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A metallo-beta-lactamase with both beta-lactamase and ribonuclease activity is linked with traduction in giant viruses

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1 **TITLE PAGE**

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3 **Full-length title: A metallo-beta-lactamase with both beta-lactamase and ribonuclease**
4 **activity is linked with traduction in giant viruses**

5 **Short title (for the running head): Metallo-beta-lactamase fold in giant viruses**

6

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17

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22

23

ABSTRACT

24
25
26 Enzymatic proteins with a metallo-beta-lactamase (MBL) fold have been essentially studied
27 in bacteria for their activity on beta-lactam antibiotics. However, the MBL fold is ancient and
28 highly conserved, and these proteins are capable of cleaving a broad range of substrates. It has
29 recently been shown that MBLs are present in a wide array of cellular organisms, including
30 eukaryotes and archaea. We show here that Tupanvirus deep ocean, a giant virus, also
31 encodes a protein with a MBL fold. Phylogeny showed its clustering with transfer
32 ribonucleases (RNases) and the presence of orthologs in other giant viruses, mainly those
33 harboring the largest sets of translation components. In addition, it suggests an ancient origin
34 for these genes and a transfer between giant viruses and *Acanthamoeba* spp., a host of many
35 giant viruses. Biologically, after its expression in *Escherichia coli*, the tupanvirus protein was
36 found to hydrolyse nitrocefin, a chromogenic beta-lactam. We also observed an hydrolysis of
37 penicillin G (10 µg/mL) and detected the metabolite of penicillin G hydrolysis,
38 benzylpenilloic acid. This was inhibited by sulbactam, a beta-lactamase inhibitor. In addition,
39 we tested the degradation of single-stranded DNA, double-stranded DNA, and RNAs, and
40 observed a strong activity on RNAs from seven bacteria with G+C varying from 42% to 67%,
41 and from *Acanthamoeba castellanii*, the tupanvirus host. This was not inhibited by sulbactam
42 or ceftriaxone. RNase activity was estimated to be 0.45 ± 0.15 mU/mg using a fluorescence-
43 based assay. Our results still broaden the range of hosts of MBL fold proteins and
44 demonstrate that such protein can have dual beta-lactamase/nuclease activities. We suggest
45 that they should be annotated according to this finding to avoid further confusion.

46

47

TEXT

48

49 INTRODUCTION

50 The metallo-beta-lactamase (MBL) superfamily encompasses a large set of enzymes,
51 including MBL and ribonuclease (RNase) Z enzymes¹. These enzymes are pleiotropic proteins
52 that can hydrolyze a wide range of substrates, among which beta-lactams, and DNA or
53 RNA^{2,3}. Such capabilities rely on an ancient and highly conserved fold, which represents a
54 stable scaffold that has evolved to perform a broad range of chemical reactions and on which
55 various catalytic, regulatory and structural activities are based²⁻⁴. This wide array of activities
56 is enabled by variations in the composition and size of loops located near the enzyme active
57 site³. A well-known catalytic activity of MBLs consists in breaking beta-lactam rings, which
58 was primarily identified in bacteria⁵. Nevertheless, this hydrolase activity is suspected to have
59 evolved in response to the environmental beta-lactams from an ancestral protein whose
60 function was not related to beta-lactams and which may have been devoid of such hydrolase
61 capability³. Concurrently to their capability to interact with various substrates that likely
62 emerged through adaptive evolution, members of the MBLs superfamily have been identified
63 in a broad range of cellular organisms, including bacteria, but also eukaryotes and archaea
64 with a beta-lactamase activity^{2,6}.

65 Giant viruses are *bona fide* microbes as their virions are visible under a light
66 microscope and they display a complexity similar to that of small cellular microorganisms^{7,8}.
67 Since their discovery in 2003, their diversity has increased considerably, with nine families
68 and more than 100 isolates cultured. Their classification alongside cellular microorganisms is
69 still debated, but their characteristics clearly distinguish them from conventional viruses^{9,10}.
70 We have investigated whether genes encoding members of the MBLs superfamily may also
71 be present in giant viruses. We found one in Tupanvirus deep ocean, a giant mimivirus

72 isolated from Brazilian Atlantic ocean sediments, and confirmed that its product harbored a
73 biologically active MBL fold with both beta-lactamase and RNase activities.

