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HflXr mediates a novel antibiotic resistance mechanism

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Abstract (less than 250 words, now : 250)

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

Significance Statement

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

\body

Introduction

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

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

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

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

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

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

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

Results

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

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

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

[illegible]

Lmo0762 is a HflX homolog

To explore the mode of action of this gene, we searched for homologs of the protein encoded by *lmo0762* using blastp and hmm protein profiles. This revealed homologs of HflX in various bacteria (Table S1), including *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli*. HflX has been described as a heat-shock stress-response GTPase protein that can split and recycle ribosomes that have become immobilized due to heat stress[15, 19, 20]. We renamed *lmo0762* to *hflXr*, for *hflX*-resistance. Surprisingly, we discovered another homolog of *hflX* in *L. monocytogenes*, *lmo1296* (Table S1). The two proteins encoded from *lmo0762* and *lmo1296* contain a GTP-binding domain and a 50S binding domain, like all other 8527 homologs of this family that were found within 8113 genomes (Table S2).

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

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

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

HflXr recycles ribosomes upon antibiotic exposure

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

In order to assess this hypothesis, we grew WT and *Δlmo0762* bacteria in the presence and absence of antibiotic, and performed a polysome profiling assay. Given that *lmo0919* may mask the effect of *hflXr* in the presence of lincomycin, we used erythromycin in this experiment. The results show that in the presence of the antibiotic, the 70S proportion is higher in *Δlmo0762* strain compared with a WT strain (Fig. 3), while in the absence of antibiotics no difference was observed (Fig S7). A similar experiment has been performed in *Escherichia coli* in the context of heat shock, and the peak difference obtained in this study is comparable to what we obtained in presence of erythromycin[19]. Such an accumulation of 70S in absence of *hflXr* show that this gene participates in ribosome recycling, probably by splitting ribosomes upon antibiotic exposure, thus recycling them to start new rounds of translation.

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

Materials and Methods

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

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Figure Legends

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

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

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

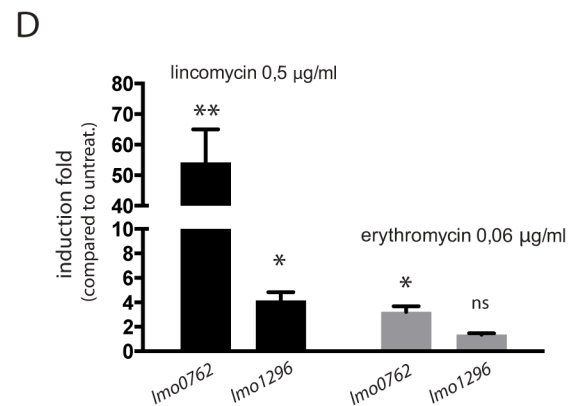
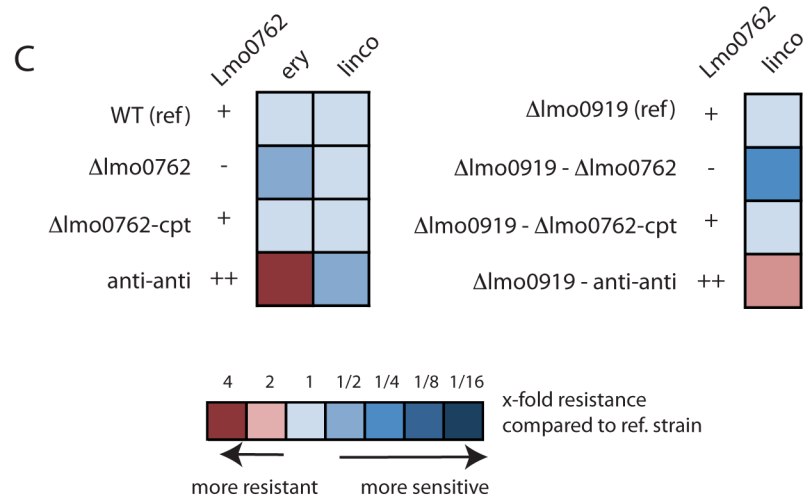
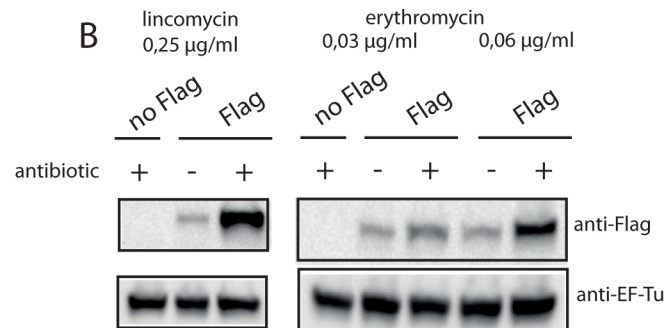
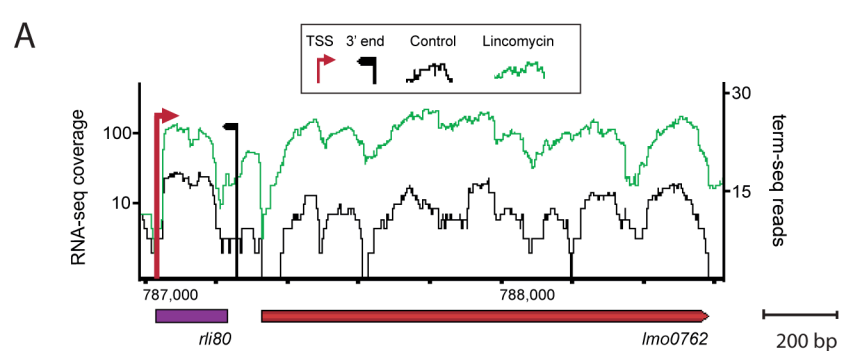
Figure 3. Lmo0762 is involved in recycling of antibiotic-stalled 70S ribosomes. Wild-type (blue) and Δ *lmo0762* (purple) bacteria were grown in BHI medium until exponential phase and erythromycin (0.18 μ g/ml) was added or not for 1h. Chloramphenicol was then added to the culture (2 min exposure at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

