c-di-GMP modulates ribosome assembly by inhibiting rRNA methylation

Siqi Yu[1,2,3#], Zheyao Hu[4#], Xiaoting Xu[1,3,5#], Xiaoran Liang[1,2,3], Jiayi Shen[1,2,3], Min Liu[1,2,3], Mingxi Lin[1,2,3], Hong Chen[6], Jordi Marti[4*], Sheng-ce Tao[6*], Zhaowei Xu[1,2,3*]

[1]Key Laboratory of Gastrointestinal Cancer (Fujian Medical University), Ministry of Education, Fuzhou, China

[2]Laboratory of Scientific Research, School of Basic Medical Sciences, Fujian Medical University, Fuzhou, China

[3]Fujian Key Laboratory of Tumor Microbiology, Department of Medical Microbiology, Fujian Medical University, Fuzhou, China

[4]Department of Physics, Polytechnic University of Catalonia-Barcelona Tech, Barcelona, Catalonia, Spain

[5]Department of Endoscopy, The First Affiliated Hospital of Fujian Medical University, Fuzhou, China

[6]Shanghai Center for Systems Biomedicine, Key Laboratory of Systems Biomedicine (Ministry of Education), Shanghai Jiao Tong University, Shanghai, China

[#]These authors contributed equally to this study.

[*]Corresponding authors. E-mail: xuzw@fjmu.edu.cn (Zhaowei Xu), taosc@sjtu.edu.cn (Sheng-ce Tao), jordi.marti@upc.edu (Jordi Marti)

Highlights

1. c-di-GMP regulates ribosome assembly in *Escherichia coli*.

2. c-di-GMP inhibits rRNA methylation activity of RlmI by inducing catalytic pocket closure.

3. c-di-GMP promotes antibiotic resistance by regulating ribosome assembly.

Abstract

Cyclic diguanosine monophosphate (c-di-GMP) is a ubiquitous bacterial secondary messenger, with diverse functions, many of which are yet to be uncovered. Stemming from an *Escherichia coli* proteome microarray, we found that c-di-GMP bound to 23S rRNA methyltransferases (RlmI and RlmE). rRNA methylation assays showed that c-di-GMP inhibits RlmI activity, thereby modulating ribosome assembly. Based on molecular dynamic simulation and mutagenesis studies, we found that c-di-GMP binds to RlmI at residues R64, R103, G114, and K201. Structural simulation revealed that c-di-GMP quenches RlmI activity by inducing the closure of the catalytic pocket. Furthermore, we revealed that c-di-GMP promotes antibiotic tolerance by regulating RlmI activity, which played a role in antibiotic-resistant strains. Finally, the binding and methylation assays showed that the effect of c-di-GMP on RlmI is conserved, at least in various pathogenic bacteria. This study discovered an unexpected functional role of c-di-GMP in regulating ribosome assembly by inhibiting rRNA methylases. This study identified an unexpected but crucial member among the c-di-GMP effectors.

Keywords: c-di-GMP; rRNA methyltransferase; ribosome assembly; antibiotic tolerance

Introduction

Cyclic diguanosine monophosphate (c-di-GMP) was first identified in *Gluconacetobacter xylinus,* where it regulated cellulose synthesis[1]. Subsequent research revealed that c-di-GMP plays a role in a wide range of bacterial biological processes, such as motility, virulence, and host-microbe symbiosis [2-5]. In a previous study, we globally screened c-di-GMP binding proteins by applying an *Escherichia coli* proteome microarray and revealed the interplay loop between c-di-GMP and protein acetylation[6]. Interestingly, the microarray assay also showed that 23S rRNA methyltransferases RlmI and RlmE act as c-di-GMP binding proteins. This suggests a functional link between c-di-GMP and ribosome assembly.

The ribosome assembly involves the processing and folding of rRNA and its concomitant assembly with ribosomal proteins. As a part of rRNA processing, rRNA methylation participates in the regulation of ribosome assembly. For example, the inactivation of RlmE is associated with a large subunit assembly defect [7], and RsmA fulfills quality control requirements in the last stages of small subunit assembly [8]. Overall, 23 ribosomal RNA methylases exist in *E. coli*, most of which have unresolved physiological functions. Consequently, studying the function and regulatory factors of rRNA methyltransferase is crucial for understanding the mechanism of ribosome assembly.

75   Ribosome biogenesis is a fundamental cellular process, equipping

76   cells with the molecular factories for cellular protein production.

77   Inhibiting ribosome assembly is considered an essential source of new

78   targets for drug development[9,10]. Therefore, studying the relationship

79   between ribosome assembly and bacterial resistance is crucial for

80   designing antibiotics targeting ribosome assembly pathways. In

81   Gram-positive bacteria, (p)ppGpp negatively impacts ribosome assembly

82   by inhibiting GTPase activity, influencing growth and antibiotic tolerance

83   [11]. However, in Gram-negative bacteria, the influence of (p)ppGpp on

84   antibiotic tolerance mainly affects nucleotide and amino acid

85   synthesis[12,13]. The regulatory relationship between ribosome assembly

86   and antibiotic tolerance in Gram-negative bacteria is yet to be understood.

87   rRNA methylation serves as a significant mechanism for bacterial

88   resistance against ribosome-targeting antibiotics. Two clinically relevant

89   examples include 16S and 23S rRNA methyltransferases, which confer

90   resistance by modifying conserved rRNA residues in site A or PTC,

91   respectively. This modification makes bacteria insensitive to

92   aminoglycosides and streptogramin B [14]. For instance, aminoglycoside

93   resistance in *E. coli* is conferred by methylation of the G1405 and A1408

94   residues in the 16S rRNA by RsmF [15] and NpmA [16], respectively. The

95   Erm macrolide-resistance methyltransferase family has been widely

96   identified in Gram-positive bacteria [17] and continued to spread and mutate

97 globally [18,19]. The chloramphenicol-florfenicol resistance (*cfr*) SAM

98 methyltransferase family shares ancestry with the housekeeping RlmN

99 methyltransferases. These methyltransferases incorporate methylation at

100 A2503 in 23S rRNA and A37 in tRNAs [20,21]. However, the upstream

101 regulatory factors and downstream mechanisms of rRNA methylation in

102 the context of antibiotic resistance remain unclear.

103   In this study, we demonstrate that c-di-GMP binds to four 23S rRNA

104 methyltransferases and that RlmI is the main effector of c-di-GMP in

105 regulating ribosome assembly. Structural analysis revealed that c-di-GMP

106 binds to RlmI at the R64, R103, G114, and K201 residues and induces the

107 closure of the catalytic pocket of RlmI. We further show that c-di-GMP

108 regulates ribosomal assembly to promote antibiotic resistance by

109 inhibiting RlmI activity. Finally, sequence comparison of RlmI

110 orthologues among bacteria indicated that some important human

111 pathogens are conserved in the c-di-GMP-based rRNA regulation

112 mechanism.

113

114 **Material & Methods**

115 ***E. coli strains and plasmids***

116 *E. coli* BW25113 as reference strain was used in this study, and pCA24N

117 and pGEX4T-1 plasmids were used to overexpress methyltransferases

118 and their mutants. Further, pET28a was used for RlmI of *S. typhimurium*,

119 *K. pneumoniae*, and *V. cholerae*. We performed recombinant RlmI

120 mutations using a QuikChange Site-Directed Mutagenesis Kit (#200518,

121 Agilent Technologies, USA).

122 The strains described earlier were grown in Vogel-Bonner medium

123 (0.81 mM MgSO$_4$·7H$_2$O, 43.8 mM K$_2$HPO$_4$, 10 mM C$_6$H$_8$O$_7$·H$_2$O, and

124 16.7 mM NaNH$_4$HPO$_4$·4H$_2$O) with 10 mM acetate at 25°C for functional

125 analysis. For kanamycin treatment, 3 μg/mL kanamycin was added to the

126 Vogel-Bonner medium.

127 For protein purification, the strains were grown in Lysogeny broth (10

128 g tryptone, 5 g yeast extract, 10 g NaCl per 1 L) medium (LB medium),

129 and then induced by 0.2 mM IPTG at 22°C for 12 h.