74

75 **RESULTS**

76 While annotating the genome of Tupanvirus deep ocean, the second isolate of a new
77 mimivirus genus, *Tupanvirus*¹¹, a gene (GenBank: AUL78925.1) that encodes a metallo-
78 hydrolase-like MBL fold was identified (Conserved Protein Domain Family Accession no.
79 cl23716)¹². This gene has a homolog in the other tupanvirus isolate (Soda Lake)
80 (AUL77644.1). Beyond, best BLASTp hits against cellular organisms included MBL fold
81 harboring proteins from an unclassified deltaproteobacterium whose genome was assembled
82 from a marine water metagenome (evalue, 5e-38; identity, 33.0; coverage, 83%), from an
83 actinobacteria (*Nonnomuraea* spp.) (1e-36; 30.0; 86%), from *Microscilla marina* (6e-34;
84 28.5%; 89%) and from *Acanthamoeba castellanii* (4e-33; 29.8%; 81%) (**Fig. 1**,
85 **Supplementary Fig. S1**). Significant BLASTp hits (evalues ranging from 1e-41 to 8e-6)
86 against viruses were also obtained with genes from putative giant viruses whose genomes was
87 assembled from metagenomes obtained from environmental samples¹³⁻¹⁵ and from Cafeteria
88 roenbergensis virus, a distant Mimivirus relative¹⁶. The 322 amino acid long tupanvirus
89 protein exhibits the conserved MBL motif "HxHxDH" in amino acid positions 60-65. A
90 search for domains using the NCBI conserved domain search (CD Search) tool¹⁷ identified a
91 MBL fold belonging to a ribonuclease Z (RNase_Z_T_toga, TIGR02650, interval= amino
92 acids 24-273, E-value= 1.81e-14; RNaseZ_ZiPD-like_MBL-fold, cd07717, interval= amino
93 acids 56-282, E-value= 1.63e-04), which is a transfer RNA (tRNA)-processing endonuclease.
94 This Tupanvirus deep ocean protein was further analyzed by three-dimensional comparison
95 against the Phyre2 web portal for protein modeling, prediction and analysis¹⁸. This analysis
96 reported a best match with 100% confidence and 85% coverage (273 amino acid residues)

97 with the crystal structure of a long form ribonuclease Z (RNase Z) from yeast (template
98 c5mtzA) (**Supplementary Fig. S2**). Proteome analysis conducted for Tupanvirus Soda Lake,
99 and Tupanvirus deep ocean, as previously described¹², did not allow the detection of these
100 proteins with a MBL fold in the virions. In addition, the dramatic RNA shutdown observed
101 during the replication of this giant virus hindered the achievement of transcriptomic analyses.
102 Interestingly, the genomes of 20 of the 21 (95%) giant viruses found to encode a MBL fold
103 protein concurrently encode tRNAs, whereas this is only the case for 46 of the 122 (38%)
104 giant viruses devoid of a MBL fold protein ($p < 10^{-3}$; Yates-corrected chi-square test)
105 (**Supplementary Fig. S3 and 4 and Table S2**). The presence of a MBL fold protein among
106 Megavirales members was correlated with the size of the gene repertoire and the number of
107 translation-associated components ($p < 10^{-3}$; Anova test). Putative proteins with a MBL fold
108 from giant viruses comprised two related phylogenetic clusters (**Fig. 1**). These clusters
109 appeared deeply rooted in the phylogenetic tree, which suggests an ancient origin for these
110 genes. In addition, one of the clusters of giant virus genes encoding MBL fold proteins
111 appeared closely related to two genes from *Acanthamoeba castellanii*, an amoebal mimivirus
112 hosts, suggesting a transfer from these giant viruses to *A. castellanii*.

113 The recombinant protein AUL78925.1 of Tupanvirus deep ocean (named TupBlac)
114 was expressed in *Escherichia coli* and was then purified, as described previously⁶. Based on
115 the phylogenetic analysis and as MBL folds can hydrolyse nucleic acids², both beta-lactamase
116 and nuclease activities of this purified protein were thereafter tested. We first evaluated the
117 beta-lactamase activity of a pure solution of TupBlac used at a concentration of 1 $\mu\text{g}/\text{mL}$ by
118 incubating it with nitrocefin, a chromogenic beta-lactam used to test the beta-lactamase
119 activity¹⁹. A significant hydrolysis activity was observed (**Fig. 2**). A concentrate of protein
120 extract (50 mg/mL) obtained from tupanvirus virions also degraded, albeit slightly, nitrocefin.
121 Thereafter, we monitored by liquid chromatography-mass spectrometry the effect of TupBlac

122 on penicillin G (10 µg/mL) and observed a significant hydrolysis activity of this compound
123 within 48h (**Fig. 3**). We also detected, in the presence of the tupanvirus protein,
124 benzylpenilloic acid, the metabolite resulting from the enzymatic hydrolysis of penicillin G²⁰.
125 Finally, we confirmed that these observations were related to a beta-lactamase activity as both
126 penicillin G degradation and benzylpenilloic acid appearance were inhibited by sulbactam, a
127 beta-lactamase inhibitor (**Fig. 3**). We further tested if pre-treatment with sulbactam had an
128 impact on the duration of the giant virus replication cycle and replication intensity. After
129 replication on *A. castellanii* strain Neff in the presence of a high concentration (10 µg/mL) of
130 sulbactam, the virions produced (10⁶/mL) were inoculated on fresh amoebae at different
131 concentrations. No differences were observed regarding viral growth in the absence or
132 presence of pre-treatment with sulbactam as assessed using high content screening
133 (**Supplementary Fig. S5**).

