Helcococcus kunzii methyltransferase Erm(47) responsible for MLSB resistance is induced by diverse ribosome-targeting antibiotics

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Helcococcus kunzii MLS_B resistance methyltransferase *erm*(47) is induced by diverse ribosome-targeting antibiotics

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Running title: Erm(47) is induced by multiple drug classes

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Abstract

Objectives: To determine the mechanism of induction of *erm*(47) and its atypical expression in the Gram-positive opportunistic pathogen, *Helcococcus kunzii*, where it confers resistance to a subset of clinically important macrolide, lincosamide and streptogramin B (MLS_B) antibiotics.

Methods: The resistant *H. kunzii* clinical isolate UCN99 was challenged with subinhibitory concentrations of a wide range of ribosome-targeting drugs. The methylation status of the *H. kunzii* ribosomal RNA at the MLS_B binding site was then determined using a mass spectrometry approach, and was correlated with any increase in resistance to the drugs.

Results: The *H. kunzii erm*(47) gene encodes a monomethyltransferase. Expression is induced by sub-inhibitory concentrations of the macrolide erythromycin, as is common for many *erm* genes, and surprisingly also by 16-membered macrolide, lincosamide, streptogramin, ketolide, chloramphenicol and linezolid antibiotics, all of which target the 50S ribosomal subunit. No induction was detected with spectinomycin, which targets the 30S subunit.

Conclusions: The structure of the *erm*(47) leader sequence functions as a hair-trigger for the induction mechanism that expresses resistance. Consequently, translation of the *erm*(47) mRNA is tripped by MLS_B compounds and also by drugs that target the 50S ribosomal subunit outside the MLS_B site. Expression of *erm*(47) thus extends previous assumptions about how *erm* genes can be induced.

Introduction

Infection with opportunistic bacterial pathogens presents a serious threat to immunocomprised individuals.[1] One such pathogen, *Helcoccocus kunzii,* is a facultatively anaerobic Gram-positive coccus that can be found as an integral part of the skin microbiota of healthy individuals.[2] This species is now being increasingly detected in skin and soft tissue infections, such as diabetic foot ulcers,[3] often in mixed cultures with other Gram-positive cocci. *H. kunzii* is generally intrinsically susceptible to many antibiotics, although a disturbing increase in the incidence of resistance to MLS_B drugs and to other clinically effective classes of antibiotics has been observed.[4] Recently, *H. kunzii* strain UCN99 was shown to have acquired a 115-kb island within its chromosome that encodes a range of antibiotic resistance determinants.[5] These include the previously uncharacterized gene *erm*(47), which confers resistance to a range of MLS_B drugs.[5]

Phylogenetically, the *erm*(47) gene belongs to the large family of *erm* genes that are found in diverse pathogenic and drug-producing bacteria.[6, 7] Each *erm* gene encodes a methyltransferase that specifically targets the N^6-position of nucleotide A2058 in 23S rRNA (*Escherichia coli* numbering),[8] which becomes modified by addition of either one or two methyl groups.[7] Type I Erm monomethyltransferases add a single methyl group to confer high resistance to lincosamides, low to moderate resistance to macrolide and streptogramin B antibiotics,[9] and no appreciable resistance to ketolides such as telithromycin[10] that are derivatives of the macrolide erythromycin.[11] Addition of two methyl groups by type II Erm dimethyltransferases confers high resistance to all MLS_B antibiotics[6, 7] and also to ketolides.[12] Consistent with these resistance phenotypes, Erm monomethytransferases are generally found in drug-producing actinobacterial species,[9] whereas pathogenic bacteria generally acquire the broader range of resistance conferred by Erm dimethyltransferases.[6, 7] However, exceptions do exist and *erm*(42) found in pathogenic Pasteurellaceae species has been shown to be a monomethyltransferase.[13]

The transfer of additional methyl groups to the rRNA has a measurable fitness cost for the bacterial cell, and thus expression of *erm* genes is often shut down in the absence of drugs.[14] Expression of *erm* can then be activated upon detection of a drug for which the resistance mechanism is required. Such induction is generally specified by a narrow range of drugs. For instance, the dimethyltransferase ErmC is induced by the macrolide erythromycin, which has a 14-membered lactone ring, but is not induced by 16-membered ring macrolides, lincosamides or streptogramin B drugs[15] despite the ability of ErmC to confer resistance to all of these compounds.[7] Furthermore, alteration of the erythromycin structure by removing its cladinose sugar results in loss of induction.[16, 17] This latter point has been central in the development of the ketolide derivatives of erythromycin where the cladinose moiety has been substituted with a keto group,[18] enabling ketolides to exert their inhibitory effects without tripping expression of MLS_B resistance.[11, 19, 20] Other structurally distinct classes of drugs such as chloramphenicol and linezolid bind at ribosomal sites that overlap those of the MLS_B antibiotics,[21, 22] but despite this, none of has previously been associated with induction of *erm*-mediated resistance.

