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1 **HflXr mediates a novel antibiotic resistance mechanism**

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3 Melodie Duval^{1,2,3}, Daniel Dar⁴, Filipe Carvalho^{1,2,3}, Eduardo P. C. Rocha^{5,6}, Rotem Sorek⁴,

4 Pascale Cossart^{1,2,3*}

5

6 1. Département de Biologie Cellulaire et Infection, Institut Pasteur, Unité des
7 Interactions Bactéries-Cellules, F-75015 Paris, France.

8 2. Inserm, U604, F-75015 Paris, France.

9 3. INRA, Unité sous-contrat 2020, F-75015 Paris, France.

10 4. Department of Molecular Genetics, Weizmann Institute of Science, Rehovot
11 76100, Israel

12 5. Microbial Evolutionary Genomics, Institut Pasteur, 75015, France.

13 6. CNRS, UMR3525, Paris, 75015, France.

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15

16 * to whom correspondence should be addressed : pascale.cossart@pasteur.fr

17

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19 molecular evolution

20

21

22 **Abstract (less than 250 words, now : 250)**

23

24 Bacteria have evolved a variety of different strategies to overcome the action of
25 antibiotics, such as drug modification, target mutation and efflux pumps. Recently, we
26 performed a genome-wide analysis of *Listeria monocytogenes* gene expression after
27 growth in the presence of antibiotics, identifying genes that are upregulated upon
28 antibiotic treatment. One of them, *lmo0762*, is a homolog of *hflX*, which encodes a heat
29 shock protein that rescues stalled ribosomes by separating their two sub-units. To our
30 knowledge, ribosome splitting has never been described as an antibiotic resistance
31 mechanism. We thus investigated the role of *lmo0762* in antibiotic resistance. First, we
32 demonstrated that *lmo0762* is a novel antibiotic resistance gene that confers protection
33 against lincomycin and erythromycin, and that we renamed *hflXr* (*hflX*-resistance). We
34 showed that *hflXr* expression is regulated by a transcription attenuation mechanism
35 relying on the presence of alternative RNA structures and a small ORF containing the
36 RLR motif, characteristic of macrolide resistance mechanisms. We provide evidence that
37 HflXr is involved in ribosome recycling in presence of antibiotics. Interestingly, *L.*
38 *monocytogenes* possesses another copy of *HflX* in the core genome, *lmo1296*, that is not
39 involved in antibiotic resistance. Phylogenies of this family show several events of gene
40 duplication and widespread presence of the *lmo0762* family in Firmicutes. Overall, this
41 study highlights a previously unknown antibiotic resistance mechanism, identifying the
42 *Listeria hflXr* as the first member of a new family of antibiotic resistance genes. The
43 resistance conferred by this gene is probably of importance in the environment and
44 within microbial communities.

45

46 **Significance Statement**

47

48 Antibiotics have been widely used to treat infections. However, bacteria have evolved
49 various antibiotic resistance mechanisms, allowing them to overcome drug exposure
50 and raising important health issues. Here, we report a new bacterial antibiotic
51 resistance mechanism, occurring via ribosome splitting and recycling, ensuring efficient
52 translation even in presence of lincomycin and erythromycin, two antibiotics that block
53 protein synthesis. This novel mechanism is mediated by a HflX-like protein, encoded by
54 *lmo0762* in *L. monocytogenes*, whose expression is tightly regulated by a transcriptional

55 attenuation mechanism. This gene increases bacterial fitness in the environment. Our
56 results raise the possibility that other antibiotic-induced resistance mechanisms remain
57 to be discovered.

58

59 \body

60 Introduction

61 To treat bacterial infections, the use of bacteriostatic or bactericidal antibiotics
62 remains the gold standard. These molecules act at many levels of the bacterial
63 metabolism to prevent replication or to promote death. In the last decade, antibiotic
64 resistance of pathogenic bacteria has become a major public health concern, and multi
65 resistant strains have become increasingly difficult to treat. In 2015, the World Health
66 Organization published an action plan on antimicrobial resistance that aims to ensure
67 prevention and treatment of infectious diseases with safe and effective medicines.
68 Beside their use as therapeutics, antibiotics are also found in the environment, since
69 micro-organisms use them as strategies to compete and survive within microbial
70 communities, and given that many antibiotic-producing organisms such as *Streptomyces*
71 *spp* live in the soil [1].

72 To overcome the action of antibiotics, bacteria have evolved a number of
73 different resistance strategies. Resistance can be intrinsic, i.e. corresponding to a natural
74 property of the bacteria, such as the presence, in Gram-negative bacteria, of an outer
75 membrane that protects peptidoglycan from vancomycin [2], or acquired, e.g. gain of
76 plasmid-mediated antibiotic resistance genes. The resistance mechanisms can be
77 classified in three major families: those that prevent the drugs from entering the cell or
78 that actively pump them out of the bacteria, those that inactivate the antibiotic, and
79 those that modify the target so that it cannot be recognized by the antibiotic[3]. The
80 genes involved in these mechanisms are often induced in presence of antibiotics, using
81 various regulatory systems[4]. One of those, called “attenuation”, relies on the presence
82 of a 5’ regulatory region that folds into alternative RNA structures controlling either
83 transcription or translation of the resistance gene.

84 *L. monocytogenes* is a food-borne pathogen responsible for listeriosis, a rare but
85 lethal disease, that affects immunocompromised individuals, as well as pregnant women
86 and elderly people[5]. Although listeriosis can be efficiently treated with ampicillin and
87 gentamicin, resistance to various antibiotics, including lincosamides, gentamicin,
88 ampicillin, streptomycin, erythromycin, kanamycin, rifampicin[6–12] has been reported
89 in both food and clinical isolates.