130

131 ***Construction of endogenous DgcZ and RlmI mutants in E. coli***

132 DgcZ and RlmI mutants were constructed using the Red-recombination

133 system based on *E. coli* BW25113 strain [22], as discussed previously [23].

134 For kanamycin-resistant *E. coli* strains, the *rlmI*[K201A] mutants were

135 constructed using the Red-recombination system with ampicillin as the

136 screening antibiotic.

137

138 ***Methyltransferase activity assay in vitro***

139 For *in vitro* methylation assays, 3 μM recombinant enzymes (RlmI and

140 RlmE) and c-di-GMP (at either 5 μM, 10 μM, or 20 μM) were incubated

141   in 20 μL reaction buffer (40 mM HEPES, 100 mM NH_4Cl, 10 mM $MgCl_2$,

142   1 mM AdoMet, pH 7.6) at 37°C for 0.5 h. Then, 5 μM rRNA substrates

143   were added and incubated at 37°C for 1.5 h. Reactions were stopped by

144   heating at 95°C for 10 min and centrifugation at 10,000 x g for 10 min at

145   4°C to remove sediments.

146   These samples were analyzed by HPLC. Briefly, the samples were

147   injected into a C-18 column (Alltima C18 4.6 250 mm^2) and analyzed by

148   reversed-phase HPLC (Shimadzu, Japan). Solution A [0.065%

149   trifluoroacetic acid in 100% water (v/v)] and solution B [0.05%

150   trifluoroacetic acid in 100% acetonitrile (v/v)] were used in a gradient

151   program (0.01 min with 5% Solution B, 20 min with 65% Solution B,

152   20.01 min with 95% Solution B, 31 min with 95% Solution B, 31.01 min

153   with 5% Solution B, 40 min with 5% Solution B, and stop in 40.01 min)

154   with a flow rate of 1 mL/min. rRNA was detected at 220 nm, and the area

155   under the curve was integrated for the relative quantification of reaction

156   products. This assay was performed in triplicate, and the results were

157   calculated using GraphPad Prism 6.

158

159   ***MeRIP-qPCR quantification of the endogenous C1962 methylation of***

160   ***23S rRNA***

161   *E. coli* strains, such as WT, Δ*dgcZ*, *rlmI*[K201A] and Δ*dgcZ rlmI*[K201A] were

162   cultivated in LB medium at 37°C overnight and transferred to

163 Vogel-Bonner medium at a 1:1000 ratio with 10 mM acetate and 3 µg/mL

164 kanamycin for 25°C for 24 h. Twenty OD cells were harvested for RNA

165 extraction using an RNA extraction kit (Sangon Biotech, China). Total

166 RNA (50 µg) was diluted in 200 µL of IP buffer (20 mM HEPES, 50 mM

167 KCl, 1 U/µL protector RNase inhibitor, pH=7.5) and treated with a

168 sonicator (Sonics and Materials, USA) (20 cycles on 35% power, 15 s

169 on/off) to prepare the RNA fragments, which were divided into 50 µL as

170 input and 150 µL for IP. Then, 2 µL of anti-m5C antibody was added to

171 150 µL of RNA fragments and incubated at 4°C for 4 h. 20 µL Protein

172 A/G beads (Thermo Fisher Scientific, USA) were added to the mixture

173 and incubated at 4°C for 1 h. The RNA was eluted with 0.1 M glycine

174 (pH = 2) and neutralized with 1.0 M Tris (pH = 8). The eluted RNA and

175 RNA fragments were purified by phenol–chloroform and quantified using

176 a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

177 Real-time RT-PCR was performed using a reverse transcription kit and

178 real-time PCR kit (Applied Biosystems, USA). The following primers

179 were used.

180

	Sequence1 (5′–>3′)	Sequence2 (5′–>3′)
C1962	CGGTCCTAAGGTAGCGAAAT	ACGGCGGCCGTAACTATA
	ACTGAGTCTCGGGTGGAGA	GCCTGGCCATCATTACGCC
23S rRNA	AGTGGAAGCGTCTGGAAAGG	ATCGTACCCCAAACCGACAC
	GCCCTACTCATCGAGCTCAC	TTCTCCCGAAGTTACGGCAC

181

Isolation and antimicrobial susceptibility test of E. coli

182

183 The soil samples were dissolved in phosphate-buffered saline, and large

184 particles were removed using static precipitation. The supernatant was

185 plated into MacConkey agar, followed by incubation at 37°C for 18 h.

186 The pink, round, medium-sized colonies were picked as *E. coli* colonies.

187 The antimicrobial susceptibility of the *E. coli* strains was determined by

188 the Kirby-Bauer assay. Briefly, *E. coli* was inoculated into

189 Mueller-Hinton agar (Sigma Aldrich, USA), and 30 μg kanamycin K

190 (Liofilchem, Italy) was used for the antimicrobial susceptibility test.

191 When the zone diameter was less than 13 mm, *E. coli* was considered

192 resistant to kanamycin. The IMViC test kit (HiMedia, India) was

193 employed to confirm the *E. coli* strains.

194 Urine samples were obtained from 8 healthy volunteers. 20 mL urine

195 was centrifuged by 8000 g for 10 minutes and the precipitate was

196 resuspended in 1 mL PBS. The method of isolation and antimicrobial

197 susceptibility test consistent with the above. The study was approved by

198 the Ethical Committee of Fujian Medical University, Fuzhou, China

199 (2022-120).

200 The minimal inhibit concentrations (MICs) of WT and RlmI^{K201A}

201 mutants were determined by ETEST (kanamycin 0.016-256 μg/mL;

202 Liofilchem).

203

Measuring the relative mRNA levels of methyltransferase

205   *E. coli* strains such as WT, $dgcZ^+$ and $dgcZ^{G206A,G207A}$ were cultivated in

206   LB medium at 37°C overnight and transferred to Vogel-Bonner medium

207   at a 1:1000 ratio with 10 mM acetate at 25°C for 12 h and then induced

208   with 0.2 mM IPTG at 25°C for 20 h. Ten OD cells were harvested for

209   RNA extraction using an RNA extraction kit (Sangon Biotech, China).

210   Real-time RT-PCR was performed using a reverse transcription kit and

211   real-time PCR kit (Applied Biosystems, USA) with three replicates. The

212   following primers were used.

	Sequence1 (5′–>3′)	Sequence2 (5′–>3′)
rlmI	GAAGCGCTGGATATTGCACG	ACGTTTGACCCGTCTGAGTC
	ATCGCGATAAGTACGCAGCA	GTCGAGGCCATCTTTTTGCG
rlmE	TGGCGCTAGAAATGTGTCGT	GGCGCTAGAAATGTGTCGTG
	ACCTTCGTAAACAGGGAGCG	CCTTCGTAAACAGGGAGCGA
gapA	GATGGCCCGTCTCACAAAGA	TTGACCTGACCGTTCGTCTG
	TGCCATTCAGTTCTGGCAGT	ACGTCATCTTCGGTGTAGCC

213

Analysis of ribosomal subunits by sucrose density gradient centrifugation

216   *E. coli* strains were cultivated in LB medium at 37 °C overnight and

217   transferred to Vogel-Bonner medium at a 1:1000 ratio with 10 mM

218   acetate and 3 μg/mL kanamycin for 25°C for 24 h. Twenty OD cells were

219   harvested and repeatedly freeze–thawed three times. The cells were

220   treated with 1 mL of lysis buffer (20 mM HEPES, 0.5 mM $MgCl_2$, 200

221   mM NH_4Cl, 1 mg/mL lysozyme, 50 units/mL benzonase, pH 7.5) at 4°C

222   for 20 min with vigorous shaking. After lysis and centrifugation at 10,000

223   $\times g$ for 10 min at 4°C. The supernatant lysate was layered on top of a

224   sucrose gradient (10%-50%, *w/v*) in lysis buffer and separated by

225   ultracentrifugation in a Beckman SW-28 Rotor at 120,000 $\times g$ for 12 h at

226   4°C. The suspension was recovered to 25 components and quantified by

227   measuring the absorbance at 260 nm on a NanoDrop 2000

228   spectrophotometer.