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

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

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

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



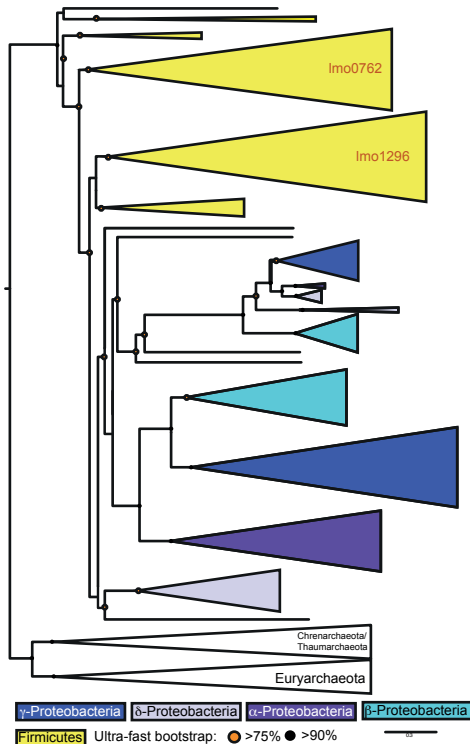
A

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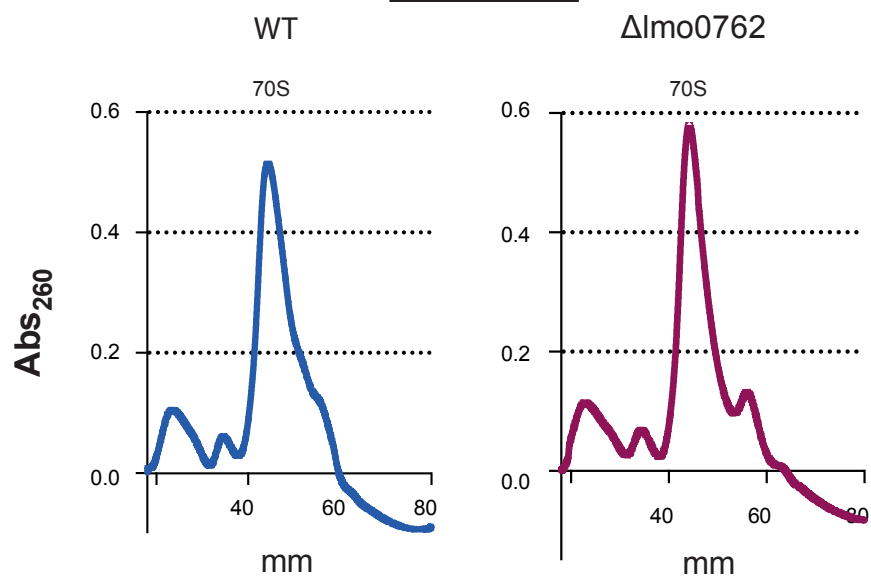
WT