90 In a previous analysis[13], we used a novel method “term-seq” to map the 3’ ends
91 of all RNAs in bacteria grown under various conditions, including in the presence or

92 absence of antibiotics. We discovered a previously unknown antibiotic resistance gene
93 in *L. monocytogenes* EGDe laboratory strain, *lmo0919*, which is induced in the presence
94 of lincomycin, an antibiotic that blocks translation by binding to the 70S ribosome in the
95 peptidyl transfer center, thereby preventing the transpeptidation step[14]. Another
96 gene, *lmo0762*, is also induced in the presence of lincomycin. This gene is located
97 downstream of *rli80*, a small RNA that encodes a putative 14 amino-acids open reading
98 frame (ORF). By sequence comparison, we found that the protein encoded by *lmo0762* is
99 homologous to *E. coli* and *S. aureus* HflX, GTPase proteins that bind to 70S
100 ribosomes[15–18] and recycle blocked ribosomes during heat shock in a GTP-
101 dependent manner[15, 19, 20]. Moreover, HflX was recently described as a RNA helicase
102 that modulates rRNA conformation of heat-damaged 50S subunits in *E. coli* [21], and
103 HflX also splits 100S disomes in *S. aureus*[20]. The specific expression activation of
104 *lmo0762* by sub-lethal doses of antibiotics suggests that this gene is involved in
105 antibiotic resistance, but to our knowledge, such a mechanism of ribosome splitting has
106 never been described in the context of antibiotic resistance, and hence *lmo0762*
107 appeared a new class of antibiotic resistance gene.

108 In this study, we assessed the role of *lmo0762* in antibiotic resistance. We first
109 confirmed its induction in the presence of lincomycin. By creating deletion and
110 overexpressing strains, we showed that the gene confers resistance to lincomycin and
111 erythromycin, two antibiotics that blocks translation. Thus, we renamed it *hflXr*, for *hflX*-
112 resistance. We analysed its expression regulation, and by combining structure
113 prediction of *rli80* and analysis of various mutants, we showed that a transcription
114 attenuation mechanism potentially leading to premature transcription termination
115 controls *hflXr* expression. This attenuation mechanism relies on the 14-aa ORF encoded
116 by *rli80* which strikingly contains the arginine-leucine-arginine (RLR) motif, a signature
117 feature for macrolide resistance gene leader peptides [22]. Moreover, by analysing the
118 ribosome “profiles” of bacteria grown in the presence of erythromycin, we provide data
119 suggesting that *Lmo0762* splits blocked 70S ribosome and promotes translation. Finally,
120 we identified in *L. monocytogenes* another homolog of *hflX*, *lmo1296*, which is not
121 involved in antibiotic resistance. By analysing the phylogeny of *hflX* genes in
122 prokaryotes, we found that this duplication happened several times independently in
123 several major clades of prokaryotes, and that many Firmicutes possess a *hflX* copy
124 closely related to *hflXr*. Hence, it is tempting to speculate that the mechanism is shared

125 by numerous bacterial species. Overall, these data reveal a new antibiotic resistance
126 mechanism in bacteria that is probably of importance for survival in the environment
127 and within microbial communities.

128

129 Results

130 ***lmo0762* is a new antibiotic resistance gene**

131 In a previous study[13], we analysed the expression profile of all *Listeria* genes in
132 the presence of sub-inhibitory concentrations of lincomycin, and discovered that
133 *lmo0762* is induced (Fig. 1A). This gene is located downstream of a small RNA, *rli80*, and
134 while *rli80* appears to be transcribed in both the absence (black) or the presence
135 (green) of lincomycin, *lmo0762* is primarily transcribed in the presence of the antibiotic.
136 This specific upregulation upon antibiotic exposure prompted us to investigate whether
137 *lmo0762* plays a role in antibiotic resistance. In order to verify *lmo0762* induction by
138 lincomycin at the protein level, we created a strain in which a Flag tag was introduced at
139 the C-terminus of the Lmo0762 protein. We extracted total proteins from *Listeria* grown
140 in BHI in the absence or presence of sub-inhibitory concentration of the antibiotic, and
141 performed a western blot experiment using an anti-Flag antibody (Fig. 1B left pannel).
142 EF-Tu was used as a loading control. The results confirmed that in presence of
143 lincomycin, Lmo0762 is induced.

144 In order to test whether *lmo0762* is an antibiotic resistance gene, we constructed
145 a mutant strain with a deleted *rli80-lmo0762* region (hereafter named Δ *lmo0762*). We
146 complemented the deletion by reintroducing the *rli80-lmo0762* region under the control
147 of its native promoter using the pAD integrative plasmid [23] (hereafter named
148 Δ *lmo0762-cpt*), and we verified by qRT-PCR that in the complemented strain, *lmo0762* is
149 induced by lincomycin at a level similar to that of the WT strain (Figure S1). We also
150 created a strain that overexpresses the gene (see below), that we named *anti-anti*. This
151 strain constitutively express Lmo0762, which results in an accumulation of the protein,
152 independently from the presence of antibiotics (Fig S2). We then performed a minimum
153 inhibitory concentration (MIC) assay on the 4 strains using various antibiotics (Fig 1C,
154 left panel and Fig S3), and we observed that the Δ *lmo0762* strain is more sensitive to
155 erythromycin than the WT strain or the complemented strain with a 2-fold effect, a
156 result consistent with effects which have been observed with several macrolide
157 resistance determinants[24]. Moreover, the overexpressing strain *anti-anti* is more

190 In order to decipher whether *lmo1296* also participates to antibiotic resistance,
191 we first analysed its induction upon antibiotic exposure. We grew the bacteria in
192 presence and absence of antibiotics, we extracted total RNA and performed qRT-PCR
193 experiment. The results show weak or no induction of *lmo1296* upon antibiotic exposure
194 in comparison to *lmo0762* (Fig 1D). Second, we deleted the gene from the EGDe strain
195 (Δ *lmo1296*), and we used this strain to perform MIC assay (Fig 2A). The results showed
196 no susceptibility for erythromycin, or any other antibiotics tested (Fig S3). We also
197 created the double deletion strain Δ *lmo0762*- Δ *lmo1296*, and no further susceptibility
198 could be observed compared with the Δ *lmo0762* strain. Finally, we re-introduced the
199 *lmo1296* gene under the control of *rli80* in the Δ *lmo0762* mutant (named Δ *lmo0762*-
200 *cpt1296*, fig. S1A). We controlled by qRT-PCR that in this strain *lmo1296* is induced by
201 the antibiotic at a similar level compared to *lmo072* in the WT or complemented strain
202 (Δ *lmo0762*-*cpt*) due to *rli80* regulation (Fig S1B), and we tested the strain in a MIC
203 assay. We observed that unlike the strain Δ *lmo0762*-*cpt* the strain Δ *lmo0762*-*cpt1296*
204 remains sensitive to erythromycin. Altogether, these data indicate that *lmo1296* is not
205 involved in antibiotic resistance.