134 Finally, as some proteins with a MBL fold can hydrolyse DNA and RNA², we tested
135 the capability of tupanvirus enzyme TupBLac to degrade synthetic single- and double-
136 stranded DNAs and bacterial RNAs. We found no effect on both DNA types. In contrast, we
137 observed a strong RNase activity (**Fig. 4**). Another set of experiments was conducted using *E.*
138 *coli* RNA as a substrate with an assessment of RNA size distribution on a bioanalyzer
139 (Agilent Technologies, Palo Alto, CA) after incubation with TupBlac. It showed a dramatic
140 degradation of RNAs by the tupanvirus enzyme (**Fig. 5a**). In contrast with the beta-lactamase
141 activity, this was not inhibited, neither by sulbactam (**Fig. 5a** and **Supplementary Fig. S6**),
142 nor by ceftriaxone (**Fig. 5b**), a cephalosporin that inhibits human SNM1A and SNM1B, that
143 are DNA repair nucleases with a MBL fold²¹. In addition, a RNase activity of the Tupanvirus
144 protein was further observed on *A. castellanii* RNA, and not inhibited either by sulbactam or
145 ceftriaxone (**Fig. 5c**). TupBLac also degraded RNA extracted from bacteria with genomes
146 with different G+C contents ranging between 41.8% and 66.6% (**Fig. 5d**), suggesting an

147 absence of influence of the G+C richness on the RNase activity. Finally, TupBlac RNase
148 activity was estimated to be 0.451 ± 0.153 mU/mg using a fluorescence-based assay, without
149 difference in the presence of sulbactam or ceftriaxone (0.520 ± 0.003 and 0.551 ± 0.024 mU/mg,
150 respectively) (*Supplementary Fig. S7*).

151

152 **DISCUSSION**

153 Hence, we found herein by several bioinformatic approaches that a gene of Tupanvirus deep
154 ocean, a recently discovered giant virus classified in family *Mimiviridae*^{11,12}, encodes for a
155 protein with a MBL fold. We further observed that this protein exhibited dual beta-lactamase
156 and RNase activities. This is the first evidence of the presence of a biologically-active protein
157 with a MBL fold in a virus. In addition, this work parallels the one on a protein detected by
158 functional screening of a metagenomic library from the deep-seep sediments²², showing that
159 the same enzyme has both beta-lactamase and RNase activities. Indeed, MBL fold proteins
160 were previously biologically-tested for either activity, but not for both. It is noteworthy that
161 the beta-lactamase activity of the MBL fold protein of Tupanvirus was inhibited by a beta-
162 lactamase inhibitor but this was not the case for the RNase activity²³. The phylogenetic study
163 of this beta-lactamase shows the presence in several other giant viruses of phylogenetically-
164 clustered counterparts, the origin of which seems very old. Interestingly, it also appears that
165 there may have been a gene transfer between these giant viruses and *Acanthamoeba* sp., the
166 amoebal host of many giant viruses. Such potential for horizontal transfer of these MBL fold
167 proteins is well-recognized³.

168 Beta-lactamases are *a priori* useless for giant viruses, which are grown in the presence
169 of various antibiotics, including beta-lactams²⁴, but our findings enhance the recent
170 reconsideration of the function of MBL fold proteins. Thus, the recent description of
171 penicillin secretion by arthropods²⁵ and the demonstration of active beta-lactamase in

172 vertebrates including humans¹⁹, as well as in archaea⁶ and fungi²⁶ show that MBL fold
173 proteins have a dramatically broad distribution. In humans, 18 genes were annotated as beta-
174 lactamases, whose activity had not been biologically-tested until recently¹⁹. In addition, MBL
175 fold proteins were highlighted to digest DNA or RNA^{2,19}. Thus, a class of enzymes, that were
176 named beta-lactamases because of their original discovery in bacteria resistant to beta-
177 lactamines, are in fact potentially versatile proteins. This differs from the drastically-
178 simplified paradigm consisting in enzymes with a beta-lactamase activity being secreted by
179 bacteria under the selective pressure of natural or prescribed antibiotics.

180 The RNase activity observed here for the Tupanvirus MBL fold protein could be
181 related to the host ribosomal shutdown observed in the presence of Tupanvirus deep ocean
182 with various protists, the mechanism of which has not been elucidated¹². This activity could
183 allow these viruses to take over on their cellular hosts by degrading cellular messenger RNAs
184 and shutting down cellular gene expression. The giant virus mRNAs should be protected from
185 such a degradation, which may be explained by the encapsidation of RNA transcripts into
186 giant virions that was detected for some of these viruses²⁷. Bioinformatic analyses suggested
187 that the tupanvirus MBL fold protein may belong to the RNase Z group that was proposed to
188 be one of the two main groups of the MBL superfamily with that encompassing MBLs¹.
189 RNase Z enzymes perform tRNA maturation by catalyzing the endoribonucleolytic removal
190 of the 3' extension of tRNA precursors that do not contain a chromosomally-encoded CCA
191 determinant²⁸⁻³⁰. The presence in giant viruses of RNases showing the greatest homology to
192 tRNases suggests a specific activity on tRNAs, which seems consistent with the presence of a
193 large set of translation components in these viruses, first and foremost Tupanvirus deep ocean
194 that is the current record holder of the number of translation components (including 70 tRNAs
195 targeting all 20 canonical amino acids). The presence of a putative tRNase in the virus that
196 currently has the most complete set of translation components of the whole virosphere is