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

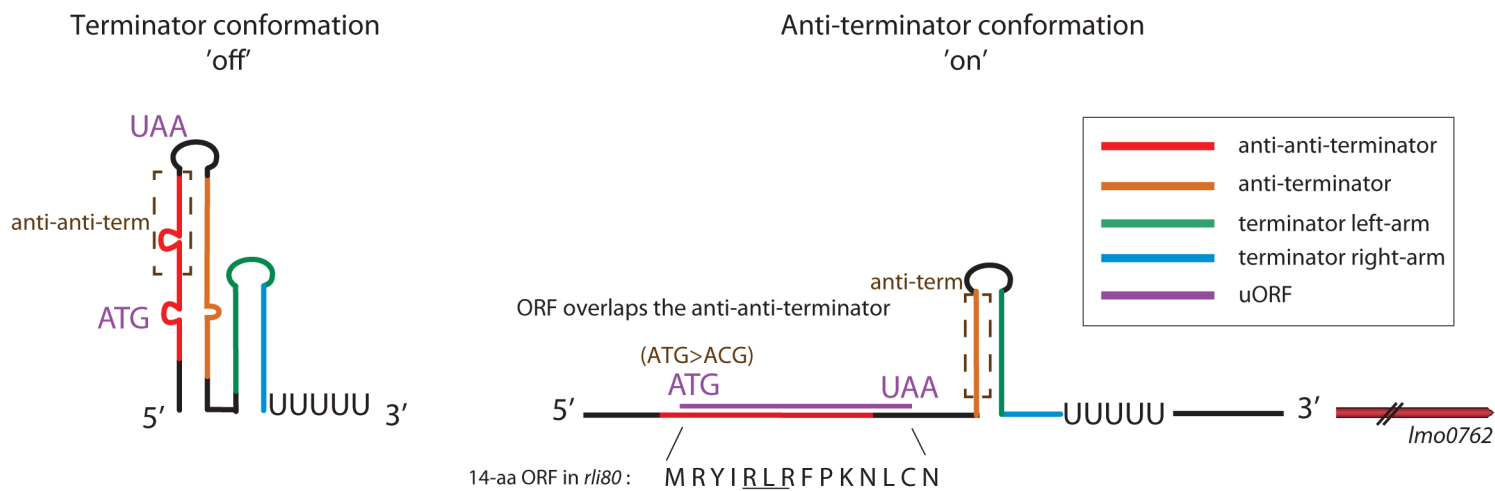
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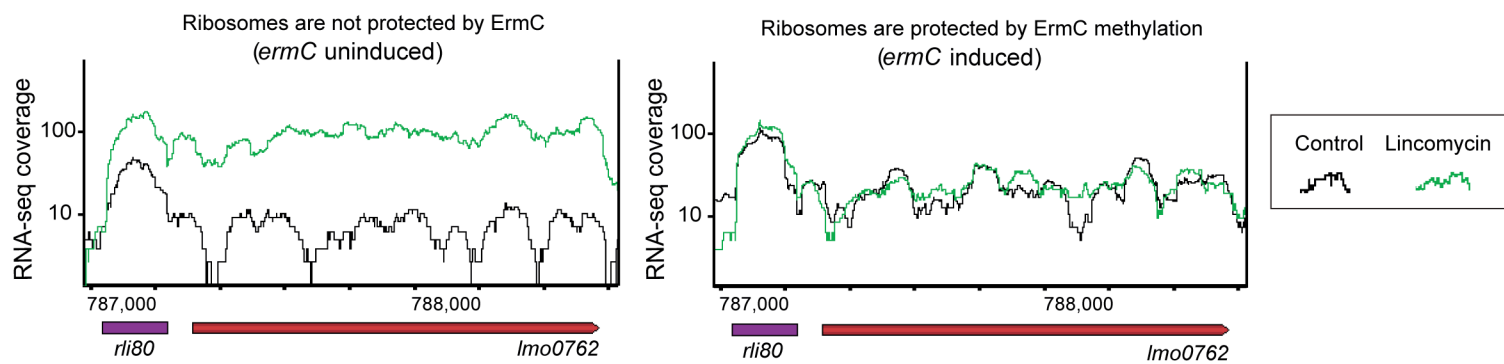
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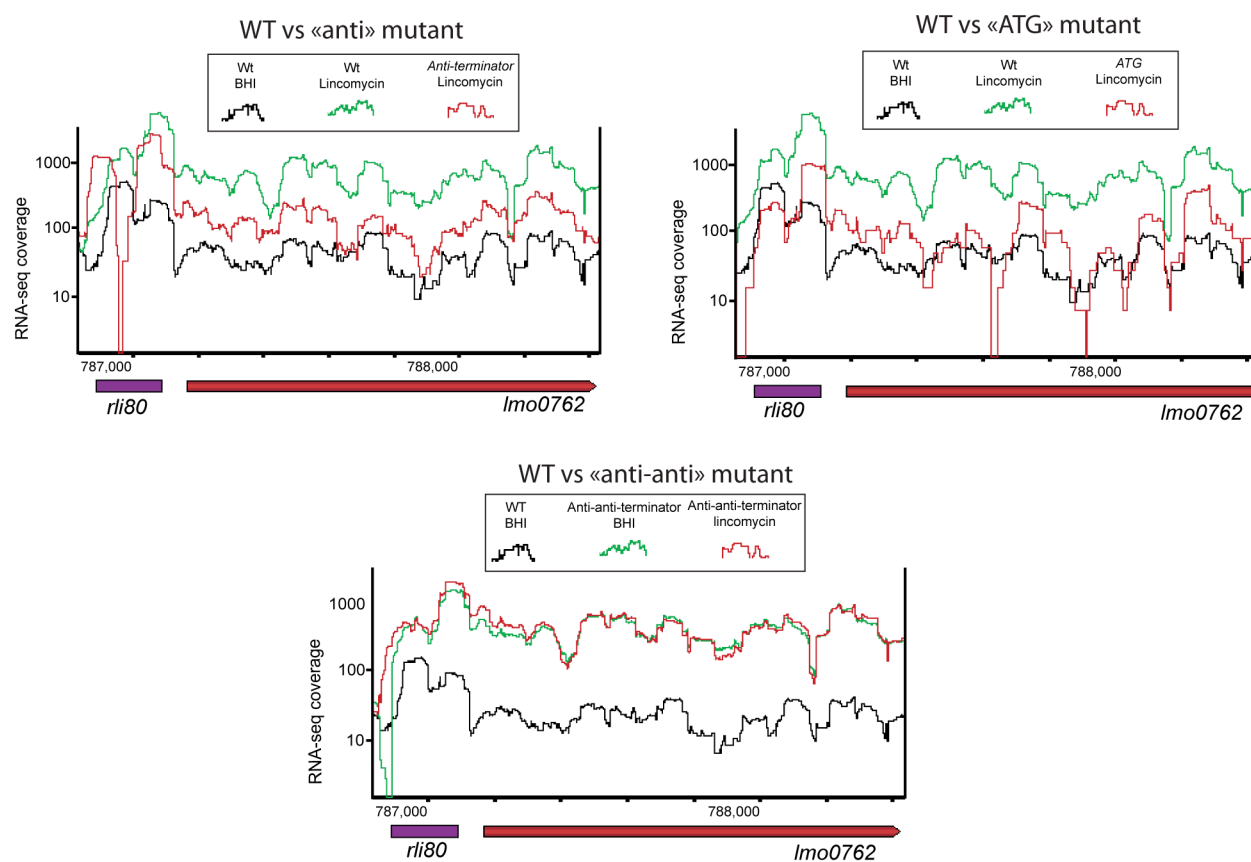