206 To unravel the evolutionary history of *hflXr* and its homologs, we reconstructed
207 the phylogeny of *hflX* genes among prokaryotes (Fig. 2B and Fig S4). Strikingly, the
208 phylogenetic tree shows that *lmo1296* and *lmo0762* are well separated in two large
209 clades, both containing almost only Firmicutes. This duplication event is probably old,
210 since it is shared by many bacteria from the clade, and the trees of the two sub-families
211 coarsely recapitulate the tree of Firmicutes (Fig. S5). Interestingly, other phyla also
212 harbour a duplication in the *hflX* genes, e.g. α , β , γ and δ Proteobacteria and Archaea.
213 These duplications occurred independently from the one of Firmicutes, highlighting the
214 importance of *hflX* duplicated genes. The analysis of 163 pan-genomes from bacteria
215 revealed that the genes of this family, when present, are in the core genome (98% in
216 >90% of the strains, Fig S6). Finally, the analysis of genetic neighbourhood of the two
217 sub-families showed much higher conservation for *lmo1296* than for *lmo0762* (Table S3
218 and S4). Based on these elements, it is tempting to speculate that the ancient duplication
219 of *hflX* led to two proteins with specialized functions in Firmicutes, explaining why
220 duplicates co-occur, one of which is involved in antibiotic resistance (*hflXr*). If correct,
221 this means that other genes of this sub-family may provide antibiotic resistance. Such

222 genes were found in important pathogens, such as *Bacillus cereus*, *Bacillus anthracis*, and
223 *Clostridioides difficile*.

224

225 **HflXr recycles ribosomes upon antibiotic exposure**

226 Ribosome stalling is a phenomenon induced by heat shock that results in halt of
227 translation. Some antibiotics can also impair translation, and resistance proteins that
228 remove the antibiotic from the stalled ribosome have previously been described. These
229 include, tetO, tetM, and ABC-F transporters[25–27]. However, a mechanism by which the
230 ribosome is split and recycled has not been described in the context of antibiotic
231 resistance, and could constitute a new class of antibiotic resistance factors.

232 In order to assess this hypothesis, we grew WT and $\Delta lmo0762$ bacteria in the
233 presence and absence of antibiotic, and performed a polysome profiling assay. Given
234 that *lmo0919* may mask the effect of *hflXr* in the presence of lincomycin, we used
235 erythromycin in this experiment. The results show that in the presence of the antibiotic,
236 the 70S proportion is higher in $\Delta lmo0762$ strain compared with a WT strain (Fig. 3),
237 while in the absence of antibiotics no difference was observed (Fig S7). A similar
238 experiment has been performed in *Escherichia coli* in the context of heat shock, and the
239 peak difference obtained in this study is comparable to what we obtained in presence of
240 erythromycin[19]. Such an accumulation of 70S in absence of *hflXr* show that this gene
241 participates in ribosome recycling, probably by splitting ribosomes upon antibiotic
242 exposure, thus recycling them to start new rounds of translation.

243

244 ***hflXr* transcription is regulated by a ribosome-dependent attenuation mechanism**

245 As shown above, *hflXr* expression is induced by lincomycin and erythromycin
246 (Fig. 1A and 1D). Moreover, *hflXr* is located downstream of *rli80*, a constitutively
247 transcribed small RNA that we hypothesized to act as a regulatory switch (Fig. 1A).
248 Using the term-seq data for *L. monocytogenes* grown in the absence of antibiotics[13],
249 we found an accumulation of 3' reads that occurred immediately downstream of the
250 *rli80* ribo-regulator, thus suggesting a regulatory mechanism relying on premature
251 termination[4] (Fig. 1A, black arrow). A hallmark of such ribo-regulators is the ability to
252 display mutually exclusive RNA folding patterns that either stabilize or destabilize the
253 transcriptional intrinsic terminator, turning the downstream gene transcription 'off' or
254 'on', respectively[4, 28, 29]. This process is named "attenuation". We thus searched for

255 such alternative RNA structures in the *rli80* sequence, using the PASIFIC algorithm[30].
256 We found that *rli80* can indeed fold into either a terminator structure, that would lead to
257 premature termination and to an accumulation of short RNAs, alternatively in a
258 structure acting as an anti-terminator, where the terminator is destabilized, allowing the
259 synthesis of full length *hflXr* mRNA (Fig. 4A and Fig. S8). In addition, we found that *rli80*
260 encodes a 14 amino-acids upstream open-reading frame (ORF) containing hallmark
261 signature arginine-leucine-arginine (RLR) motif (Fig. 4A), which is commonly found in
262 small ORFs of other macrolide sensing attenuators and controls translation arrest by
263 disturbing the transpeptidation step due to the amino-acid geometry [22], thus
264 promoting the expression of the downstream macrolide resistance gene[31, 32] (Table
265 1).