229

230   ***Molecular dynamics simulations***

231   The computational tool has been microsecond-scale molecular dynamics

232   (MD) simulation [24]. In MD simulation, the corresponding Newton's

233   equations of motion are integrated numerically after choosing reliable

234   force fields, allowing us to monitor each individual atom in the system in

235   a wide variety of setups, including liquids at interfaces, in solid walls or

236   biological membranes, among others[25,26]. The number of particles and the

237   pressure and temperature of the system were fixed, whereas the volume

238   was adjusted accordingly. MD simulation can model hydrogens at the

239   classical or quantum levels[27]. Besides its energetic and structural

240   properties, it provides access to time-dependent quantities such as

241   diffusion coefficients or spectral densities, enhancing its applicability. In

242 the present study, we conducted MD simulations of c-di-GMP bound at

243 rRNA methyltransferases (RlmI). The simulation system contained one

244 rRNA methyltransferase and 7 c-di-GMP molecules fully solvated by

245 21,818 TIP3P water molecules in potassium chloride solution at the 0.15

246 M concentration, yielding a system size of 68,532 atoms. In each of the

247 two statistically independent MD simulations, one single c-di-GMP

248 molecule was placed at the center of the simulation box, near domains I,

249 II and III of RlmI, whereas the remaining 6 free c-di-GMPs were

250 randomly distributed around the RlmI protein according to the default

251 settings of Charmm-GUI "Multicomponent Assembler". All MD

252 simulation inputs were generated using the CHARMM-GUI platform [28,29],

253 and the CHARMM36m force field [30] was adopted for interactions

254 between rRNA methyltransferases and c-di-GMP. The force field used

255 also included the parameterization of the species c-di-GMP. All bonds

256 involving hydrogens were set to fixed lengths, allowing fluctuations of

257 bond distances and angles for the remaining atoms. The crystal structure

258 of rRNA methyltransferases was downloaded from RCSB PDB Protein

259 Data Bank (file name "3c0k"). The system was energy-minimized for

260 50,000 steps and well-equilibrated (NVT equilibration in Figure 16 of SI)

261 for 250 ps before the production of MD simulation. Production runs were

262 performed with an NPT ensemble for 2 µs. The pressure and temperature

263 were set at 1 atm and 310.15 K, respectively, to simulate the human body

264   environment. The GROMACS 2021 package was employed in all MD

265   simulations [31]. Time steps of 2 fs were used in production simulations,

266   and the particle mesh Ewald method with a Coulomb radius of 1.2 nm

267   was employed to compute long-range electrostatic interactions. The

268   cutoff for Lennard–Jones interactions was set to 1.2 nm. The pressure was

269   controlled with a Parrinello-Rahman piston with a damping coefficient of

270   5 ps^{-1}, whereas the temperature was controlled using a Nosé-Hoover

271   thermostat with a damping coefficient of 1 ps^{-1}. Periodic boundary

272   conditions in three directions of space were considered. We employed the

273   "gmx-sham" tool of the GROMACS 2021 package to perform the Gibbs

274   free energy landscape analysis. Moreover, the software VMD [32] and

275   UCSF Chimera[33] were used for trajectory analysis and visualization.

276   The radius of gyration R_g, used as a reaction coordinate in the

277   computation of Gibbs free energy landscapes, was determined as follows:

278
$$Rg = \sqrt{\frac{\sum_i \|r_i\|^2 m_i}{\sum_i m_i}} \quad (1)$$

279   where m_i is the mass of atom i, and r_i is the position of the same atom

280   with respect to the center of mass of the selected group. RMSD was

281   calculated as follows:

282
$$RMSD(t) \equiv \sqrt{\frac{1}{N}\sum_{i=1}^{N} \delta_i^2(t)} \quad (2)$$

283   where δ_i is the difference in distance between atom i [located at $x_i(t)$] of

284   the catalytic domain and the equivalent location in the crystal structure.

285   RMSF values were obtained as follows:

$$RMSFi \equiv \sqrt{\sum_{t_j=1}^{\Delta t} (x_i(t_j) - \tilde{x}_i)^2},$$

286   where $\tilde{x}_i$ is the time average of x_i, and Δt is the time interval at which

287   the average was taken.

288

289   ***ITC assay***

290   In the ITC assay, c-di-GMP (#C057 of Biolog), RlmI, and its mutants

291   were prepared in titration buffer (20 mM Tris, 50 mM NaCl, 200 mM

292   KCl, pH 7.0). Protein concentrations were measured based on Coomassie

293   brilliant blue staining. The ITC titrations were performed using a

294   MicroCal iTC200 system (GE Healthcare, PA, USA) at 25°C. Each

295   titration process involved 22 injections with 5 μL c-di-GMP. The stock

296   solution of c-di-GMP at 0.5 mM was titrated into WT or mutant RlmI (25

297   μM) in sample cells of 200 μL volume individually. c-di-GMP was

298   titrated into 200 μL titration buffer as a control for subsequent data

299   processing. The resulting titration curves were processed using the Origin

300   7.0 software program (OriginLab) according to the "one set of sites"

301   fitting model.

302

303   ***Streptavidin blotting assay***

304   In this assay, RlmI (0.1 mg/mL) and its mutants were incubated with 10

305   μM biotin-c-di-GMP in a reaction buffer (20 mM Tris, 50 mM NaCl, 200

306   mM KCl, pH 7.0) at 37°C for 1 h. These samples underwent

307   UV-crosslinking on ice for 0.5 h to further link c-di-GMP to RlmI. These

308   linked samples were divided into two parts for analysis by western

309   blotting. After incubation with IRDye 800CW Conjugated Streptavidin

310   (#926-32230; LI-COR Biosciences, USA) at room temperature for 2 h,

311   another membrane was incubated with anti-His antibody (05-949,

312   Millipore, USA) at 4°C for 12 h and then incubated with an IRDye 800

313   secondary antibody for 1 h. The resulting membranes were visualized

314   with an Odyssey Infrared Imaging System (LI-COR Biosciences).

315

316   ***Isolation and quantification of c-di-GMP in E. coli***

317   The c-di-GMP isolation was performed as discussed previously[6,34].

318   Briefly, *E. coli* cells with 50 OD were harvested and resuspended in 2 mL

319   of ddH$_2$O. Further, 8 mL of the mixture containing 50% methanol and 50%

320   acetonitrile was added to extract intracellular c-di-GMP. Meanwhile, 1

321   μM cGMP was added as the internal reference. For c-di-GMP absolute

322   quantification, the density of *E. coli* sediment is defined as 1 mg/mL. The

323   extracts were analyzed by ultra-high-performance liquid chromatography

324   coupled with ion mobility mass spectrometry (UPLC-IM-MS).

325   UPLC-IM-MS was performed using a Waters UPLC I-class system

326   equipped with a binary solvent delivery manager and a sample manager

327 coupled with a Waters VION IMS Q-TOF mass spectrometer equipped

328 with an electrospray interface (Waters Corporation, CT, USA).

329

Determination of the strain growth curve in Vogel-Bonner medium

331 As discussed above, the strains WT, $\Delta dgcZ$, $rlmI^{K201A}$ and $\Delta dgcZ$

332 $rlmI^{K201A}$ were grown in Vogel-Bonner medium with 10 mM acetate at

333 25°C. For kanamycin treatment, 0, 1.5, 3, 6 and 9 μg/mL kanamycin was

334 added to the Vogel-Bonner medium, respectively. These cell

335 concentrations were measured at OD_{600} using NanoDrop 2000

336 spectrophotometer at 8, 12, 16, 24, and 32 h. Then, the growth curve was

337 drawn using GraphPad Prism 6.

338

Statistical analysis

340 Pairwise comparisons were performed using two-tailed Student's *t*-test,

341 and statistical significance was set at $*p < 0.05$, $**p < 0.01$. Error bars

342 represent the mean ± range.

