

243 **5.6. Ribo-Seq**

244 Libraries were prepared as previously described in [4]. *S. pneumoniae* cultures were
 245 grown to log-phase. Next, cells were pretreated for 2 min with 100 µg/mL
 246 chloramphenicol before being pelleted by centrifugation. After decanting the
 247 supernatant, the cell pellets were resuspended in 2.5 mL of resuspension buffer (10 mM
 248 MgCl₂, 100 mM NH₄Cl, 20 mM Tris, pH 8.0, and 1 mM chloramphenicol). Then, cells
 249 were sonicated on ice and centrifuged, and 25 Abs₂₆₀ ribosome units (1 A₂₆₀ = 12 µg/µL)
 250 were digested with MNase (Roche). Digested samples were carefully loaded onto
 251 sucrose gradients and centrifuged at 124,700 × *g* for 8 hr at 4°C. After centrifugation,
 252 ribosome footprint-associated fractions were pooled, and RNA was purified using the
 253 SDS/hot-acid/phenol method. The ribosome footprint samples were resolved on a
 254 denaturing polyacrylamide gel, and a band between 20 and 45 nucleotides (nt) was
 255 excised. RNA was recovered using the ZR small-RNA PAGE recovery Kit (Zymo
 256 Research). After dephosphorylation of the 3' ends of the recovered RNA using T4
 257 polynucleotide kinase (T4 PNK; NEB), RNA was ligated to Linker-1 (5'-App
 258 CTGTAGGCACCATCAAT ddC-d') using the following reaction components: 20%
 259 (wt/col) PEG, 10% DMSO, 1 × T4 Ligase reaction buffer, 20 U SUPERase[•] In, and 10
 260 U T4 Ligase 2, truncated (NEB). Ligated products were resolved on a 10% TBE-Urea
 261 gel, and a band between 30 and 70 nt was excised. Ligated RNA was recovered using

the ZR small-RNA PAGE Recovery kit. After phosphorylation of the 5' ends of the 3'-ligated samples using T4 PNK, Linker-2 (5'-GAGTCTGCGTGTGATTCGGGTTAGGTGTTGGGTTGGGCCA-3') was ligated using T4 RNA Ligase1 (NEB). Reaction mixtures were resolved on a 10% TBE-Urea gel, and a band between 90 and 120 nt was excised. Ligated RNA was recovered using the ZR small-RNA PAGE Recovery kit. cDNAs were synthesized using SuperScript III Reverse Transcriptase (Invitrogen) and Linker-1-RT (5'-ATTGATGGTGCCTACAG-3') as a primer. RNA products were hydrolyzed by adding 1 mM NaOH to a final concentration of 0.1 mM and incubating for 15 min at 95°C. The cDNA products were resolved from the unextended primer on a 10% TBE-Urea gel, and a band between 90 and 120 nt was excised. DNA was recovered using the ZR small-RNA PAGE Recovery Kit. The resulting cDNA was PCR amplified with Q5 High Fidelity Polymerase (NEB) using Linker-2-partial (5'-TTAGGTGTTGGGTTGGGCCA-3') and Linker-1 as the primers. Amplified PCR products were purified using AMPure Bead (Beckman Coulter). KAPA Hyper Prep Kits for Illumina (KK8500) were used to construct the library, and the resulting DNA libraries were sequenced using the Illumina HiSeq 1500 system. The Illumina libraries were preprocessed by clipping the adapter sequences (Linker-1 and Linker-2) using CutAdapt v.2.10 [22] for the linker sequences, and

280 Trimmomatic v.0.39 [20] for the Illumina adapters. Then, the sequencing reads were
281 aligned to the S1 genome sequences [7] using HASAT2 v.2.2.1 [21]. Next, the S1 gene
282 feature file [7] was used to determine the CDS region. Sequencing data were deposited
283 in the DDBJ database with the accession number DRA011224.

284 **5.7. Definition of the ribosome density**

285 The ribosome density (RD) was calculated as previously described [23], except that
286 ribosome footprints between 24 and 30 nt were used for the calculation. The rationale
287 for choosing this range was to identify as many footprints as possible to improve the
288 statistical power and to exclude those that were suspected not to be responsible for the
289 footprints.

290 **5.8. Definition of A-site peaks**

291 First, we determined the A-site corresponding to each read by an offset of $[(15/27) \times (L)]$
292 from the 5' end of the read, where L is the length of each read. We used the normalized
293 A-site count (ribo/mRNA) as A-site peaks.

294 **5.9. Metagene analysis for ribosome stalling**

295 To obtain the metagene profile illustrated in Figure 3B, an A-site density higher than
296 eight was scaled by its own mean RD. Each normalized RD profile was aligned by their
297 respective start codon and averaged across each position.

298 **5.10. Definition of the stalling peptides**

299 The stalling P-values were calculated as previously described [11]. Peptides with the
300 P-value less than 0.0001 are termed here as stalling peptides.

301 **5.11. Enrichment of over- and underrepresented peptide sequences**

302 The over- and underrepresented peptide sequences were identified as previously
303 described [11], except that the cutoff of the P-value was 0.0001 for the underrepresented
304 peptides and 0.9999 for the overrepresented peptides.

305

306 **6. Declarations**

307 **Ethics approval and consent to participate**

308 Not applicable.

309 **Consent for publication**

310 Not applicable.

311 **Availability of data and materials**

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

314 **Competing interests**

315 The author declare that they have no competing interests.

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320 **Author contributions**

321 ST contributed to the conception and overall design of the work, all of the experiments,
322 bioinformatics analysis, and interpretation of Ribo-Seq data and drafting and revision of
323 the manuscript. The author read and approved the final manuscript.