266 Based on this model as well as on the induction by antibiotics, we hypothesized
267 *hflXr* transcription is controlled either by direct binding of the antibiotic to the mRNA
268 (riboswitch) or by a ribosome-mediated attenuation mechanism due to ribosomal
269 stalling on the *rli80* ORF. To discriminate between these two possibilities, we performed
270 RNA-seq to measure the lincomycin-dependent induction of *hflXr* in a *Listeria* strain
271 expressing the 23S rRNA methyltransferase ErmC[33]. In these bacteria, the rRNA 23S is
272 methylated at position A2058, thus rendering the ribosomes insensitive to
273 lincomycin[33]. The results show that in ErmC-expressing bacteria, the expression of
274 *lmo0762* was no longer activated in response to the antibiotic (Fig. 4B), suggesting that
275 the ribo-regulation depends on stalled ribosomes rather than by direct binding of the
276 antibiotic to the RNA. We further tested this hypothesis by validating that the small ORF
277 is indeed translated *in-vivo* by generating a translational GFP fusion to the C-terminus of
278 the small peptide (Fig. 4C), that showed fluorescence when visualised under microscope.
279 In order to validate the attenuation mechanism, which involves regulatory structures
280 and the *rli80* ORF, we created a series of mutants. First we mutated the ATG of the ORF
281 (ATG>ACG), as well as regions that are controlling the formation of the terminator, and
282 are named anti-terminator region and anti-anti-terminator region (Fig. 4A, brown
283 dashed squares). We analysed the RNA-seq profile of the *rli80-lmo0762* locus in these
284 different mutant strains in the absence and presence of lincomycin (Fig. 4D). Strikingly,
285 and in agreement with the above hypothesis, mutating the anti-terminator region or the
286 ORF ATG, both of which are predicted to stabilize the 'off' conformation of *rli80*,
287 prevented the ribo-regulator from activating expression during antibiotic exposure and

288 led to increased antibiotic sensitivity in MIC experiment (Fig S3) and to fewer mRNA
289 abundance in qRT-PCR assay (Fig. S9). In contrast, mutating the anti-anti-terminator
290 region, which is predicted to maintain the 'on' conformation of the ribo-regulator, led to
291 constitutive readthrough and induction of *hflXr*, regardless of the presence of
292 lincomycin, as well as enhanced antibiotic resistance (Fig 1C, Fig S3 and Fig S9). Taken
293 together, these results show that *rli80* controls the expression of *hflXr* via a ribosome-
294 dependent transcription attenuation mechanism, such that HflXr protein expression is
295 induced in response to ribosome inhibition and stalling.

296

297 Discussion

298 In this work, we describe a novel antibiotic resistance mechanism to lincomycin
299 and erythromycin in *Listeria monocytogenes* which is mediated by Lmo0762, an HflX
300 homolog, that we renamed HflXr, for HflX-resistance. We showed that deletion of the
301 gene renders the bacteria more sensitive to erythromycin and lincomycin whilst its
302 overexpression renders the bacteria more resistant. The induction of the gene in
303 presence of antibiotics is mediated by an attenuation mechanism that involves a small
304 ORF containing a RLR sequence. This RLR motif is a signature of macrolide resistance
305 genes, since it is commonly found in small ORFs in leader regions that regulate their
306 expression (Table 1) [31, 32]. Moreover, by analysing the ribosome "profiles" of bacteria
307 grown in presence or absence of antibiotic, we provide evidence that the proportion of
308 70S ribosome increases upon erythromycin treatment in a strain depleted from *hflXr* in
309 comparison to the WT strain, which led us to hypothesise that HflXr mechanism of
310 action is to split ribosomes. Surprisingly, *in vitro* experiments showed that erythromycin
311 and lincomycin can inhibit the GTPase activity of the *E. coli* HflX[15]. Given that
312 ribosome splitting was described as a GTP-dependent mechanism[15, 19], further
313 investigations will be required to reconcile these data with our results.

314

315 Interestingly, *L. monocytogenes* encodes another *hflX* homolog, *lmo1296*, which is
316 not involved in antibiotic resistance. Many Firmicutes possess two copies of *hflX*
317 homologs, and similar independent duplications are observed in other clades of
318 prokaryotes (Fig 2B). Given that many Firmicutes possess a *hflX* gene that belongs to the
319 sub-family of *hflXr*, it seems reasonable to think that the antibiotic resistance
320 mechanism that we described in *Listeria* for *hflXr* could be conserved in many other

321 bacteria. This duplication represents an example of how bacteria can employ common
322 stress response factors as antibiotic resistance genes. In addition, our work is now
323 strengthened by two studies that were recently published where functional
324 metagenomic databases constructed from antibiotic rich environments pointed *hflX*
325 from *Simkania negevensis* and *Emergencia timonensis* as a putative resistance genes [24,
326 34]. Based on our phylogenetic analysis, *hflX* from *Eubacterium spp*, which is closely
327 related to *E. timonensis*, belongs to *hflXr* family, which is in agreement with our claim
328 that this gene family is involved in antibiotic resistance. *S. negevensis* is not a Firmicute
329 and its *hflX* belongs to sub-families which have not been studied for antibiotic resistance.
330 These observations reinforce the conclusions of our study and further suggest that this
331 new antibiotic resistance mechanism is likely spread in the environment. The level of
332 resistance conferred by *hflXr* which may appear weak in clinical settings according to
333 EUCAST breakpoints, is probably of importance in the environment and within
334 microbial communities, conferring resistance to antibiotics that may be found in the soil.

335

336 The benefit provided by *hflXr* in bacteria exposed to lincomycin and to
337 erythromycin seems different, since it was necessary to delete *lmo0919*, another
338 lincomycin resistance gene, to observe the effect of the *hflXr* deletion. Lincomycin
339 belongs to the lincosamide antibiotic family, whose members bind the ribosome at the
340 peptidyl-transferase center and inhibit peptide bond formation. Erythromycin is a
341 macrolide antibiotic that also binds at the vicinity of the peptidyl transfer center, in the
342 peptide exit tunnel channel, nearby to the lincomycin target site. Both antibiotics
343 prevent translation at early stages of elongation[33]. The remaining stalled ribosomes
344 need to be recycled in order to start a new round of translation. It is interesting to note
345 that in order to show the involvement of *lmo0762* in lincomycin resistance, we had to
346 delete *lmo0919* gene, while the effect of *hflXr* could be directly visualized for
347 erythromycin. Our careful analysis has shown *lmo0919* gene is encoding an “ABC-F
348 transporter” (Fig. S10) although we previously considered it encodes an antibiotic efflux
349 pump. Recent studies have shown that antibiotic resistance genes annotated as “ABC-F
350 transporters” have the capability of displacing ribosome-bound antibiotics, *in-vitro*[25,
351 27]. We thus propose that HflXr could act in concert with Lmo0919 in the presence of
352 lincomycin: HflXr would split the ribosome, while the Lmo0919 would displace the
353 antibiotic, thus recycling the ribosome to re-start translation. Interestingly, Lmo0919 is

354 only produced in the presence of lincomycin, and is not activated by the presence of
355 erythromycin[13], while other ABC-F transporters confer macrolide resistance, such as
356 MsrA[27]. Thus, we hypothesize that in the presence of erythromycin, HflXr acts in
357 combination with a protein with macrolide displacement activity to rescue stalled
358 ribosome and restart translation. This hypothesis is presented in Fig. 5.