197 likely not fortuitous. Furthermore, it was described for *Escherichia coli* that its RNase Z had
198 endoribonucleasic activity on messenger RNAs, being responsible for their decay in *in vitro*
199 experiments²⁹. This further argues that MBL fold proteins may contain a wide range of
200 activities. PNGM-1, a MBL fold protein whose sequence was recently described from a deep-
201 sea sediment metagenome by detection of its beta-lactamase activity³¹, was also found to
202 harbor dual beta-lactamase and RNase activities²². MBL fold proteins from giant viruses are
203 clustered with this protein in the phylogenetic analysis. Interestingly, PNGM-1 was suspected
204 to have evolved from a tRNase Z²². In conclusion, our data still broaden the range of
205 biological hosts of MBL fold proteins and demonstrate that such proteins can display dual
206 beta-lactamase and nuclease activities. Therefore, we reannotated the tupanvirus MBL fold
207 protein as a beta-lactamase/nuclease.

208

209 **MATERIALS AND METHODS**

210 **Bioinformatics**

211 Searches for Tupanvirus deep ocean protein AUL78925.1 homologs were performed using
212 the BLAST tool³². Phylogeny reconstruction was performed after amino acid sequence
213 alignment with the Muscle program³³ and the Maximum-Likelihood method using FastTree³⁴,
214 and tree visualization used MEGA 6 software³⁵. The amino acid sequences analyzed are
215 Tupanvirus deep ocean protein AUL78925.1 and its homologs with the greatest BLASTp
216 scores from the NCBI GenBank protein sequence database, our sequence database of giant
217 virus genomes, and previously described draft genome sequences from 14 *Acanthamoeba*
218 species³⁶; a set of previously described MBL fold proteins¹⁹; and a set of sequences from the
219 UniProtKB database¹ previously used for phylogeny reconstructions. Three-dimensional
220 comparisons for protein modeling, prediction and analysis were carried out against the Phyre2
221 web portal¹⁸. The set of translation components from each representative of the proposed

222 order Megavirales³⁷ was obtained through a BLASTp search³² with their repertoire of
223 predicted proteins against clusters of orthologous groups of proteins (COGs) involved in
224 translation (category J)³⁸, using 10^{-4} and 50 amino acids as thresholds for e-values and
225 sequence alignment lengths, respectively. The set of tRNAs from each virus was collected
226 using the ARAGORN online tool (<http://130.235.244.92/ARAGORN/>)³⁹. Hierarchical
227 clustering was performed using the MultiExperiment Viewer software⁴⁰ based on the patterns
228 of presence/absence of MBL fold protein, numbers of translation-associated components
229 (number of tRNAs, aminoacyl tRNA-synthetases, other tRNA-associated proteins, other
230 translation-associated proteins) and size of the gene repertoires for Megavirales members
231 (**Supplementary Table S2**). For each item, the maximum value was determined, and values
232 for each virus were considered relatively to these maximum values, being therefore comprised
233 between 0 and 100%.

234 **Cloning, expression and purification**

235 The Tupanvirus deep ocean gene bioinformatically predicted to encode a beta-lactamase
236 superfamily domain (AUL78925.1¹²) was designed to include a Strep-tag at the N-terminus
237 and optimized for its expression by *Escherichia coli*. It was synthesized by GenScript
238 (Piscataway, NJ, USA) and ligated between the NdeI and NotI restriction sites of a pET24a(+)
239 plasmid. *E. coli* BL21(DE3)-pGro7/GroEL (Takara Shuzo Co., Kyoto, Japan) grown in ZYP-
240 5052 media were used for the expression of the recombinant protein. When the culture
241 reached an O.D._{600 nm} = 0.6 at 37°C, the temperature was lowered to 20°C and L-arabinose
242 (0.2% m/v) was added in order to induce the expression of chaperones. After 20 hours, cells
243 were harvested by centrifugation (5,000 g, 30 min, 4°C) and the pellet was resuspended in
244 washing buffer (50 mM Tris pH 8, 300 mM NaCl) and then stored at -80°C overnight. Frozen
245 *E. coli* were thawed and incubated on ice for 1 hour after having added lysozyme, DNase I
246 and PMSF (phenylmethylsulfonyl fluoride) to final concentrations of 0.25 mg/mL, 10 µg/mL