Growth studies of *H. kunzii* strain UCN99 showed that *erm*(47) confers resistance to a range of MLS_B antibiotics and that this phenotype is transferable to other bacterial species.[5] In the present study, we investigate the mechanism of *erm*(47) induction and determine whether the resultant rRNA modification falls into the type I or type II group of methyltransferases. We have applied Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry to determine the methylation status of *H. kunzii* rRNA after being challenged with a range of clinically relevant drugs that target the bacterial ribosome. We show that *H. kunzii erm*(47) is an inducible monomethyltransferase that contravenes many of the previous assumptions about how *erm* genes are induced. Although, as might have been expected, full induction is obtained in the presence of sub-inhibitory amounts of erythromycin, we show that *erm*(47) expression is also induced by a

range of compounds that include the 16-membered macrolide spiramycin, the lincosamide lincomycin, the type-B streptogramin quinupristin, the ketolide telithromycin, as well as the non-MLS_B drugs chloramphenicol and linezolid.

Materials and Methods

Bacterial strains and growth conditions

The susceptible strain *H. kunzii* ATCC51366 and the resistant strain UCN99 containing *erm*(47) were grown at 37 °C in cation-adjusted Mueller-Hinton broth (CA-MHB) supplemented with 5% lysed horse blood and 20 mg/L nicotinamide adenine dinucleotide.[5] Minimal inhibitory concentrations (MICs) of the antibiotics listed below were determined by the broth microdilution method according to EUCAST guidelines (http://www.eucast.org/). The UCN99 strain was grown with one-fourth the MICs of erythromycin (64 mg/L), spiramycin (64 mg/L), lincomycin (64 mg/L), telithromycin (2 mg/L), dalfopristin/quinupristin (0.03 mg/L), chloramphenicol (1 mg/L), linezolid (0.25 mg/L) or spectinomycin (2 mg/L) to test the effects of these drugs on induction of the *erm*(47) gene.

RNA purification

H. kunzii cells were grown to late log phase in 50 mL medium and were harvested by centrifugation at 10000 g for 20 min. Cells were washed by twice resuspending in 100 mL buffer A (50 mM Tris-Cl pH 7.2, 10 mM $MgCl_2$, 100 mM NH_4Cl) and pelleted by centrifugation. Cells were lysed by sonication at 4 °C in 10 mL buffer A. Cell debris containing the chromosomal DNA was removed by centrifugation at 15000 g for 10 min. and the supernatent was extracted with phenol/chloroform to remove proteins. Cellular RNA was recovered by ethanol precipitation, and was redissolved in 100 µL H_2O.

MALDI mass spectrometry analysis of rRNAs

The 23S rRNA region around A2058 was isolated using a hybridization method.[23, 24] A mixture of 150 pmol of *H. kunzii* rRNA and 500 pmol of an oligodeoxynucleotide complementary to the

nucleotide sequence G2032-G2084 (Fig. 1A) was heated for 5 min at 90 °C in 100 µl of 50 mM Tris.Cl pH 7.5 followed by slow cooling to 37 °C. The mixture was incubated for a further 60 min at 37 °C with 0.5 µg RNase A, and nuclease digestion was stopped by extracting with phenol/chloroform. The RNA-DNA hybrid was recovered by ethanol precipitation and run on a denaturing 13% polyacrylamide gel to release the rRNA fragment that had been protected from RNase A digestion. The rRNA fragment of approximately 57 nucleotides ran slightly slower on the gel than its complementary DNA oligonucleotide, and was excised and extracted.