343

Results

Ribosomal RNA large subunit methyltransferases are c-di-GMP

effectors

347 In a previous study, we screened c-di-GMP-binding proteins in *E. coli*

348 using a proteomic microarray and found that the rRNA

349   methyltransferases RlmI and RlmE were potential c-di-GMP effectors[6]

350   **(Fig. 1A)**. Based on the demonstration of binding between c-di-GMP and

351   the methyltransferases, we speculated that c-di-GMP might affect rRNA

352   methylation activity. To test this hypothesis, we examined the activity of

353   four methyltransferases in the presence of c-di-GMP, using rRNA

354   methylation as our indicator. For this purpose, we synthesized

355   unmethylated 23S rRNA positions 1932-1991 and 2522-2581 for

356   m5C1962 by RlmI [35] and m2U2552 by RlmE [36], respectively. The

357   methyltransferases can catalyze the production of methylated rRNA and

358   exhibit specific peaks in HPLC **(Fig. 1B-C)**. However, when c-di-GMP

359   was introduced, methylation activities were significantly inhibited in a

360   dose-dependent manner. We compared the effect of additional c-di-GMP

361   on methylation products, using the group without c-di-GMP treatment as

362   the reference value. Specifically, 5 μM c-di-GMP inhibited the activity of

363   RlmI and RlmE by 49% and 31%, respectively **(Fig. 1D)**.

364

365   ***c-di-GMP inhibited ribosome assembly, with RlmI as the main effector***

366   rRNA methylation is a prerequisite for the accurate assembly of

367   ribosomes. We hypothesized that c-di-GMP might affect ribosomal

368   assembly in *E. coli* (*E. coli* BW25113 as reference strain) by inhibiting

369   methylation activity. To investigate the regulatory role of c-di-GMP, we

370   constructed the strains of *dgcZ* knockout and overexpression. Compared

371 with wild-type (WT) strains, the c-di-GMP level in *dgcZ* overexpressing

372 strains increased by 12.2 times **(Fig. S1A)**. Then, we used a sucrose

373 density gradient (SDG) assay to detect the ribosome particle, and the

374 results showed that both knockout and overexpression of *dgcZ* could not

375 affect the ribosome abundance of 50S compared with WT strain without

376 antibiotic treatment **(Fig. 2A)**. c-di-GMP is a stress response factor.

377 Therefore, we hypothesized that the regulation of ribosome assembly by

378 c-di-GMP might occur under antibiotic stress. We treated *E. coli* cells

379 with kanamycin, a ribosome-targeted antibiotic that can also elevate the

380 cellular c-di-GMP level in *E. coli* by increasing the mRNA levels of *dgcZ*

381 [6,37]. This elevation was controlled by the RNA-binding protein *csrA* [38,39].

382 Following kanamycin treatment, the c-di-GMP concentrations in WT

383 cells were 6.2-fold higher than in untreated cells **(Fig. S1A)**. Notably, the

384 c-di-GMP levels in *dgcZ*-defective cells were not responsive to

385 kanamycin **(Fig. S1A)** because DgcZ serves as an effector for

386 kanamycin-induced elevation of c-di-GMP levels. An SDG assay

387 revealed that the ribosome disintegrated into 30S and 50S particles at low

388 Mg^{2+} concentrations, and ~45S particles[11] were observed in

389 kanamycin-treated WT cells and *dgcZ* replenishment strain (Δ*dgcZ::dgcZ*)

390 **(Fig. 2A)**. However, the strain with *dgcZ* inactivation mutation did not

391 show the presence of 45S particles (Δ*dgcZ::dgcZ*[G206A,G207A]). The results

392 indicated that the increase in c-di-GMP levels inhibited the assembly of

393   ribosomal large subunits in *E. coli*. In addition, we found c-di-GMP

394   inhibited the activity of four methyltransferases and downregulated the

395   methylation of 23S RNA *in vitro* (**Fig. 1D**). Then, the four

396   methyltransferases were overexpressed in kanamycin-treated WT cells to

397   examine the role of the methylation enzymes in c-di-GMP-regulated

398   ribosome assembly. We found that the overexpression of the methylases

399   did not affect c-di-GMP levels (**Fig. S1B**), and the overexpression of

400   RlmI weakened the effect of c-di-GMP on ribosomal assembly (**Fig. 2B**).

401   Thus, RlmI was the major effector of c-di-GMP in regulating ribosome

402   assembly.

403   c-di-GMP binds to its effectors via Arg residues[40]. We mutated all

404   Arg residues to Ala in RlmI to determine the binding sites on RlmI. Then,

405   we developed the *in vitro* assay in which purified RlmI mutants were

406   incubated with biotin-c-di-GMP, ultraviolet (UV)-crosslinked, and

407   probed with fluorescent streptavidin [41,42]. We observed that RlmI mutants

408   with R64A and R103A exhibited a significantly weakened interaction

409   with c-di-GMP (**Figs. 2C and S2**). Furthermore, when we determined the

410   activity of RlmI mutants, both RlmI^{R64A} and RlmI^{R103A} displayed

411   methylation activities slightly lower than that of RlmI under 5 μM rRNA

412   substrate. Upon treatment with 20 μM c-di-GMP, the methylation activity

413   decreased by 80%, 6%, and 32% in RlmI, RlmI^{R64A}, and RlmI^{R103A},

414   respectively (**Fig. 2D**). The effect of c-di-GMP for RlmI was significantly

415 weakened under R64A and R103A mutations in comparison to that of

416 WT, which validated that R64 and R103 are the key sites to the binding

417 of c-di-GMP to RlmI.

418   We eliminated the effect of c-di-GMP on RlmI by mutation of the

419 binding sites R64A and R103A to validate whether RlmI was the main

420 effector of c-di-GMP in ribosome assembly. With kanamycin treatment,

421 WT, $rlmI^{R64A}$, $rlmI^{R103A}$, and $\triangle rlmI$ cells were assayed by SDG. Under

422 kanamycin stimulation, elevated c-di-GMP in WT cells inhibited RlmI,

423 resulting in missing rRNA methylation. This inhibition of RlmI impairs

424 ribosomal assembly and leads to the production of 45S precursors. The

425 *rlmI* knockout strains serve as a positive control for demonstrating *rlmI*

426 functional defects. In addition, the R64A and R103A mutants abolished

427 the effect of c-di-GMP on RlmI. The results showed that the ~45S

428 particle was observed in WT and Δ*rlmI* cells but not in R64A and R103A

429 mutants **(Fig. 2E)**. Thus, RlmI played a key role in ribosomal assembly

430 under kanamycin stress, and c-di-GMP regulated its activity.

431

432 ***c-di-GMP induces the closure of the catalytic pocket of RlmI***

433 To reveal the structural mechanism of c-di-GMP regulating RlmI enzyme

434 activity, we investigated the conformational changes of RlmI during its

435 interaction with c-di-GMP in an aqueous ionic solution using molecular

436 dynamics (MD) simulation. In MD simulation, root mean square

437   deviation (RMSD) indicated the fluctuations and stability of the

438   conformations of RlmI and root mean square fluctuation (RMSF)

439   revealed flexibility during the full simulation period. By RMSD and

440   RMSF, we found that residues 160-170, 302-320, and 370-390 were

441   mainly involved in the conformational fluctuation of RlmI **(Fig. 3A)**. We

442   labeled residues 160-170 as "Domain-I" (DM-I), residues 302-320 as

443   "Domain-II" (DM-II), and residues 370-390 as "Domain-III" (DM-III).

444   DM-I and DM-III are the regions of the protein that correspond to the

445   RNA-binding area, whereas DM-II is located near the

446   S-adenosyl-L-methionine (AdoMet) binding-related area. An overall

447   view of the evolution of RlmI fluctuations revealed a distinct

448   conformational fluctuation around 0.8 µs during the simulation (average

449   of Trajectory #1 and Trajectory #2) **(Fig. 3B)**. Combining RMSD, RMSF,

450   and trajectory analysis results, the RlmI was found to exist in two states,

451   State-I and State-II, while interacting with c-di-GMP. c-di-GMP can

452   interact with the RNA-binding area (State-I), and the domain DM-III shut

453   down after 0.8 µs. The results suggested that (1) c-di-GMP can interact

454   with the RNA-binding domains and then induce the closure of DM-III,

455   and (2) it was corroborated that the "on-off" of the RNA-binding area

456   was mainly embodied by DM-III to a large extent **(Fig. 3C and Video**

457   **1-2)**. The dynamic process of c-di-GMP-induced conformational

458   rearrangements in the active domain of RlmI was similar to that of other

459   c-di-GMP effectors such as YcgR [43], FleQ [44], and CheR1 [45].