247 and 0.1 mM, respectively. Partially lysed cells were then disrupted by 3 consecutive cycles of
248 sonication (30 seconds, amplitude 45) performed on a Q700 sonicator system (QSonica).
249 Cellular debris were discarded following a centrifugation step (10,000 g, 20 min, 4°C). The
250 Tupanvirus protein was purified with an ÄKTA avant system (GE Healthcare, Bucks, UK)
251 using Strep-tag affinity chromatography (wash buffer: 50 mM Tris pH 8, 300 mM NaCl, and
252 elution buffer: 50 mM Tris pH 8, 300 mM NaCl, 2.5 mM desthiobiotin) on a 5 mL StrepTrap
253 HP column (GE Healthcare). Fractions containing the protein of interest were pooled. Protein
254 purity was assessed using 12.5% SDS-PAGE analysis (Coomassie staining). Protein
255 expression was confirmed by performing MALDI-TOF MS analysis on gel bands previously
256 obtained by SDS-PAGE. Protein concentrations were measured using a Nanodrop 2000c
257 spectrophotometer (Thermo Scientific, Madison, WI, USA).

258 **Spectrophotometry assay for the detection of beta-lactamase activity in Tupanvirus** 259 **virions**

260 Tupanvirus purified virions in solution were centrifuged at 5,000 RPM in order to collect 1g
261 of humid matter. Virions were then suspended into 2 mL of a phosphate-buffered saline
262 (PBS) solution at pH 7.4 prepared in water from a commercial salt mixture (bioMerieux,
263 Marcy-l'Etoile, France). Virions were broken after five freeze-thaw cycles followed by 10
264 minutes of sonication (Q700 sonicator with a Cup Horn, QSonica, Newtown, Connecticut,
265 USA). Integrity of virions was checked by scanning electron microscopy (TM 4000, Hitachi
266 High-Technologie Corporation, Tokyo, Japan). Debris were discarded following a
267 centrifugation step (15,000 g, 10 minutes). The clear supernatant was lyophilized and then
268 reconstituted in 100 µL of PBS (corresponding to a final concentration of 50 mg/mL of
269 soluble proteins). A pure solution of Tupanvirus protein was buffer-exchanged in PBS and the
270 concentration was adjusted to 1 mg/ml. The degradation of nitrocefin (1 mM in PBS), a
271 chromogenic cephalosporin substrate, was monitored as previously described after the

272 addition of virion protein extract or Tupanvirus protein to the solution⁶.

273 **Beta-lactam antibiotic degradation monitoring by liquid chromatography-mass**
274 **spectrometry (LC-MS)**

275 Penicillin G and sulbactam stock solutions at 10 mg/mL were freshly prepared in water from
276 the corresponding high purity salts (Sigma Aldrich). A total of 30 μ L of tupanvirus protein
277 solution at 1 mg/mL was spiked with penicillin G and sulbactam at a final concentration of 10
278 μ g/mL, before incubation at room temperature. Each time point corresponded to triplicate
279 sample preparations. Negative controls consisted of PBS spiked with penicillin G and
280 sulbactam. Then, 70 μ L of acetonitrile were added to each sample, and tubes were vortexed
281 10 minutes at 16,000 g to precipitate the proteins. The clear supernatant was collected for
282 analysis using an Acquity I-Class UPLC chromatography system connected to a Vion IMS
283 Qtof ion mobility-quadrupole-time of flight mass spectrometer, as previously described⁶.

284 **Assessment of the effect of a beta-lactamase inhibitor on Tupanvirus growth**

285 To evaluate the effect of a beta-lactamase inhibitor sulbactam on Tupanvirus growth, we
286 tested Tupanvirus replication on *A. castellanii* pre-incubated with a high dose of sulbactam.
287 Tests were performed in triplicate and amoebae cultivated in trypticase soy medium¹². Four 1
288 mL culture wells containing $5 \cdot 10^5$ *A. castellanii* were incubated at 32°C, one of which
289 contained 500 mg/L of sulbactam. After 24 hours, Tupanvirus was added at a multiplicity of
290 infection (MOI) of 1 in the well with sulbactam. Two other wells were inoculated with
291 Tupanvirus, including one in which 500 mg/L of sulbactam was added. The last well was
292 used as control of amoeba survival. After 24h, amoebae were counted and Tupanvirus was
293 titrated by qPCR as previously described¹². In order to assess whether sulbactam could have
294 affected newly formed virions, tupanviruses produced on amoebae incubated with sulbactam
295 were inoculated on fresh amoebae at different concentrations. Their growth was monitored
296 using high content screening microscopy every 8h for 48h⁴¹. Viral replication was compared

297 to that of tupanviruses produced on amoebae non-treated with sulbactam at the same MOIs.