359

360 *hflX* transcription is regulated by an attenuation mechanism that relies on the
361 upstream regulatory RNA, rli80, which folds into alternative regulatory structures and
362 contains a small ORF that harbours the RLR motif. This attenuation mechanism involves
363 the pausing antibiotic-stalled ribosomes on the ORF, which in turn prevents the
364 formation of the terminator hairpin structure, thus permitting the transcription the full-
365 length *hflXr* mRNA. This allows the bacteria to fine-tune the expression of *hflXr* in
366 response to two antibiotics that block translation after incorporation of few amino acids.
367 As a consequence, the regulation also works as a feed-forward loop, by shutting down
368 the expression of HflXr when the antibiotic is cleared. Indeed, in the absence of drug, the
369 ribosome does not pause on the regulatory region, and this in turn prevent *hflXr*
370 transcription. These findings are recapitulated in our model (Fig. 5). Such an attenuation
371 mechanism involving RNA structures and a small ORF has been found for different
372 antibiotic resistance genes e.g. *ermC* gene[4, 31]. It is interesting to note that in this
373 latter example, a translation attenuation modulates the availability of the ribosome
374 binding site of the resistance gene, whilst our regulation mechanism modulates the
375 transcription of *lmo0762* via a transcriptional attenuation.

376

377 Overall, we described here a novel antibiotic resistance mechanism in *Listeria*
378 *monocytogenes*, that uses HflXr protein to recycle ribosomes in presence of antibiotics.
379 The gene *hflXr* seems widely spread across species, and it is to be expected that *hflXr*
380 genes will be employed in other bacteria species for antibiotic resistance in the
381 environment and within bacterial communities. This study also allows us to highlight
382 how bacteria can take advantage of general stress factors e.g., a heat shock factor, by
383 using them in a different context, in order to improve fitness and growth in a broad
384 range of conditions.

385

386 Materials and Methods

387

388 Materials and Methods can be found in the Supplementary Information sheet.

389

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505

506

507 Figure Legends

508

509 **Figure 1. Lmo0762 expression is induced in the presence of lincomycin and**
510 **erythromycin.** (A) *L. monocytogenes* was grown until exponential phase in BHI, and
511 bacteria were collected before (black RNA-seq coverage), or after 15 min exposure to
512 sub-inhibitory concentration (0.25 µg/ml) of lincomycin (green). The RNA was
513 extracted and sequenced, and the result for *rli80-lmo0762* is shown here. RNA-seq
514 coverage was normalized by the number of uniquely mapped reads in each sequencing
515 library. Black and green curves represent RNA-seq coverage, black arrow represents
516 term-seq reads. (B) Bacteria EGDe WT and carrying a flag tag at the C-terminus of
517 Lmo0762 (Flag) were grown for 1h in presence or in absence of various concentrations
518 of lincomycin and erythromycin, and total protein were extracted. Western blot analysis

519 using anti-flag antibody revealed an induction of Lmo0762-flag in presence of the
520 antibiotic. EF-Tu was used as a loading control. (C) Schematic representation of MIC
521 experiment. The colors indicates that the tested strain is more sensible (blue) or more
522 resistant (red) to the antibiotic compared to the reference (ref) strain. Lmo0762
523 induction is also represented. (D) RNA was extracted from wild-type bacteria grown in
524 BHI, before and after 15 min exposure to lincomycin or erythromycin. Induction of
525 *lmo0762* and *lmo1296* was calculated by comparison with their endogenous level before
526 the addition of the antibiotic. Data are represented as mean \pm s.e.m. We used a one-way
527 anova on Δ Ct values for statistics, using biological replicates as pairing factors ($p < 0.05$).

528

529 **Figure 2. Phylogeny of HflX homologs.** (A) Schematic representation of MIC
530 experiments, similar to 1C. (B) Schematic representation of the phylogenetic tree of *hflX*
531 homologs among prokaryotes. A detailed tree is available on Fig. S4, S5 and on
532 additional on-line file S1.

533

534 **Figure 3. Lmo0762 is involved in recycling of antibiotic-stalled 70S ribosomes.**
535 Wild-type (blue) and Δ *lmo0762* (purple) bacteria were grown in BHI medium until
536 exponential phase and erythromycin (0.18 μ g/ml) was added or not for 1h.
537 Chloramphenicol was then added to the culture (2 min exposure at 5 mM) in order to
538 stabilize the polysomes, and bacteria were pelleted and flash-frozen. The cellular
539 content was extracted, and 15K units of A_{260nm} was loaded on a 5-50% sucrose gradient.
540 After ultracentrifugation, the samples were collected from top (0mm) to bottom
541 (80mm) of the tubes and the A_{260nm} absorbance was monitored using a UV lamp. The
542 baseline was corrected and the results were normalized based on the area under the
543 curve.