RNA aliquots of 2.5 pmol in 1 µL were mixed with 0.5 µL 3-hydroxypicolinic acid (0.5 M in 50% acetonitrile) and digested in a total volume of 3 µL with either RNase A or RNase T1 (Sigma-Aldrich) for 3 h at 37 °C. The resultant oligonucleotides were analysed in positive ion mode by MALDI mass spectrometry as previously described.[23, 24]

Secondary structure calculations erm(47) mRNA stability

Models for RNA folding based on minimum free energy structures were predicted using publically assessible online tools[25] and included mfold (http://www.bioinfo.rpi.edu/applications/mfold/). The free energies (ΔG) of structures were estimated from the stacking interaction contributions of different RNA basepairs determined experimentally at 25 °C.[26]

Results

Resistance phenotypes

The MICs of a range of drugs targeting the ribosomal 50S subunit were determined for the susceptible and resistant *H. kunzii* strains (Table 1). The UCN99 strain exhibited appreciably higher resistance to the macrolides erythromycin and spiramycin and to the lincosamide lincomycin. No resistance was conferred to the ketolide telithromycin, the streptogramin A/B combination of dalfopristin/quinupristin, chloramphenicol nor to the oxazolidinone linezolid. As expected, there was no resistance to the 30S subunit-targeting drug spectinomycin (Table 1).

Erm(47) is a monomethyltransferase

The MALDI mass spectrometry approach used here measures the masses of RNA oligonucleotides to within 0.2 Da.[27] Thus the addition of one methyl group (plus 14 Da) or two methyl groups (plus 28 Da) is readily detected in rRNA fragments of known sequence. After RNase T1 digestion of rRNA from the susceptible strain, nucleotide A2058 of the macrolide/lincosamide binding site (in bold, Fig. 1A) resides in the fragment **A**AAGp with a mass/charge (*m/z*) of 1351 (Fig. 1B). Digestion of the rRNA region with RNase A yields a larger fragment of GG**A**AAGACp (*m/z* 2675) containing this nucleotide (Fig. 1C).

Sub-inhibitory amounts of erythromycin produced no change in the rRNA of the susceptible ATCC51366 strain (Fig. 2A), as would be predicted for a strain lacking resistance determinants. Equivalent treatment of the resistant UCN99 strain with erythromycin (addition of one-fourth MIC, Table 1) caused a shift in the AAAGp fragment from *m/z* 1351 to *m/z* 1365 (Fig. 2C), corresponding to the addition of a single methyl group. No peak was detected at *m/z* 1379 under these (or any other) drug conditions, showing that no dimethylation of A2058 takes place and that Erm(47) is exclusively a monomethyltransferase. Under noninducing condition, a minor amount of

A2058 monomethylation was observed (Fig. 2B), indicating that expression of *erm*(47) in *H. kunzii* is not completely shut down in the absence of drug.

Induction by other drugs

Other drugs that target the 50S ribosomal subunit were tested for their ability to induce *erm*(47) expression. After establishing the MICs for telithromycin, lincomycin, spiramycin, dalfopristin/quinupristin, chloramphenicol and linezolid, one-fourth of the inhibitory concentrations added to cell cultures (Table 1). Telithromycin (Fig. 2D), lincomycin (Fig. 2E) and spiramycin induced *erm*(47) to a level that results in full monomethylation of nucleotide A2058. Lower, but nevertheless significant, levels of *erm*(47) expression were induced by chloramphenicol (Fig. 2F), the streptogramin A/B pair, dalfopristin/ quinupristin (Fig. 2G), and linezolid (Fig. 2H).

Comparable control studies were carried out with the 30S ribosomal subunit drug, spectinomycin. Methylation profiles remained identical to those from cells with no drug (Fig. 2B), showing that spectinomycin was not an inducer of *erm*(47).

Discussion

We show here that *erm*(47) encodes a methyltransferase that adds a single methyl group to 23S nucleotide A2058 of *H. kunzii*. This rRNA modification confers high resistance to lincosamide antibiotics[9] and moderate to high macrolide resistance, but no appreciable resistance to ketolides such as telithromycin.[10] Although the resistance observed here to the 16-membered ring macrolide spiramycin is somewhat higher than might have been expected, the resistance phenotype conferred by Erm(47) (Table 1) otherwise matches well with that seen for other Erm monomethyltransferases in a wide range of bacteria.[6, 7] Methylation of rRNA by Erm variants, and ensuing effects on the translational machinery, incur a measurable cost to the host bacterium,[14] and thus expression of these enzymes is often shut down in the absence of drug. When antibiotic appears in the environment and the resistance mechanism is needed, expression of *erm* can then be rapidly induced at the translational level.[28]