324

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397 **Figure legends**

398 **Fig. 1 Structure of TEL binding to the ribosome 50S subunit.**

399 The TEL molecule is shown by a stick-and-ball model. The residues of 23S rRNA A752
400 and L22 K94 are shown. Dashed lines show the distances from the oxygen atom in TEL
401 to the hydrogen atom of the amino group in the side chain of L22 K94.

402

403 **Fig. 2 Effect of m¹G748/L22 K94 on growth.**

404 (A) Growth of strains S1 (closed circle), Sp375 (Sp52/pRlmA^{II}; closed square), Sp52
405 (open square), and Sp36 (closed triangle) in BHI-Y broth at 37°C and 5% CO₂. (B)
406 Growth competition between Sp375 (Sp52/pRlmA^{II}; open circle) and Sp52 (closed
407 circle). Sp375 was mixed with an equal quantity of Sp52 cells and grown as described
408 in Materials and Methods. The Sp375 cells were assayed by plating on BHI-Y agar
409 supplemented with 5% horse blood plus kanamycin, whereas the sum of the Sp375 and
410 Sp52 cells were obtained by plating on BHI-Y agar supplemented with 5% horse blood.
411 The percent of Sp375 or Sp52 cells remaining at each sub-culture was calculated, and
412 the percent abundance of each strain was plotted. Dilution between cycles was 1:20.
413 Mean values and standard deviations from at least three independent experiments are
414 given in (A) and (B).

415

416 **Fig. 3 Ribosome profiling in *S. pneumoniae* reveals ribosome stalling.**

417 (A) Ribosome density plot of the *erm(B)* operon shows the difference in ribosome
 418 occupancy among WT, L22 mutant, Double mutant, and RlmA^{II} mutant strains. (B)
 419 Metagene profile of the ribosome stalling in WT or L22 mutant (left panel), WT or
 420 Double mutant (middle panel), and WT or RlmA^{II} mutant (right panel). The A-site
 421 density across the stalling genes was aligned relative to the start codon.

422

423 **Fig. 4 Effect of m¹G748/L22 K94 on the interactions between the ribosomal exit**
 424 **tunnel and the nascent peptide.**

425 (A) Venn diagram for stalling peptide sequences in WT, L22 mutant, Double mutant,
 426 and RlmA^{II} mutant strains. The number of stalling peptide sequences are shown in
 427 parentheses. (B) The average distribution of stalling peptide sequences along the length
 428 of the tunnel. The positions along the tunnel (x-axis) represent the first position the
 429 peptide occupies. The arrow underneath the x-axis stands for the direction of NPET. The
 430 height of the bar is the probability that this position is occupied (average probability
 431 over all peptide sequences defined in the title). Bars are colored red/blue if their
 432 corresponding probabilities were significantly ($P < 0.05$) higher/lower than random,
 433 respectively; other bars ($P > 0.05$) appear in gray.

434

435 **Fig. 5 Role of m¹G748/L22 K94 in the association between ribosome stalling and**
 436 **proteome composition.**

437 (A) Distribution of the number of stalling peptide sequences with respect to proteome
 438 representation in WT, L22 mutant, Double mutant, and RlmA^{II} mutant; the P-value
 439 represents the statistical significance of the over- and underrepresented proportion. (B)
 440 A phylogenetic tree based on average linkage clustering of the selected gram-positive
 441 bacteria using the random vector produced by proteome representation. The proteome
 442 representation segregated into two groups in a manner largely consistent with the
 443 combination of the residue at position 90 in L22 and the status of G748 methylation.
 444 The amino acids residues at position 90 in L22 are shown in parentheses after the name
 445 of the organism followed by the status of G748 methylation.

446 **Supplementary tables**

447 **Supplementary Table 1: Bacterial strains**

Strain	Relevant characteristics	Reference of source
<i>Streptococcus pneumoniae</i>		
S1	TEL-resistant clinical isolate	[6]
Sp36	TEL-resistant mutants of S1 isolated from 8	[6]

	μg/mL TEL-containing BHI-Y agar plates	
Sp52	TEL-resistant mutants of Sp36 isolated from	This study
	128 μg/mL TEL-containing BHI-Y agar plates	
Sp274	<i>ΔtlrB::aad(9)</i> in S1	[6]
Sp284	Sp274 harboring pTKY1111	[4]
Sp288	Sp52 harboring pTKY1127	This study
Sp375	Sp52 harboring pTKY1111	This study
Sp378	Sp52 harboring pTKY1111	This study
Sp379	Sp274 harboring pTKY1127	[4]

448

449 **Supplementary Table 2: Plasmids**

Plasmid	Relevant characteristics	Reference of source
pUC18	Cloning vector	Lab. collection
pLZ12-Km2	Shuttle vector	[24]
pTKY862	pLZ12-Km2 with Sp resistant cassette <i>aad(9)</i>	[25]
pTKY1041	pLZ12-Km2 with 1401 bp <i>erm(B)</i> fragment from S1	[6]

pTKY1109	pUC18 with 328 bp <i>tlrB</i> fragment	[6]
pTKY1110	pUC18 with disrupted <i>tlrB</i> fragment by insertion of an Sp resistant cassette <i>aad(9)</i>	[6]
pTKY1111	pLZ12-Km2 with 1065 bp <i>tlrB</i> fragment from S1	[6]
pTKY1127	pLZ12-Km2 with 1065 bp <i>tlrB</i> fragment from Sp44	[6]

450

(A)

(B)