298 **Nuclease activity assessment**

299 Nuclease activity was assessed using double-stranded DNA, (+) and (-) single-stranded
300 DNAs, and single-stranded RNAs as substrates. Single-stranded DNAs were synthetic
301 polynucleotides (*Supplementary Table S3*); double-stranded DNA was obtained by annealing
302 (+) and (-) single-stranded DNAs in a thermocycler at temperatures decreasing from 95°C to
303 25°C over 1h. RNAs used as substrate were from *Escherichia coli*, from different bacteria that
304 differ by the G+C content of their genomes (*Streptococcus parasanguinis* (41.8%), *Vibrio*
305 *parahaemolyticus* (45.4%), *Vitreoscilla massiliensis* (49.4%), *Aeromonas salmonicida*
306 (58.5%), *Aeromonas hydrophila* (61.5%) and *Pseudomonas aeruginosa* (66.6%)), and from
307 *Acanthamoeba castellanii*. RNAs were purified using RNeasy columns (Invitrogen, Carlsbad,
308 CA, USA). Enzymatic reactions were performed by incubating each polynucleotide (2 µg)
309 with 15 µg of the expressed Tupanvirus protein TupBlac in Tris-HCl buffer 50 mM, pH 8.0,
310 sodium chloride 0.3 M, using a final volume of 20 µL at 30°C for 2 h. After incubation, the
311 material was loaded onto denaturing polyacrylamide gel electrophoresis (dPAGE) at 12% or
312 analysed using the Agilent RNA 6000 Pico LabChip kit on an Agilent 2100 Bioanalyzer
313 (Agilent Technology, Palo Alto, CA, USA). Controls were carried out under the same
314 conditions. The action of TupBlac on RNAs was also assayed in the presence of ceftriaxone,
315 an inhibitor of human metallo β-lactamase fold DNA repair nucleases SNM1A and SNM1B²¹.
316 To do this, enzymatic reactions were conducted at 30°C by incubating *E. coli* RNA (1 µg)
317 with TupBlac (15 µg) in the presence of ceftriaxone at 200 µM. At different times, reactions
318 were stopped by addition of proteinase K (10 µg) and incubated 1h at 37°C. For a quantitative
319 assessment of the RNase activity of the TupBlac enzyme, we used the RNaseAlert QC
320 System kit (Fisher Scientific, Illkirch, France) according to the manufacturer's protocol. This
321 assay uses as substrate a fluorescence-quenched oligonucleotide probe that emits a fluorescent

322 signal in the presence of RNase activity. RNase activities were assayed in the absence or
323 presence of sulbactam (10 µg/mL) or ceftriaxone (200 µM). Negative controls were made
324 with all the reagents used (RNase free water, enzyme buffer, sulbactam and ceftriaxone).
325 Fluorescence was monitored continuously at 37°C for 1h by a Synergy HT plate reader
326 (BioTek Instruments SAS, Colmar, France) with a 485/528 nm filter set. RNase activities of
327 TupBlac were estimated using supplied RNase A used as a standard (10 mU/mL). Two
328 independent experiments were conducted.

329

330

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341

342 **Author contributions** Conceived and designed the study: DR, PC, PP, BLS. Designed and/or
343 performed experiments: DR, PC, LP, SA, NA, EC, BLS, PP. Analyzed and interpreted data:
344 DR, PC, EC, BLS, PP. Wrote the manuscript: PC and DR. All authors read and approved the
345 final manuscript.