The premise of such an induction mechanism is that it confers a selective advantage to the bacterium by preventing nonproductive gene expression in the absence of drug while facilitating rapid implementation of resistance upon drug detection.[29] Full induction of *erm*(47) is seen here with erythromycin and lincosamine (Fig. 2) conferring high level resistance to these drugs (Table 1). Expression of *erm* is not normally initiated by 16-membered macrolides[6, 7] and although induction of *erm*(47) by spiramycin was unexpected, the subsequent resistance conferred to this macrolide does offer a clear functional advantage to *H. kunzii* (Table 1). In the case of the dalfopristin/quinupristin compounds, the streptogram B moiety quinupristin contacts nucleotide A2058,[21, 22] and conceivably promotes *erm*(47) induction. Here, the accompanying streptogramin A component, dalfopristin, would act in synergy to aid quinupristin binding, and no resistance is conferred to this drug combination despite monomethylation of the rRNA (Table 1). No induction of *erm*(47) was seen with the 30S subunit-targeting drug spectinomycin, which is also consistent

212 with the view that drugs which bind to other ribosomal targets outside the MLS_B site do not act as
213 inducers of *erm* genes.
214 However, the other 50S-targeting drugs chloramphenicol and linezolid bind outside the MLS_B
215 site,[21, 22] but nevertheless induce a significant degree of *erm*(47) expression. Neither
216 chloramphenicol nor linezolid has previously been linked with *erm* induction, and the expression of
217 *erm*(47) seen here offers no apparent advantage to the bacterial cell. These observations challenge
218 the classic model for induction and prompts the question of how *erm*(47) differs from other
219 inducible *erm* genes.
220 At a first glance, the *erm*(47) operon is organized in a manner similar to other inducible *erm*
221 genes, where translation occurs on a bicistronic mRNA together with an upstream leader
222 sequence.[28] As in the classic model for other *erm* operons, the *erm*(47) mRNA can fold into a
223 conformation (Fig. 3A), where the groundstate in the absence of antibiotic occludes the ribosome
224 binding site and the start codon of the methyltransferase cistron. In the absence of antibiotics, the
225 inherent helicase activity of the translating ribosome[30] would disrupt the 1:2 helix to read through
226 the *erm* leader sequence and then dissociate from the mRNA after synthesizing the short leader
227 peptide. Here, the ribosomal binding site and first codon of the *erm*(47) cistron would remain
228 inaccessible within the 3:4 helix. However, when an antibiotic such as erythromycin causes the
229 ribosome to stall on region 1 of the leader sequence, helix 1:2 would remain unraveled and enable
230 mRNA refolding to expose the downstream start site where another ribosome would enter and
231 initiate methyltransferase synthesis (Fig. 3B).
232 The propensity of ribosomes to stall on the *erm* leader is determined by both the type of antibiotic
233 and by the encoded sequence of the leader peptide. The macrolides erythromycin and azithromycin
234 stall ribosomes on *E. coli*[31] and *Staphylococcus aureus* mRNAs[32] by a similar mechanism that is
235 accentuated by specific tripeptide sequences such as Arg-Leu-Arg. This sequence is present in the

236 *erm*(47) leader (Fig. 3) and several other *erm* leader sequences.[33] Stalling by telithromycin is also
237 promoted at Arg-X-Arg triplets,[31] consistent with *erm*(47) induction by this drug. Furthermore, the
238 *erm*(47) leader also contains runs of serine codons that have been shown to augment ribosome
239 stalling in the presence of chloramphenicol or linezolid.[34] Pausing of the ribosome in at any of these
240 sites in the leader could lead to rearrangement of the mRNA secondary structure as described above
241 (Fig. 3B) and result in *erm*(47) expression.
242 Also particular to the *erm*(47) operon is the small but reproducible amount of *erm*(47) expression
243 seen in drug-free cells (Fig. 2B). Generally in *erm* mRNAs, the overall stability of the occluded
244 structure in the absence of drugs (Fig. 3A) has to be greater than that an alternative structure
245 facilitated by ribosome stalling. However, free energy estimates suggest that the groundstate folding
246 of the *erm*(47) operon might favor a conformation with helix 2:3 extending into a paired structure
247 between region 1 and region 4 (Fig. 3C). Although such a configuration would occlude the
248 initiation site of the *erm*(47) methyltransferase, each translational event on the leader sequence
249 would briefly expose the downstream initiation site to leave a narrow window for expression of
250 *erm*(47) even in the absence of drug.
251 A mechanism favoring the Fig. 3C structure, would explain the minor amount of *erm*(47)
252 expression in drug-free cells. Furthermore, this leakiness could conceivably be exacerbated by
253 drugs such as chloramphenicol and linezolid that do not normally induce *erm* genes but which, in
254 the sub-inhibitory amounts used here, clearly provoke *erm*(47) expression. Although the mechanism
255 for this remains unclear, the lack of induction by the 30S subunit inhibitor spectinomycin points
256 towards a 50S-specific effect, and might be linked to the proximity of the chloramphenicol and
257 linezolid binding sites to that of the MLS_B compounds. Taking these points together, we conclude
258 that the *erm*(47) operon is activated by a much wider range of drugs than previously have been
259 associated with the induction of *erm*-encoded resistance.