544

545 **Figure 4. Lmo0762 expression is regulated by a transcription attenuation**
546 **mechanism.** (A) The predicted rli80 RNA-structures were analysed using the PASIFIC
547 algorithm, and two alternative conformations were predicted, one with an intrinsic
548 terminator (left) that leads to a short transcript, and one with an anti-terminator (right)
549 that leads to a long transcript that encodes *lmo0762*. Key regulatory regions were
550 identified (anti-anti-terminator in red, anti-terminator in orange, terminator in
551 green/blue) and a short ORF of 14 amino acids (purple) is encoded in a region that

552 encompasses the anti-anti-terminator region. Different mutants were created where
553 regulatory regions were removed (dashed brown squares) in order to decipher the
554 regulatory mechanism. (B) Wild-type bacteria in which *ermC* expression was induced or
555 not were grown as described in the legend to figure 1A and RNA was extracted and
556 sequenced. The RNA-seq profile of the *rli80-lmo0762* locus is presented here. (C) The *L.*
557 *monocytogenes lmo0762* ribo-regulator (*rli80*) was modified by a chromosomal in-frame
558 fusion of a GFP reporter protein that lacks the initiation codon, to the 14 aa ORF. Left
559 and right panels show the phase contrast and fluorescence images, respectively, and
560 demonstrate that the ORF is translated *in-vivo*. (D) Wild-type and mutant bacteria were
561 grown as described in the legend to figure 1A and RNA was extracted. The RNA-seq
562 profile of the *rli80-lmo0762* locus is presented here.

563

564 **Figure 5. Model of the combined action of Lmo0762 and Lmo0919 to protect**
565 **bacteria against lincomycin and erythromycin.**

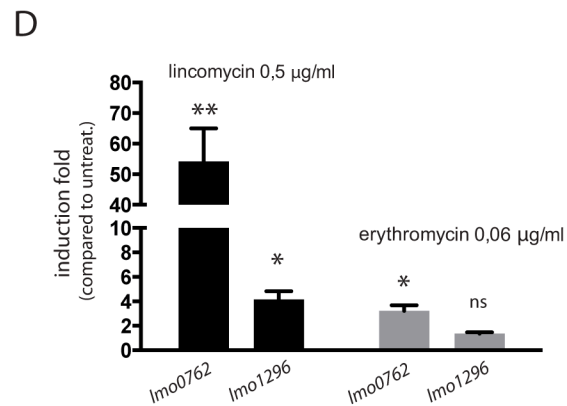
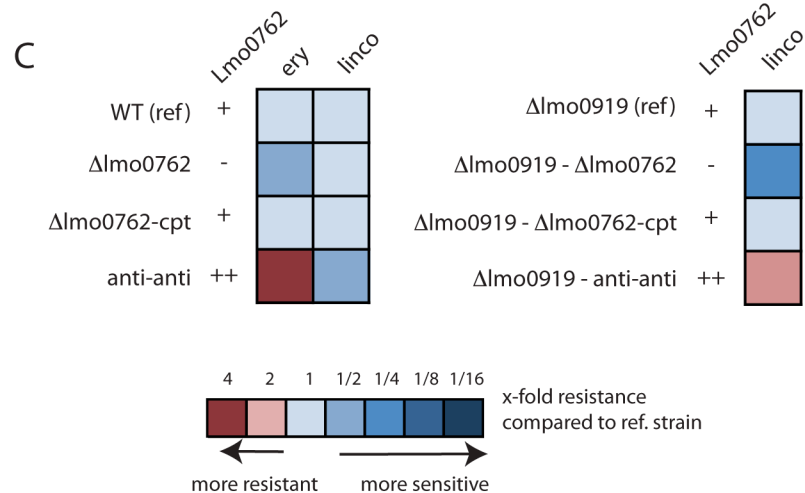
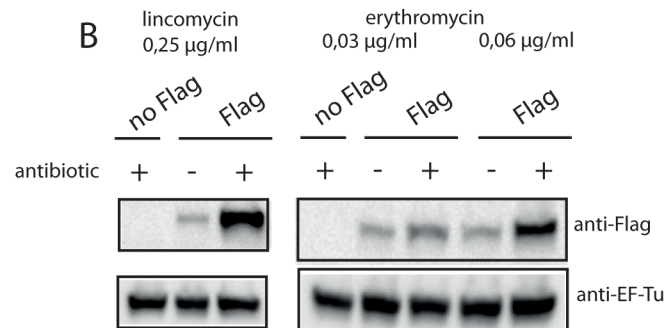
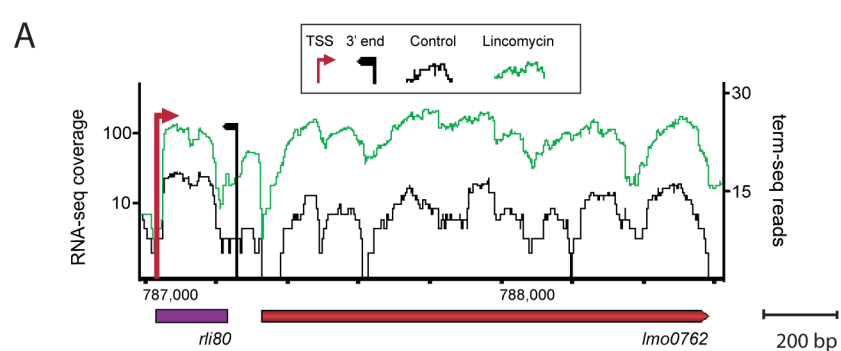
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567

568 **Table 1. RLR motif is found upstream of macrolide resistance genes.** Many
569 macrolide resistance genes are located downstream a small ORF which contains the RLR
570 motif. We selected few examples of these genes (list modified from[31]), a complete list
571 is available in[32]