460

R64, R103, G114, and K201 residues of RlmI interact with bound c-di-GMP

463   We employed Gibbs free energy analysis to identify the dominant

conformation of RlmI and c-di-GMP complex. The Gibbs free energy

surfaces were shown for the two runs and their average **(Fig. 4A),** using

RMSD and radius of gyration as the variables. We identified the free

energy basin, the one with the lowest free energy (set to 0 kJ/mol) **(Fig. 4A, yellow point)**, and found that the corresponding regions were almost

overlapping for the three sets **(Fig. 4B)**. Thus, the results indicated the

two independent simulated trajectories as convergent and physically

equivalent.

To explore the binding sites of c-di-GMP and RlmI, we superimposed

the stable-state configurations of RlmI and c-di-GMP for the three sets

(Fig. 4B). Two independent trajectories #1 and #2 were taken into

consideration for the computational analysis and the average was selected

for convergence and physical equivalence analysis. The average

conformation showed that R64 and G114 together stabilize the guanosine

moiety of c-di-GMP. Correspondingly, R103 and K201 act to stabilize the

negatively charged region of c-di-GMP. It is evident that R103 and K201

formed a stable hydrogen bond with the oxygen atom of the phosphate

481   group of c-di-GMP.

482   Noncovalent interactions, including hydrogen bonds, coordination

483   bonds, and salt bridges, are crucial for maintaining the tertiary structure

484   of proteins. Benefiting from the all-atom-level precision of molecular

485   dynamics simulations, we analyzed the hydrogen bond interaction map of

486   c-di-GMP with RlmI using time-dependent atomic site distances between

487   selected atomic sites to uncover the interaction mode of c-di-GMP with

488   RlmI, providing guidance for further experimental verification. Atomic

489   detail sketches of c-di-GMP and the main residues described in this

490   section are provided in **Figures S3 and S4**. While labeling the amino acid

491   residues in the hydrogen bond interaction map of c-di-GMP with RlmI,

492   we also labeled the lifetime of hydrogen-bonding interactions between

493   c-di-GMP and corresponding amino acid residues. Considering that our

494   molecular dynamics simulation spanned a timeframe of 2 μs, we

495   subsequently performed site mutation verification on residues with

496   hydrogen bond interaction lifetimes exceeding 400 ns. Six amino acid

497   residues from RlmI were selected as the potential binding sites for

498   c-di-GMP: R64, R103, E108, G114, T116, and K201 **(Fig. 4C)**.

499   Atom-atom distances as a function of time and bond lifetimes are

500   presented in **Fig. S5-S15**.

501   We employed the streptavidin blotting assays to determine the

502   interaction between c-di-GMP and RlmI mutants, aiming to validate the

503   results obtained from the molecular dynamics simulations. The result

504   revealed that amino acid residues R64, R103, G114, and K201 were

505   crucial for the binding of c-di-GMP to the RlmI. In addition, E108A and

506   T116A of RlmI slightly affected c-di-GMP binding **(Fig. 4D)**. We next

507   performed isothermal titration calorimetry (ITC) titrations with these

508   mutants and determined K_d values of 1.3, 102.3, 76.5, 148.6, and 401.2

509   μM for RlmI, RlmI^{R64A}, RlmI^{R103A}, RlmI^{G114A}, and RlmI^{K201A}, respectively

510   **(Figs. 4E and S16)**. We found that the stoichiometries have not

511   significant different between RlmI and its variants, which showed 1:1

512   binding ratio with c-di-GMP **(Fig. 4E)**. Further, we observed that the

513   activity of RlmI^{K201A} showed no significant change with 20 μM c-di-GMP

514   treatment compared with c-di-GMP free group. **(Fig. 4F)**. The results

515   suggested that R64, R103, G114, and K201 residues of RlmI were the

516   critical sites for c-di-GMP binding.

517

518   ***c-di-GMP regulates RlmI to promote antibiotic resistance***

519   As a close correlation exists among c-di-GMP, ribosomal assembly, and

520   antibiotic resistance[46,47], we hypothesized that c-di-GMP regulated

521   ribosomal assembly to promote antibiotic resistance by inhibiting RlmI

522   activity. To test this hypothesis, we interfered with the interaction of

523   c-di-GMP and RlmI by introducing the K201 mutation in endogenous

524   RlmI (*rlmI*^{K201A}), *dgcZ* depletion (△*dgcZ*), *dgcZ* overexpression (△*dgcZ*

525    $dgcZ^+$) and both mutants ($\triangle dgcZ\ rlmI^{K201A}$). Then, we determined the

526   c-di-GMP level and methylation level of 23S rRNA C1962. The results

527   showed that the cellular c-di-GMP levels and C1962 methylation levels

528   did not significantly change in WT, $\triangle dgcZ$, $rlmI^{K201A}$, and $\triangle dgcZ$

529    $rlmI^{K201A}$ without kanamycin treatment (**Figs. 5A and 5B**). However, the

530   c-di-GMP levels of WT and $rlmI^{K201A}$ increased about six times compared

531   with $\triangle dgcZ$ and $\triangle dgcZ\ rlmI^{K201A}$ when treated by kanamycin, and the

532   c-di-GMP levels of $\triangle dgcZ\ dgcZ^+$ increased about eleven times

533   compared with WT (**Fig. 5A**). Furthermore, the methylation analysis

534   showed that elevated c-di-GMP (WT, $\triangle dgcZ\ dgcZ^+$) significantly

535   decreased C1962 methylation, and the K210A mutation ($rlmI^{K201A}$)

536   abolished the effect of c-di-GMP (**Fig. 5B**).

537   We measured the growth curves and MICs of the four strains, *i.e.*, WT,

538    $\Delta dgcZ$, $rlmI^{K201A}$, and $\Delta dgcZ\ rlmI^{K201A}$. We tested serial concentrations (0,

539   1.5, 3, 6 and 9 μg/mL) of kanamycin for strain growth curve. The strains

540   cannot growth in treatment with 9 μg/mL kanamycin, so we determined

541   the growth curve for other four concentrations. The curves showed that

542   growth of the four strains were consistent when kanamycin was not added

543   (**Fig. 5C**). However, the growth of the $\Delta dgcZ$ strains decreased compared

544   with that of the WT strains with 1.5, 3 and 6 μg/mL kanamycin treatment,

545   indicating that c-di-GMP promoted the antibiotic tolerance of *E. coli*.

546   Furthermore, we mutated the c-di-GMP binding site K201 of RlmI

547 ($rlmI^{\text{K201A}}$) and found that $rlmI^{\text{K201A}}$ strains had greater sensitivity to

548 antibiotics and slower growth rate compare to that of WT, which suggests

549 that the interaction of c-di-GMP and RlmI enhances the antibiotic

550 tolerance **(Fig. 5 C-D)**.

551 Kanamycin is an antibiotic that targets ribosomes and can

552 endogenously increase the c-di-GMP level. Therefore, we speculate that

553 c-di-GMP has a promoting effect on kanamycin resistance. To test this,

554 we isolated 40 *E. coli* strains from soil (named S1-S20) and urine (named

555 C1-C20) samples resistant to kanamycin and determined the cellular

556 c-di-GMP levels to investigate the role of c-di-GMP and RlmI axis in

557 kanamycin-resistant *E. coli*. The results revealed that 13/40 strains (S3,

558 S4, S7, S12, S16, S20, C1, C2, C4, C8, C14, C15, C18) showed

559 significant increase in c-di-GMP levels compared with the BW25113

560 strains **(Fig. S17A)**. Furthermore, we mutated the endogenous RlmI in

561 K201 to alanine and determined the minimal inhibitory concentration

562 (MIC). We obtained 12 of 13 K201 mutant strains, excluding the C12

563 strain, which might be a multidrug-resistant strain. Drug susceptibility

564 results showed that the MICs of the S4, S16, S20, C1 and C2 strains

565 substantially decreased following the mutation at K201 of RlmI **(Fig. 5E)**.