346

347

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FIGURES

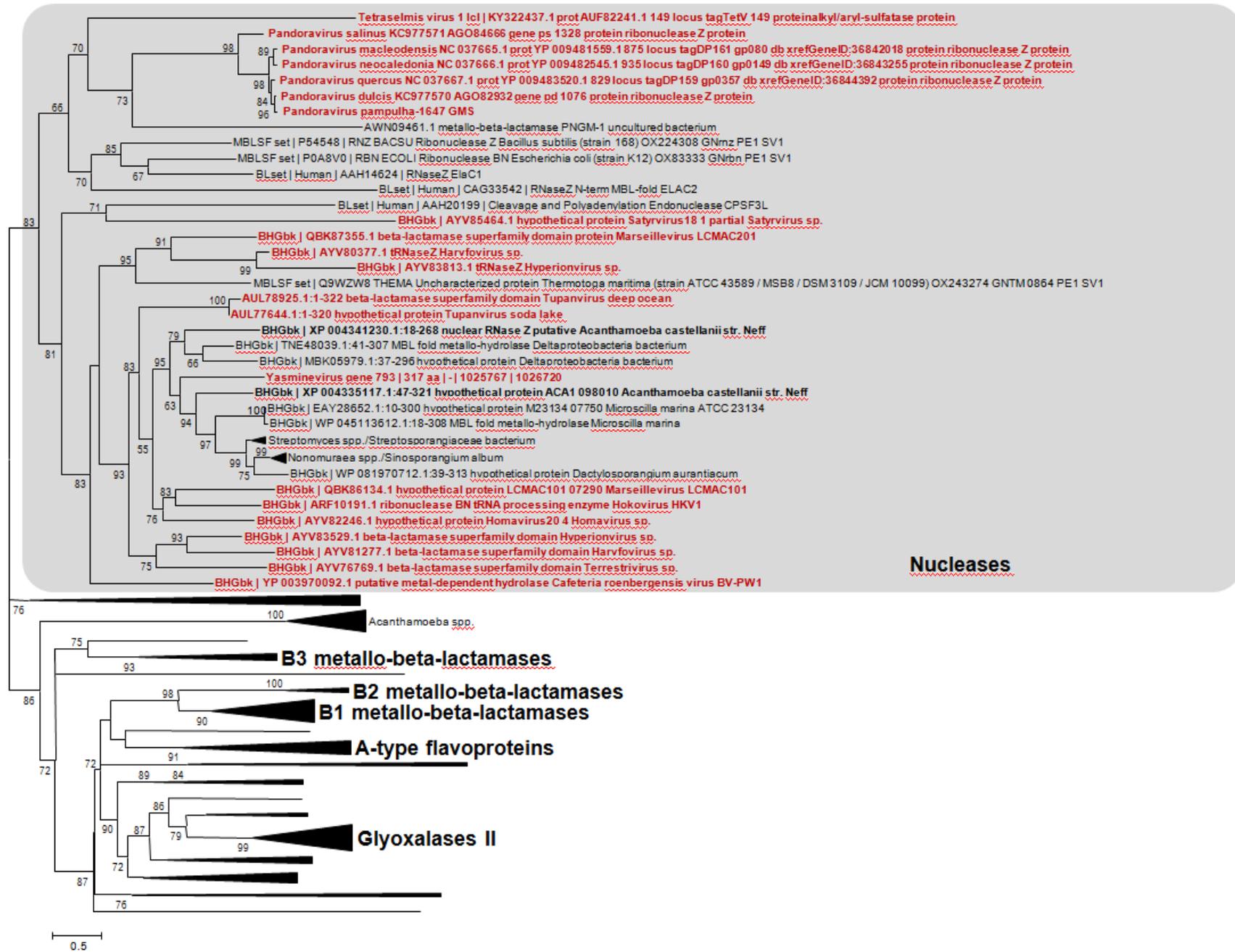
440

441 **Figure 1 | Phylogeny reconstruction based on metallo-beta-lactamase (MBL) fold** 442 **proteins**

443 Phylogeny reconstruction was performed after amino acid sequence alignment with the
444 Muscle program³³ with the Maximum-Likelihood method using FastTree³⁴, and tree was
445 visualized with the MEGA 6 software³⁵. The amino acid sequences analyzed are Tupanvirus
446 deep ocean protein AUL78925.1 and its homologs with the greatest BLASTp scores from the
447 NCBI GenBank protein sequence database (nr) (see *Supplementary Table S1*), our sequence
448 database of giant virus genomes, and previously described draft genome sequences from 14
449 *Acanthamoeba* species³⁶; a set of previously described MBL fold proteins¹⁹; and a set of
450 sequences from the UniProtKB database¹, previously used for phylogeny reconstructions.
451 Extended tree is available in *Supplementary Figure S1*.

452

453



455 **Figure 2 | Effect on nitrocefin of expressed Tupanvirus protein (TupBlac).**