572

573



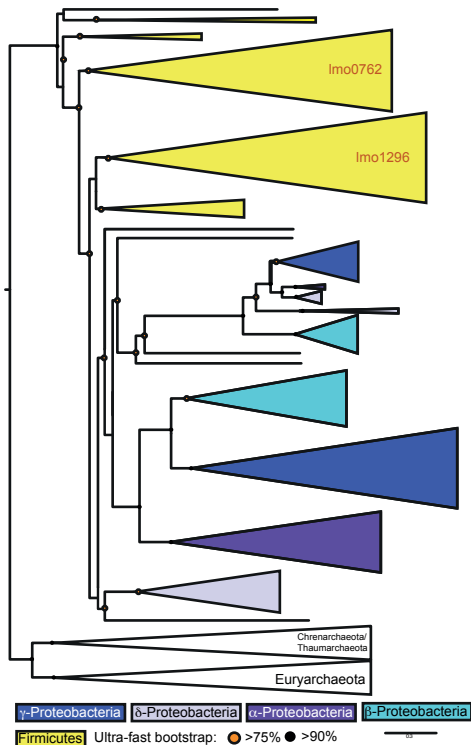
A

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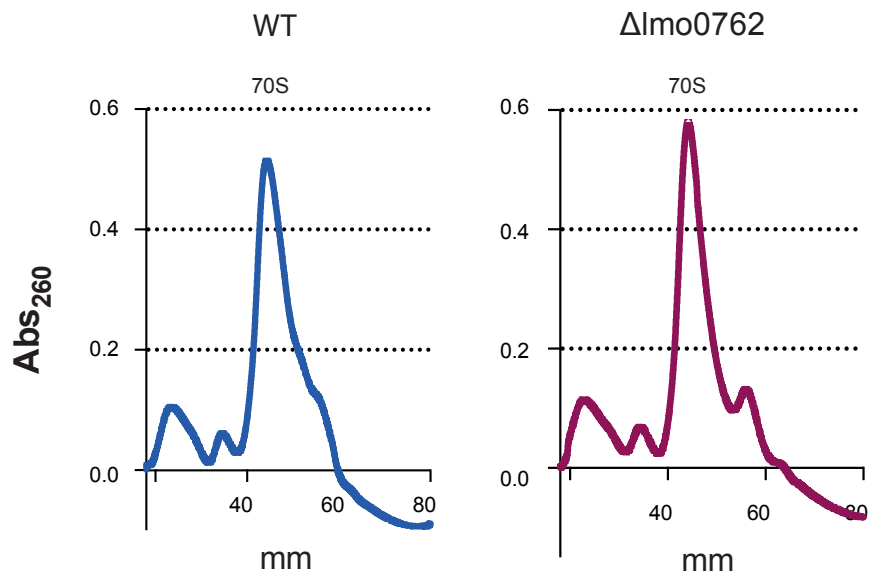
WT