566 Thus, c-di-GMP regulated RlmI activity to promote antibiotic tolerance,

567 and this pathway played a key role in a portion of antibiotic-resistant *E.*

568 *coli*.

569

Effect of c-di-GMP on RlmI is conserved in multiple pathogenic bacteria

c-di-GMP is a ubiquitous bacterial secondary messenger, and RlmI is highly conserved in bacteria. Thus, we hypothesized that the binding and inhibition of c-di-GMP with RlmI from *E. coli* was the same for the RlmI homologues in other bacteria. RlmI protein sequences from a series of highly diverse bacteria were aligned with testing this hypothesis, and we found that the c-di-GMP binding region was reasonably well conserved in these bacteria (**Fig. 6A**). Then, we selected *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Vibrio cholerae* as the exemplary members of this conserved set. We found that RlmI$^{S.\ typhimurium}$, RlmI$^{K.\ pneumoniae}$, and RlmI$^{V.\ cholerae}$ could bind to c-di-GMP and that the binding could be abolished when the lysine in RlmI was mutated (**Fig. 6B**). Furthermore, the *in vitro* activity analysis showed that similar to RlmI$^{E.coli}$, the aforementioned three RlmI homologues exhibited methylase activity for 23S rRNA and this activity could be inhibited by c-di-GMP (**Fig. 6C**). Thus, the effect of c-di-GMP on RlmI is conserved in multiple pathogenic bacteria and might also play a role in antibiotic resistance.

588

Discussion

c-di-GMP is a key secondary messenger in prokaryotes. rRNA

591 methylation occurs in both prokaryotes and eukaryotes. This study

592 discovered that c-di-GMP binds to four rRNA methyltransferases and

593 inhibits their activities. RlmI was found to be the major effector of

594 c-di-GMP in ribosome assembly. The molecular dynamics simulation

595 analysis revealed the binding sites and models of c-di-GMP and RlmI.

596 The MIC assay further demonstrated that c-di-GMP inhibits ribosome

597 assembly, promoting antibiotic resistance in *E. coli*. Thus, we established

598 a regulatory pathway among c-di-GMP and ribosome functions and

599 revealed its role in antibiotic resistance.

600   Previous studies have reported that c-di-GMP regulates mature

601 ribosome function through RimK in *Pseudomonas*[48,49], EF-P in

602 *Acinetobacter baumannii* [50], Vc2 riboswitches in *V. cholerae* [51]. c-di-GMP

603 regulates the glutamate ligase RimK, which catalyzes glutamate residues

604 to the C-terminus of ribosomal protein RpsF to affect ribosomal

605 function[48,49]. The binding of c-di-GMP enhances the function of EF-P,

606 promoting translation efficiency and modulating bacterial physiology and

607 virulence [50]. Additionally, c-di-GMP binds to the Vc2 riboswitch,

608 inducing structural changes that result in the switch-OFF and switch-ON

609 states of translational initiation [51]. This study revealed that c-di-GMP

610 affects ribosome assembly, offering a new perspective on c-di-GMP's

611 role in ribosome regulation. Numerous accessory factors play a role in

612 guiding the ribosome assembly process, including GTPases, rRNA

613   modification enzymes, helicases, and maturation factors [52]. Our findings

614   establish c-di-GMP as an upstream regulatory signal for rRNA

615   modification, creating a connection between environmental stimuli and

616   ribosome function.

617   RlmI is a large ribosomal RNA subunit methyltransferase that

618   methylates cytosine specifically at position 1962 (m5C1962) of 23S

619   rRNA. In previous studies, RlmI depletion did not lead to abnormal

620   ribosome assembly or growth arrest of *E. coli* at 20°C and 37°C[53]. Indeed,

621   we found that RlmI depletion did not affect the ribosome abundance of

622   50S compared with WT strains when not treated with antibiotics.

623   However, with kanamycin treatment, the ~45S particle was observed in

624    $\triangle$*rlmI* cells **(Fig. 2E)**. Thus, RlmI plays a key role in ribosomal assembly

625   under kanamycin stress. As deletion of most ribosomal

626   methyltransferases does not cause significant phenotypic changes, these

627   studies have demonstrated that the function of methylases under different

628   growth conditions may help understand the physiological significance of

629   ribosome assembly.

630   rRNA methylation is a fundamental mechanism contributing to

631   bacterial resistance against ribosome-targeting antibiotics. Genes

632   encoding corresponding methyltransferases have been identified in

633   clinical isolates of pathogenic bacteria [54]. In 16S rRNA, methylation of

634   C1405 and A1408 confer high-level resistance to aminoglycosides, which

635   is catalyzed by the RsmF and Npm families, respectively. The

636   phylogenetic analysis suggests that m^7G1405 and m^1A1408

637   methyltransferases share a common ancestor with

638   aminoglycoside-producing soil bacteria. In 23S rRNA, the SAM

639   methyltransferase family shares ancestry with the housekeeping RlmN

640   methyltransferases, which incorporate the methylation of A2503 in 23S

641   rRNA. However, the connection between RlmI and antibiotic resistance

642   has not previously been reported. We also detected the transcriptional

643   levels of RlmI for the drug-resistant strains and did not find any intrinsic

644   change in expression levels **(Fig. S17B)**. Because RlmI is not the direct

645   target of most of the known antibiotics, and its enzymatic activity could

646   be regulated through the binding of c-di-GMP. We speculate that RlmI

647   may function as an effector of the stress factor c-di-GMP, and the

648   pathway of c-di-GMP regulating RlmI may play the role in intrinsic

649   tolerance.

650   Bacteria mainly acquire resistance by altering resistance genes,

651   reducing intracellular antibiotic concentrations, protecting antibiotic

652   targets, and inactivating antibiotics[55]. c-di-GMP promotes the biofilm to

653   reduce the permeability of antibiotics [46,56]. Other mechanisms by which

654   c-di-GMP promotes bacterial resistance remain to be studied. We found

655   that the level of c-di-GMP increased in some drug-resistant *E. coli* and

656   interfering with the effect of c-di-GMP on ribosome methylation in these

657   bacteria could increase their sensitivity to antibiotics. c-di-GMP has

658   essential physiological functions in drug-resistant bacteria. However, not

659   all drug-resistant bacteria have elevated intracellular c-di-GMP

660   concentrations, and c-di-GMP regulates other drug-resistance-related

661   pathways, such as biofilms. The question of whether the resistance

662   pathway regulated by c-di-GMP has a synergistic or independent effect is

663   worth exploring. Therefore, more data on drug-resistant strains are

664   needed to examine the role of c-di-GMP in bacterial resistance.

665   In summary, we identified rRNA methyltransferases as the novel

666   c-di-GMP effectors from *E. coli* proteome microarray assay. The

667   functional analysis revealed an unexpected role of c-di-GMP in regulating

668   ribosome assembly by inhibiting rRNA methylases, highlighting the

669   physiological function of the regulatory axis in bacterial drug resistance.

670

Acknowledgments

678

Author contributions

Z.W.X., S.C.T. and J.M. conceived the idea. S.C.T. provided key reagent. S.Q.Y., X.R.L., J.Y.S., and H.C. performed protein mutation and interaction assay. X.T.X. performed the bacterial culture and protein purification. S.Q.Y., M.L., and X.T.X. performed an enzyme activity assay. X.T.X., X.R.L., X.T.X., and L.X.Z. performed the bacterial drug sensitivity test. Z.Y.H. and J.M. performed molecular dynamic simulation and structure analysis. Z.W.X., S.Q.Y., Z.Y.H., and J.M. prepared the figures. Z.W.X., S.Q.Y., Z.Y.H., and J.M. wrote the manuscript.