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Transparency declarations

Nothing to declare.

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Figure Legends

Figure 1 (A) Schematic of the 3´-half of *H. kunzii* 23S rRNA based on secondary structures at the Gutell lab comparative RNA site (http://www.rna.icmb.utexas.edu). The sequence around the Erm methyltransferase target at nucleotide A2058 (bold line within the boxed area of the schematic) was isolated for mass spectrometry analysis. The *E. coli* numbering system for rRNA nucleotides[35, 36] is used throughout. (B) MALDI mass spectrum of this rRNA sequence after digestion with RNase T1. The target nucleotide A2058 resides in the tetranucleotide **A**AAGp at *m/z* 1351.2 prior to methylation. (C) Mass spectrum of the same rRNA sequence after RNase A digestion, where A2058 is in the larger oligonucleotide GG**A**AAGACp at *m/z* 2675.4. The calculated singly protonated masses of the oligonucleotides (inserted tables) match the experimentally measured *m/z* values (shown on the spectral peaks) to within 0.1 Da.

Figure 2 Enlargement of RNase T1 spectral region containing nucleotide 2058 (boxed on spectrum in Fig. 1B). (A) RNA from the susceptible ATCC51366 strain without *erm*. Unmethylated A2058 is in the AAAGp fragment at *m/z* 1351; the neighboring fragment CUAGp at *m/z* 1304 remains unchanged under all conditions and is shown for reference. (B) RNA prior to addition of antibiotic from the resistance UCN99 strain containing *erm*(47). (C – H) The UCN99 strain after *erm*(47) induction with one-fourth the MICs of the antibiotics indicated. After induction with erythromycin, lincosamine and telithromycin, the *m/z* 1351 peak is shifted to *m/z* 1365 indicating that nucleotide A2058 has been modified with a single methyl group. No spectral peak was evident at *m/z* 1379 showing that no dimethylation of this nucleotide takes place. Chloramphenicol, dalfopristin/quinupristin and linezolid also induce the expression of *erm*(47) to varying degrees.

Figure 3 Putative secondary structures of the *erm*(47) leader mRNA. (A) The classic ground-state structure for inducible *erm* operons[28] in which the base-paired segments 1:2 and 3:4 are formed. In this conformation, translation of the methyltransferase would be prevented due to sequestering of

379 the ribosome binding site (Shine-Dalgarno, in blue) and the initiation codon (green) within the
380 secondary structure formed by segments 3 and 4 (insert). (B) Drug-induced pausing of ribosomes
381 around the series of hydrophobic residues (Arg-Leu-Arg, bold) encoded in region 1 of the leader
382 sequence frees region 2 to form a stable helical structure with region 3. This in turn opens up the
383 Shine-Dalgarno and start codon sequences in region 4 enabling translation of the *erm*(47)
384 methyltransferase to be initiated. We note that the sequence at the 3´-end of *H. kunzii* 16S rRNA
385 restricts its interaction to the AGGU (blue) nucleotides of the Shine-Dalgarno regions whereas in
386 numerous other bacteria this interaction would be significantly more extensive and (for instance in
387 *E. coli*) include five additional nucleotides upstream of the leader cistron (boxed in blue). (C) An
388 alternative ground-state structure that also sequesters the translational start site of the *erm*(47)
389 cistron. Free energy (ΔG) estimations indicate that this structure is more favorable than that in panel
390 A. After drug-induced pausing of a ribosome in region 1, this structure requires no conformational
391 rearrangement of regions 2 and 3 in order to free the methyltransferase initiation site.
392

Table 1. Resistance phenotypes conferred by *erm*(47) in *H. kunzii*.