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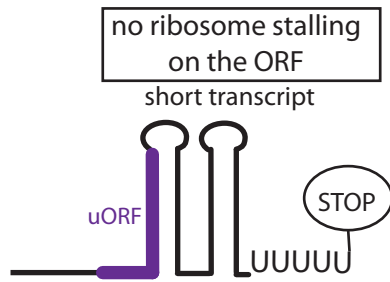
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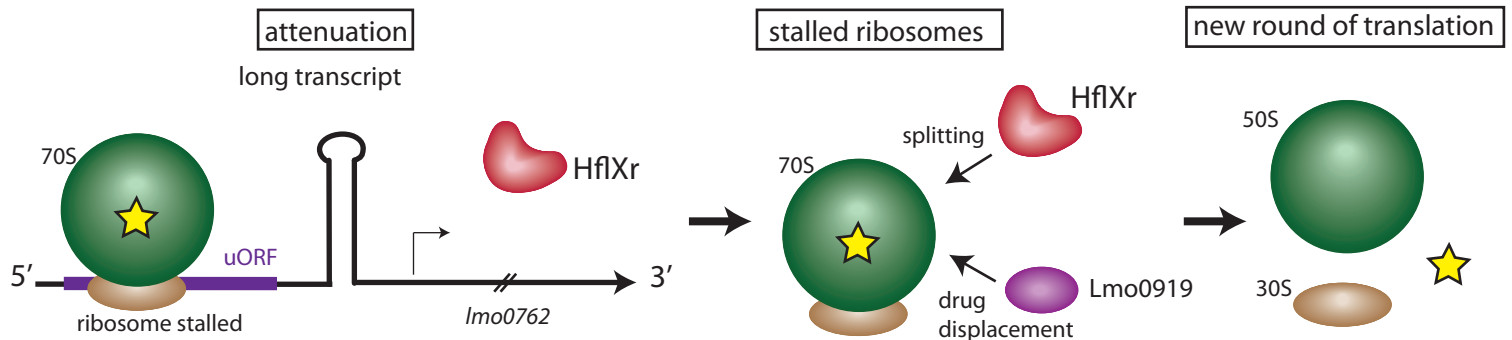


no antibiotics



 no HflXr
production

+ lincomycin ★



+ erythromycin ★