Data and software availability

The crystal structure files, MD simulation files (input files, parameter files, topology files, etc.), and structures of c-di-GMP are available on the website https://github.com/Zheyao-Hu/RlmIcdiGMP. Moreover, all the software (free to use) packages used in this study were the official release version without any modification. The raw protein microarray data have been published in the Protein Microarray Database (www.proteinmicroarray.cn/) with the accession number PMDE226 (http://www.proteinmicroarray.cn/index.php/experiment/detail?experimen t_id=226).

Conflicts of interest

701   The authors declare no conflicts of interest.

702

703   **Figure legends**

704   **Figure 1. Ribosomal RNA large subunit methyltransferases are**

705   **c-di-GMP effectors.**

706   (A) *E. coli* proteome microarrays were probed with biotin-c-di-GMP and

707   biotin. Obvious binding differences of RlmI and RlmE on the microarrays

708   incubated with biotin-c-di-GMP and biotin were observed. Two spots per

709   protein were observed, and the positive signal-to-noise ratio [(SNR) (+)]

710   represented the average SNR of the two duplicate spots. (B-C) *In vitro*

711   methylation reaction. The synthesized rRNA fragments were used for *in*

712   *vitro* methylation enzyme activity testing. The HPLC peaks are derived

713   from RlmI (B) and RlmE (C) with the treatment of 0, 5, 10 and 20 μM

714   c-di-GMP, respectively. (D) Quantitative results of HPLC peak. The

715   methylated rRNAs were detected by HPLC and quantified by area under

716   the curve. The bar chart shows the relative enzyme activity with the data

717   points, using the reaction without the addition of c-di-GMP as the

718   baseline (three preparations, mean ± range; **$p < 0.01$, two-tailed

719   Student's t-test).

720   **Figure 2. c-di-GMP inhibits ribosome assembly, with RlmI as the**

721   **main effector.**

722   (A) SDG assay for the strains with elevated c-di-GMP. c-di-GMP was

723   elevated by treatment with kanamycin or overexpression of DgcZ, and

724   the ribosome particle was assayed by SDG. The corresponding three

725   peaks represent the ribosome particles of 30S, pre-50S, and 50S. (B) SDG

726   assay for the strains overexpressing two methyltransferases. RlmI and

727   RlmE were overexpressed under the conditions of kanamycin treatment,

728   and the ribosome particle was assayed by SDG. (C) Streptavidin blotting

729   assays for WT and mutant RlmI. The arginine on RlmI was mutated to

730   alanine, and the interaction of c-di-GMP and RlmI mutants was

731   determined. The results indicated that R64A and R103A weakened the

732   binding of c-di-GMP and RlmI. Streptavidin represents the interaction

733   signals, and α-His represents the protein levels. The bar chart shows the

734   relative intensity of streptavidin with the data points (three preparations,

735   mean ± range; **$p < 0.01$, two-tailed Student's t-test). (D) *In vitro*

736   methylation assay of the two RlmI mutants. The synthesized rRNA

737   fragments were used as substrates, and the reaction products were

738   analyzed by HPLC. The bar chart shows the relative activity of RlmI with

739   the data points (three preparations, mean ± range; ns: no significant

740   difference, *$p < 0.05$, **$p < 0.01$, two-tailed Student's t-test). (E) SDG

741   assay for the strains with RlmI depletion and mutation. Strains with

742   endogenous depletion and mutation of RlmI were constructed using the

743   Red-recombination system. These strains were treated with kanamycin,

744   and the ribosome particle was assayed by SDG.

Figure 3. Binding model of c-di-GMP and RlmI.

(A) RMSD value of RlmI during interaction with c-di-GMPs. The arrow marks the main changes at the residue level. The solid and dashed lines represent two simulated trajectories, and the degree of overlap represents the convergence of two trajectories. (B) RMSF value of RlmI during interaction with c-di-GMP. The arrow marks the main changes on the timeline. (C) Representative snapshots of the transition between RlmI State-I and State-II. The arrow marks the main changes in DM-III.

Figure 4. Determination of the binding sites of c-di-GMP on RlmI.

(A) Gibbs free energy landscapes illustrating the binding of c-di-GMP to RlmI. I#1, I#2, and I represent trajectories 1, 2, and average, respectively. (B) Interaction model of c-di-GMP and RlmI. The key binding sites R64, R103, G114, and K201 are highlighted. I#1, I#2, and I represent trajectories 1, 2, and average, respectively. (C) Interaction landscapes of c-di-GMP and RlmI. The interaction sites between c-di-GMP and RlmI in MD simulation are marked in the plane diagram of c-di-GMP. The annotated duration represents the interaction time between c-di-GMP and RlmI in the 10 μs MD simulation, with green boxes highlighting residues with interaction times greater than 400 ns. (D) Streptavidin blotting assays comparing WT and mutant RlmI. The potential binding sites within RlmI were mutated to alanine, and the interaction with c-di-GMP was determined. Streptavidin represents the interaction signals, and α-His

767 represents the protein levels. The intensity of WT was used as the

768 reference for statistical analysis of the variants. The bar chart shows the

769 relative intensity of streptavidin with the data points (three preparations,

770 mean ± range; $*p < 0.05$, $**p < 0.01$, two-tailed Student's t-test). (E) ITC

771 analysis of the binding between c-di-GMP and RlmI. c-di-GMP was

772 titrated into RlmI and its mutants by three repetitions. (F) *In vitro*

773 methylation assay of the RlmI mutants. The bar chart shows the relative

774 activity of RlmI with the data points (three preparations, mean ± range; ns:

775 no significant difference, two-tailed Student's t-test).

776 **Figure 5. c-di-GMP regulates ribosome assembly to promote drug**

777 **resistance.**

778 (A) Relative c-di-GMP level of WT, $\Delta dgcZ$, $\Delta dgcZ \, dgcZ^+$, $rlmI^{\text{K201A}}$ and

779 $\Delta dgcZ \, rlmI^{\text{K201A}}$ strains. The intracellular c-di-GMP concentrations were

780 determined by UPLC-IM-MS. The bar chart shows the relative

781 quantification of c-di-GMP with the data points (three preparations, mean

782 ± range; $**p < 0.01$, two-tailed Student's t-test). (B) Methylation level of

783 C1962 in 23S rRNA of WT, $\Delta dgcZ$, $\Delta dgcZ \, dgcZ^+$, $rlmI^{\text{K201A}}$ and $\Delta dgcZ$

784 $rlmI^{\text{K201A}}$ strains. The endogenous methylation level in C1962 was

785 determined by meRIP-qPCR in triplicate. The bar chart shows the relative

786 quantification of methylated rRNA with the data points. (C) Growth

787 curves of the WT, $\Delta dgcZ$, $rlmI^{\text{K201A}}$ and $\Delta dgcZ \, rlmI^{\text{K201A}}$ strains. The *E.*

788 *coli* strains were cultured in Vogel-Bonner medium with 0, 1.5, 3 and 6

789 μg/mL kanamycin treatment, respectively. The growth was measured after

790 8, 12, 16, 20, 24, and 32 h in triplicate (three preparations, mean ± range).

791 (D) The MICs for the WT, Δ*dgcZ*, *rlmI*^{K201A} and Δ*dgcZ rlmI*^{K201A} strains.

792 The bar chart shows the MICs, which were determined by ETEST. The

793 bar chart shows the value of MICs with the data points (three preparations,

794 mean ± range; ns: no significant difference, **$p < 0.01$, two-tailed

795 Student's t-test). (E) The MICs of the resistant strains. The 12

796 kanamycin-resistant strains were mutated from RlmI K210 to alanine, and

797 the MICs were determined by ETEST. The fold changes of MIC

798 (MIC_*rlmI*^{K201A}/MIC_WT) were annotated for the strains with

799 statistically different MICs upon the mutation of K201 to Alanine. The

800 bar chart shows the value of MICs with the data points (three preparations,

801 mean ± range; *$p < 0.05$, **$p < 0.01$, two-tailed Student's t-test).

802 **Figure 6. c-di-GMP binds RlmI and inhibits its activity conserved in**

803 **multiple pathogenic bacteria.**

804 (A) CLUSTALW alignment of the binding sites of c-di-GMP and RlmI.