***H. kunzii* strains**	**Minimal inhibitory concentrations (mg/L)**							
	erythro-mycin	**linco-mycin**	**telithro-mycin**	**spira-mycin**	**dalfopristin /quinupristin**	**chloram-phenicol**	**linezolid**	**spectino-mycin**
ATCC 51366	1	2	2	4	0.25	4	1	8
UCN99 [*erm*(47)]	≥256	≥256	1	≥256	0.12	4	1	8

Table 1. MICs for the susceptible ATCC 51366 strain and the resistant UCN99 strain of *H. kunzii* harbouring *erm*(47) were determined by broth microdilution. The MIC values were used to establish growth conditions for the UCN99 strain, where one-fourth of the MIC for each drug was used to test for *erm*(47) induction.

FIG 1

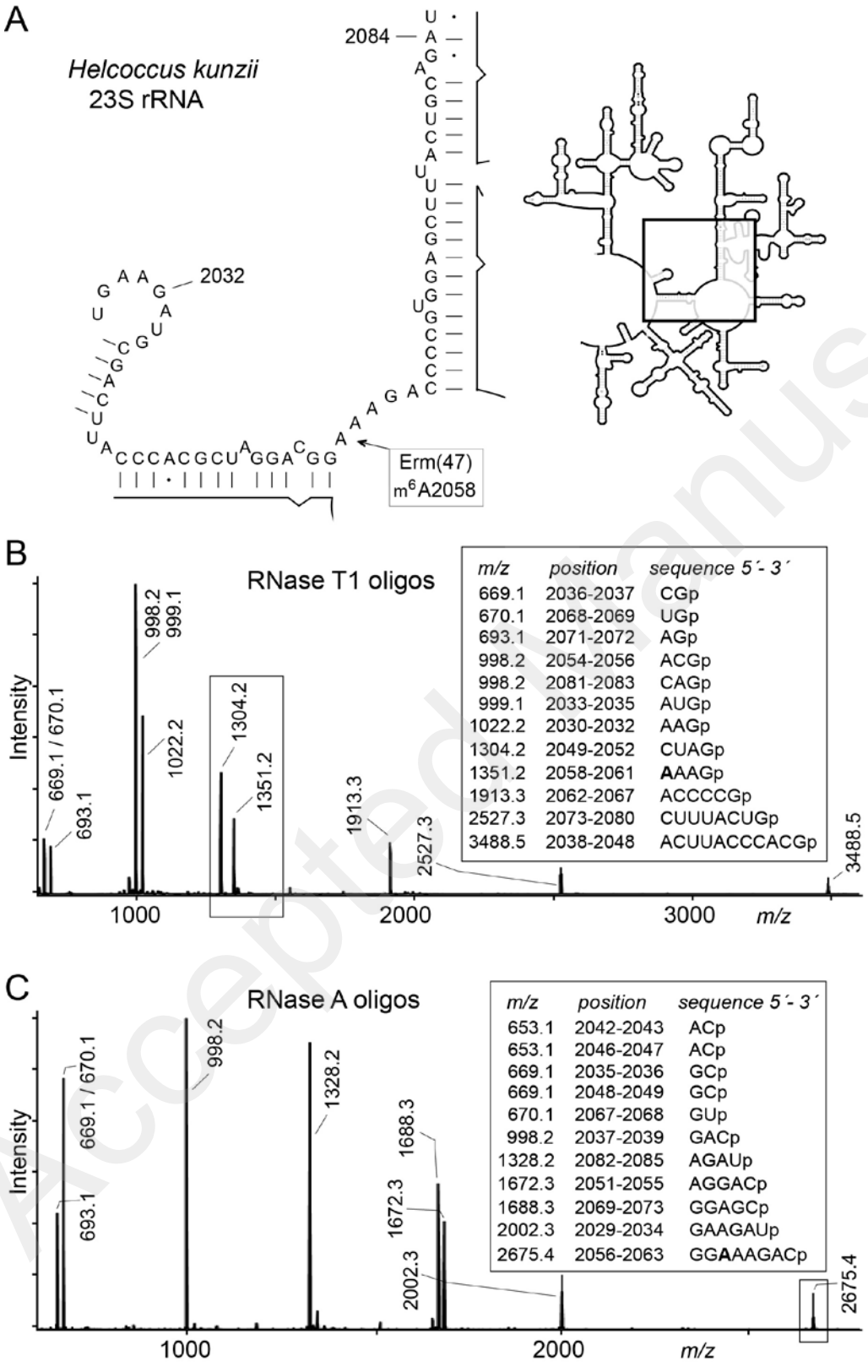

FIG 2

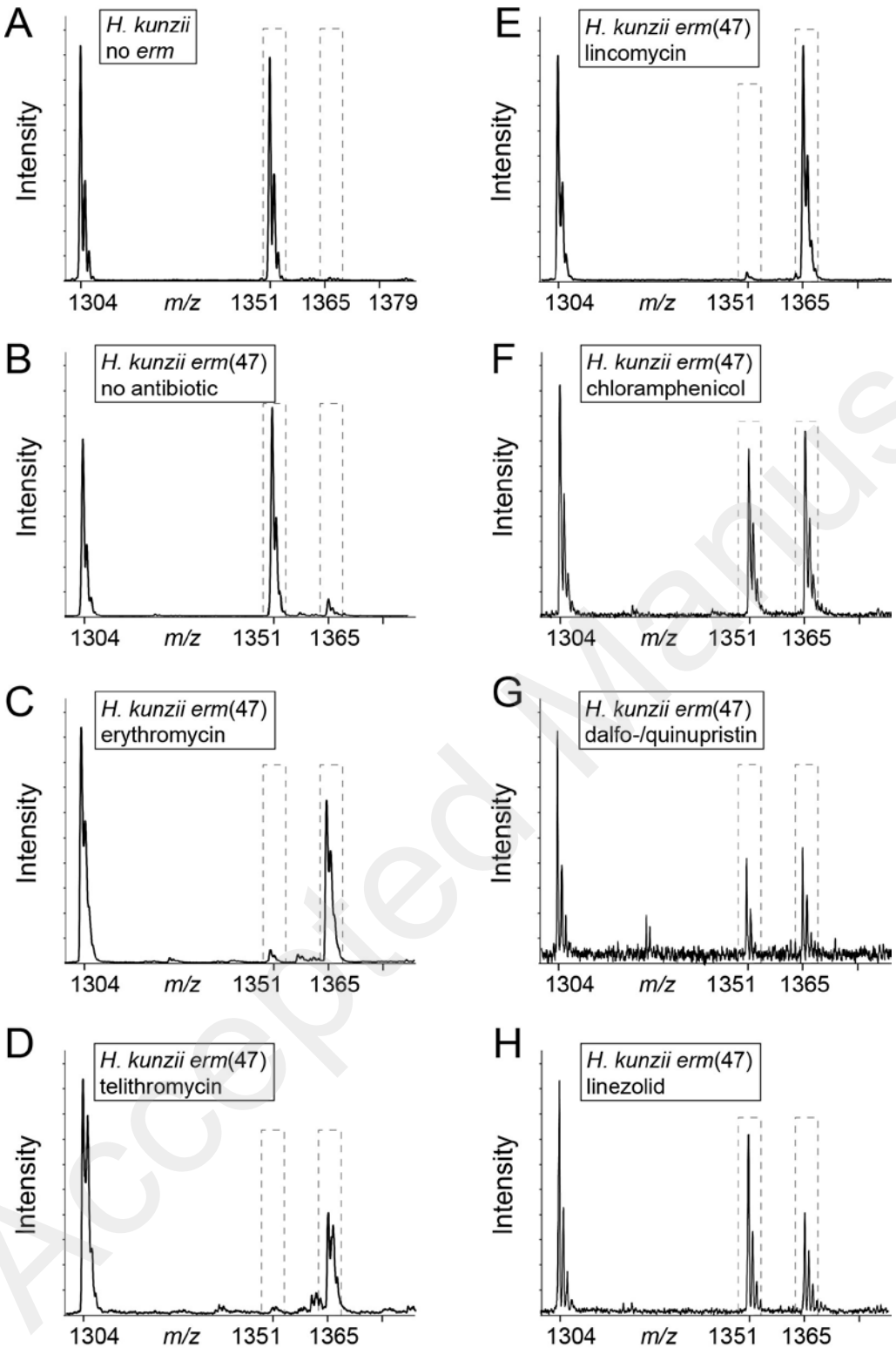

FIG 3