 Δ Imo1296 Δ Imo0762 Δ Imo1296 Δ Imo0762 Δ Imo0762-cpt Δ Imo0762-cpt1296

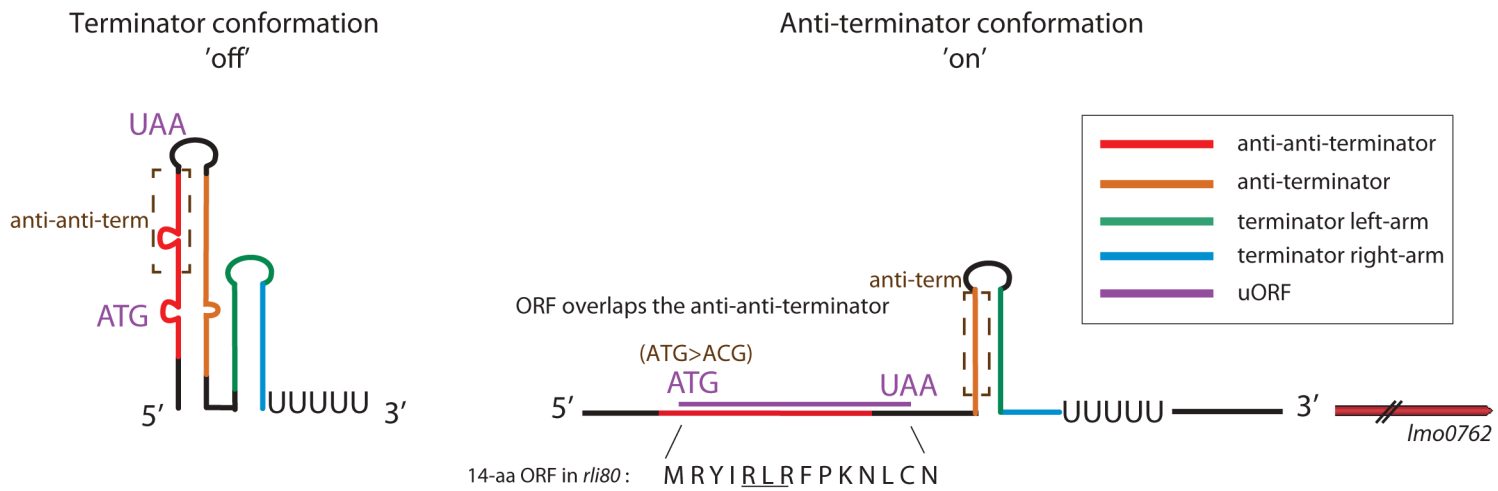
B



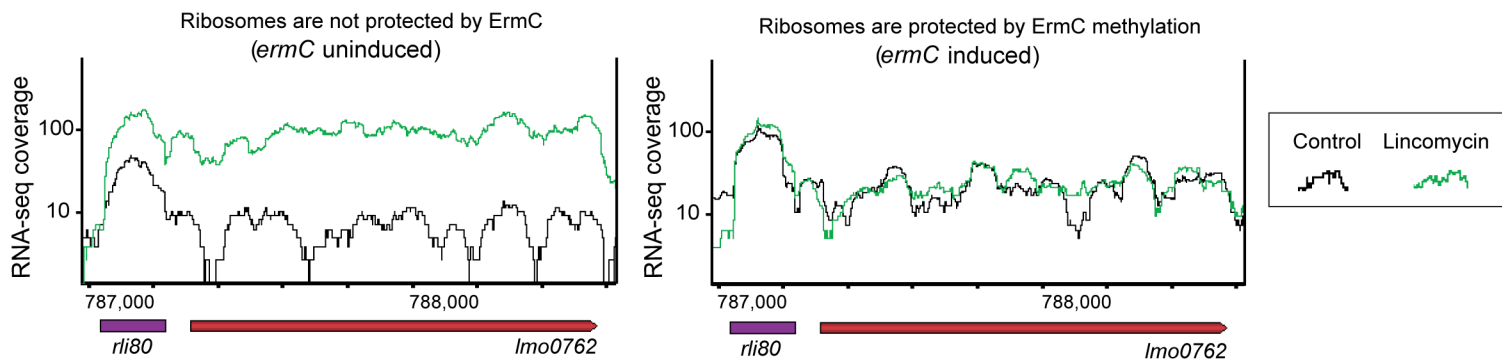
Erythromycin



A



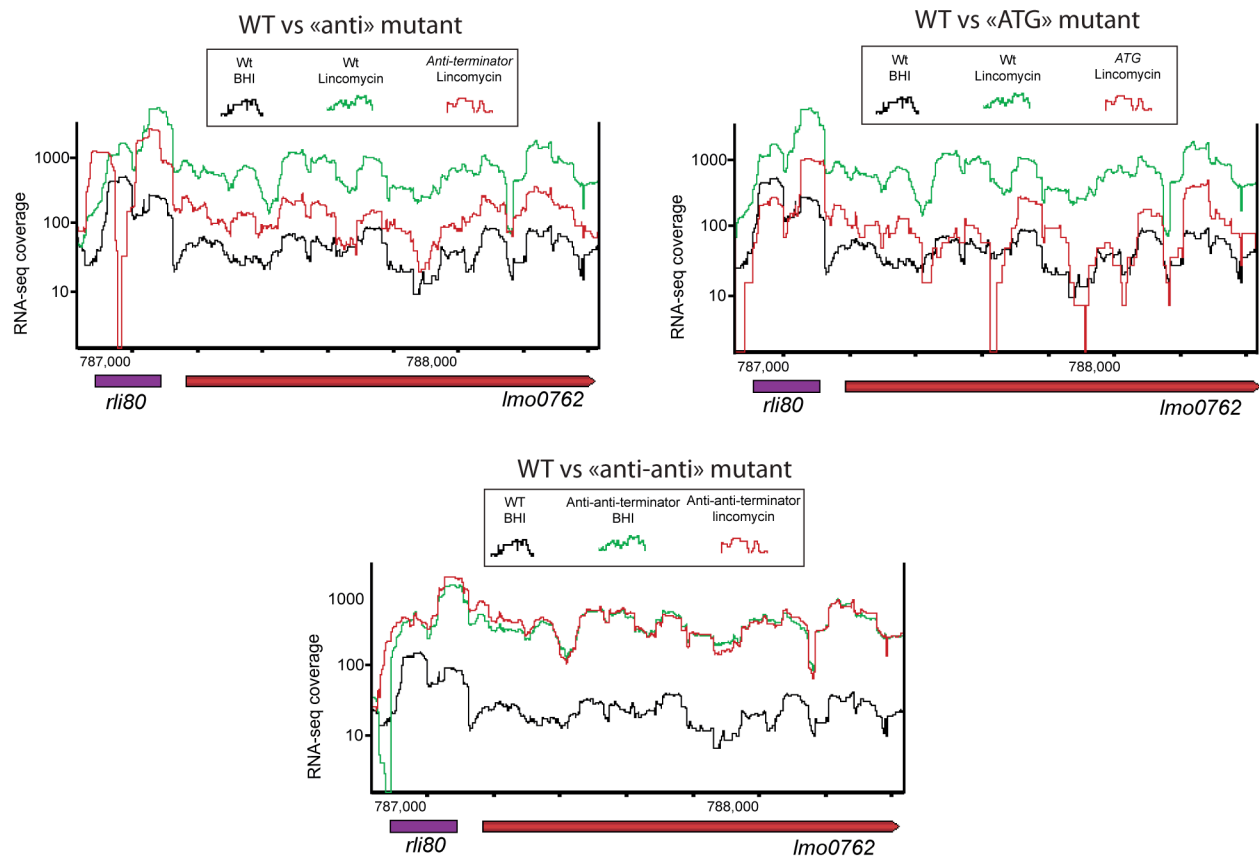
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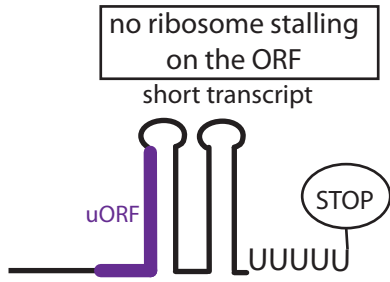
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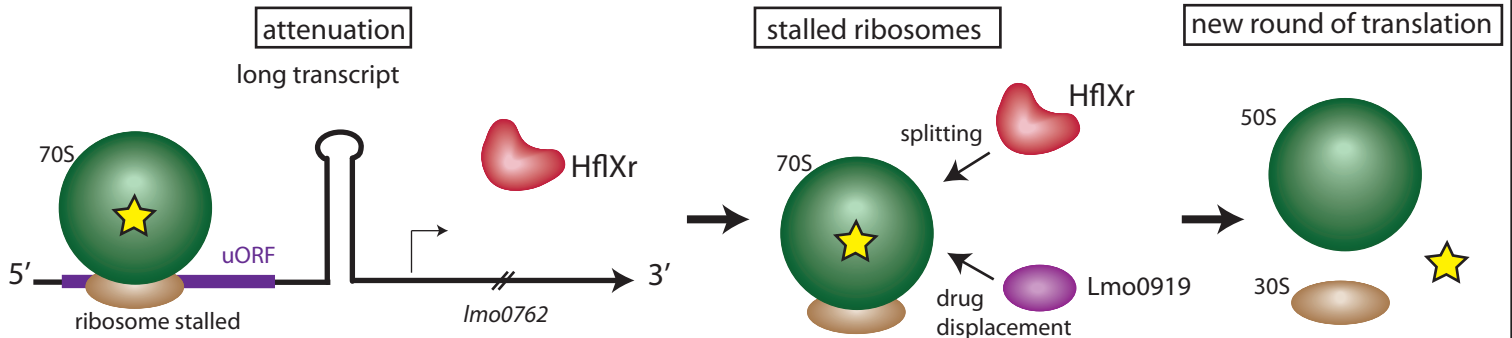


no antibiotics



~~no HflXr production~~

+ lincomycin ★



+ erythromycin ★