805 Residues involved in c-di-GMP binding (R64, R103, G114, and K201)

806 are marked by red arrows. (B) Streptavidin blotting assays for RlmI of

807 four species. The WT and mutant RlmI interacted with biotin-c-di-GMP

808 and were crosslinked by UV. Streptavidin and α-His present interaction

809 signals and protein levels, respectively. (C) *In vitro* methylation assay for

810 the RlmI of four species. The experiment was performed in triplicate. The

811   bar chart shows the quantitative results of methylation products with the

812   data points (three preparations, mean ± range; ns: no significant

813   difference, *$p < 0.05$, **$p < 0.01$, two-tailed Student's t-test).

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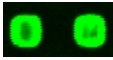
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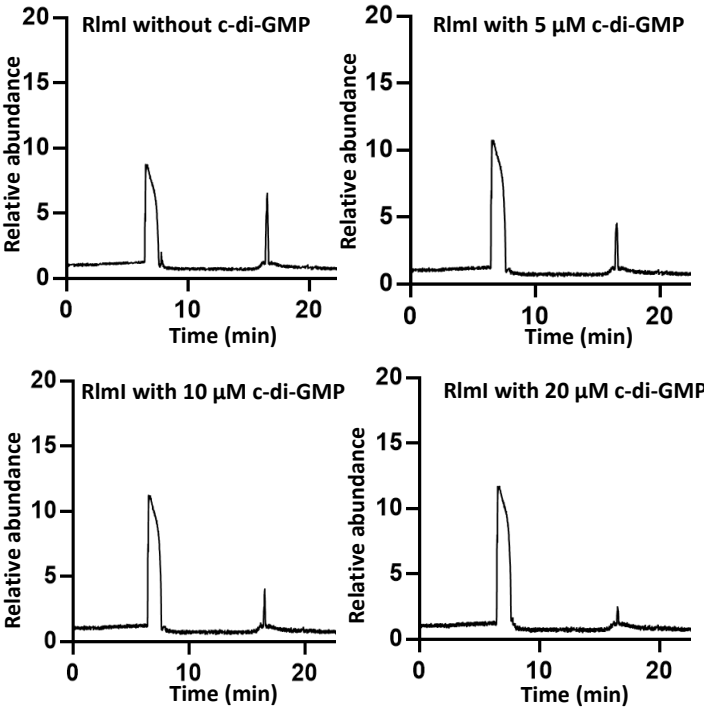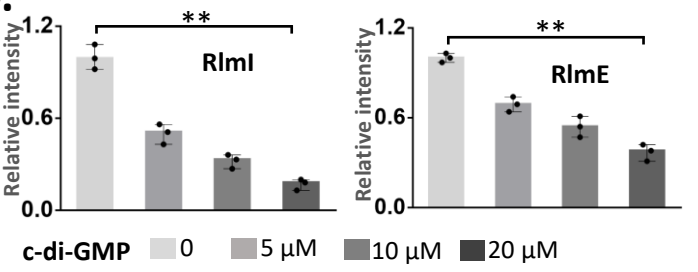
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Figure 1

A.

Protein	Bio-c-di-GMP	Biotin	SNR(+)	*P* value
RlmI			2.63	2.41×10^{-7}
RlmE			2.25	8.63×10^{-7}

D.

B.

C.

Figure 2

Figure 3

A.

B.

C.

Figure 4

A.

Gibbs Energy Landscape (three panels, with I#1, I#2, I labeled)

B.

(structural models with I#2, I, I#1 labeled; right panels showing I#1, I#2, I interactions)

Right panel I#1: ARG64(H2), ARG64(H4), O2, N4, GLY114(H3), ARG103(H2), O11, O14, LYS201(H2), LYS201(H1), with distances 1.89, 1.99, 1.55, 1.55, 1.69, 1.81

Right panel I#2: ARG64(H2), N4, O11, O2, GLY114(H3), ARG103(H2), O5, O14, LYS201(H2), LYS201(H1), with distances 1.84, 1.93, 1.63, 2.15, 1.62

Right panel I: ARG64(H2), N4, O11, O2, GLY114(H3), ARG103(H2), O14, LYS201(H2), LYS201(H1), with distances 1.92, 1.93, 1.58, 1.90, 1.75

C.

ARG64: 470ns
GLU108: 432ns
GLY114: 1148ns
SER130: 83ns

ARG64: 419ns

HIS376: 71ns

LYS201: 1235ns

HIS376: 185ns

GLU384: 153ns

GLY385: 106ns

TYR205: 69ns

ARG64: 504ns
GLU108: 361ns

THR116: 665ns

GLN372: 24ns

ARG103: 902ns
LYS201: 982ns
LYS201: 147ns

GLN372: 94ns

ASP207: 245ns

HIS200: 17ns
TYR205: 136ns
ASP207: 342ns

LYS303: 58ns

D.

WT R64A R103A E108A G114A T116A K201A

Streptavidin

RlmI

α-His

Relative intensity (bar graph): WT, R64A**, R103A**, E108A*, G114A**, T116A*, K201A**

E.

	K_d (μM)	n
WT	1.3±0.09	1.1±0.1
R64A	102.3±7.16	0.9±0.1
R103R	76.5±3.92	0.9±0.1
G114A	148.6±9.35	0.8±0.2
K201A	401.2±69.23	0.8±0.2

Injectant (kcal mol⁻¹) vs Molar ratio

F.

RlmI relative activity vs:

RlmI	WT	WT	K201A	K201A
c-di-GMP (μM)	0	20	0	20

ns

Figure 5

Figure 6

A.

----- K I L	----- YRWI	----- PT I I	----- HKTG -----	*Methanocaldococcus jannaschii*
----- FRA	----- HRLV	----- PGLV	----- QKTG -----	*Thermus thermophilus*
----- LRV	----- YRVL	----- PGLV	----- QKTG -----	*Deinococcus radiodurans*
----- GWT	----- FRLF	----- GGVT	----- AMTG -----	*Bacillus subtilis*
----- VRM	----- FRWI	----- PGVT	----- HKTG -----	*Alteromonas mediterranea*
----- VRI	----- YRLI	----- PGVT	----- HKTG -----	*Pseudoalteromonas atlantica*
----- ARV	----- FRLC	----- PGVT	----- KTGF -----	*Pseudoalteromonas translucida*
----- IRV	----- YRLI	----- PGVT	----- HKTG -----	*Colwellia psychrerythraea*
----- VRI	----- YRLV	----- PGIT	----- HKTG -----	*Shewanella baltica*
----- ARV	----- YRLI	----- PGIT	----- HKTG -----	*Erwinia tasmaniensis*
----- ARV	----- YRLI	----- PGVT	----- HKTG -----	***Klebsiella pneumoniae***
----- ARV	----- YRLI	----- PGVT	----- HKTG -----	*Cronobacter sakazakii*
----- ARV	----- YRLI	----- PGVT	----- HKTG -----	***Salmonella typhimurium***
----- ARV	----- YRLI	----- PGIT	----- HKTG -----	*Citrobacter koseri*
----- ARV	----- YRLI	----- PGIT	----- HKTG -----	***Escherichia coli***
----- ARV	----- YRLI	----- PGIT	----- HKTA -----	*Shigella flexneri*
----- ARV	----- YRLI	----- PGIT	----- HKTG -----	*Serratia proteamaculans*
----- ARV	----- YRLI	----- PGIT	----- HKTG -----	*Yersinia pestis*
----- ARV	----- YRLC	----- PGLT	----- HKTG -----	*Aeromonas salmonicida*
----- ARV	----- YRLI	----- PGIT	----- HKTG -----	***Vibrio cholerae***
----- ARV	----- YRLI	----- PGIT	----- HKTG -----	*Aliivibrio fischeri*
----- IRV	----- YRLI	----- PGIT	----- HKTG -----	*Photobacterium profundum*
----- LRV	----- YRLV	----- PGIT	----- HKTG -----	*Tolumonas auensis*

B.

C.

Graphical Abstract

c-di-GMP Inhibits Ribosomal RNA Methylation under Antibiotic Stimulation