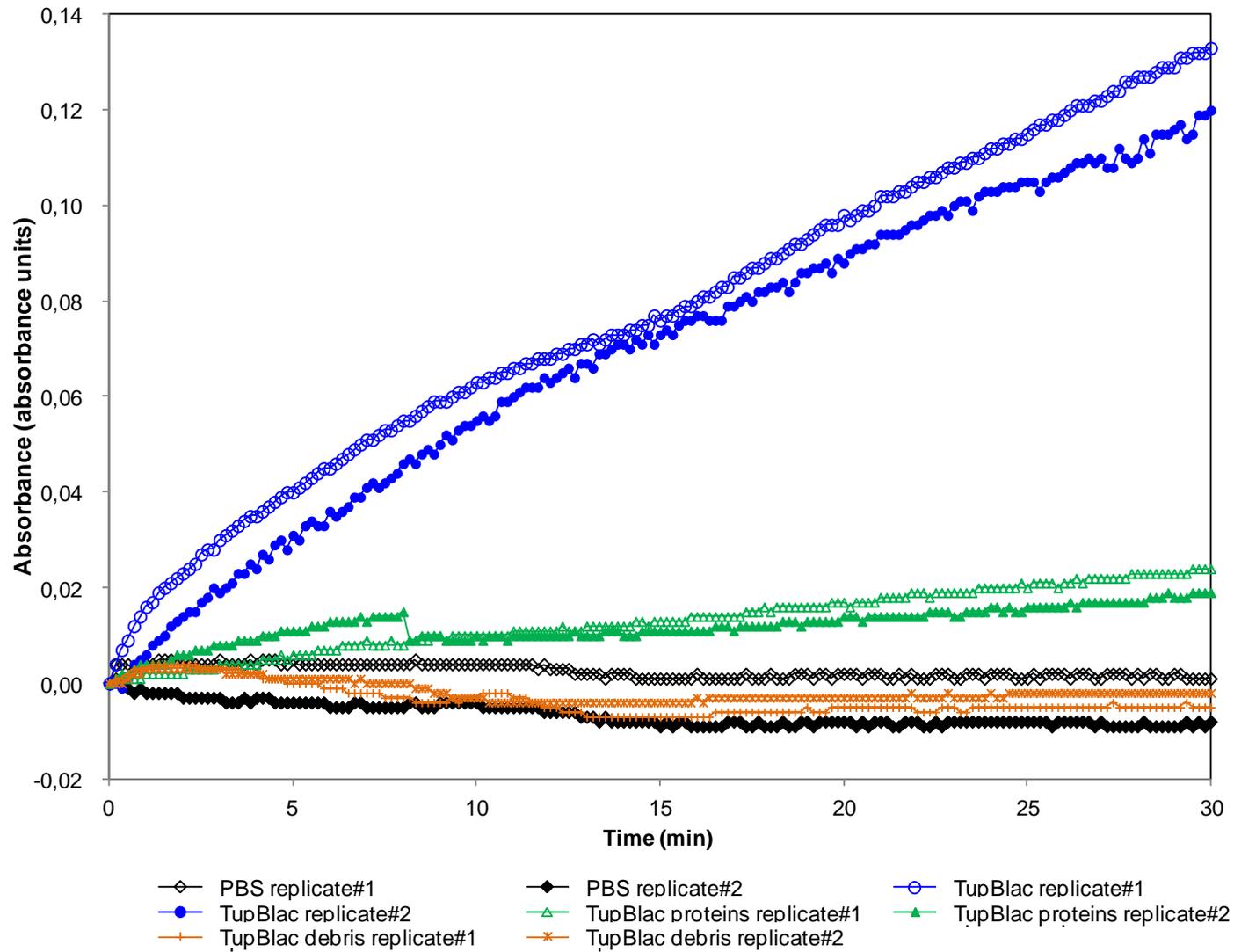
456 The effect on nitrocefin of the expressed Tupanvirus protein (TupBlac) was assessed by

457 monitoring the degradation of nitrocefin, a chromogenic cephalosporin substrate.

458 PBS, Phosphate-Buffered Saline; TupBlac, tupanvirus expressed protein

459

460



462 **Figure 3 | Effect on penicillin G of expressed Tupanvirus protein (TupBlac).**

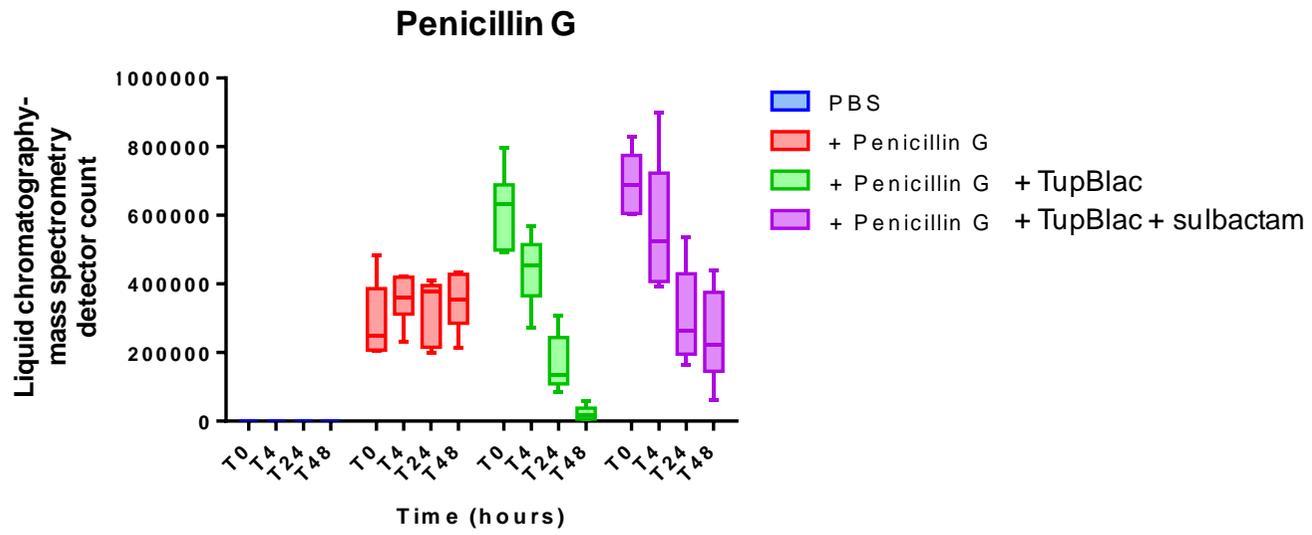
463 The effect on penicillin G of the expressed Tupanvirus protein (TupBlac) and its inhibition by
464 sulbactam were assessed by monitoring by liquid chromatography-mass spectrometry (LC-
465 MS) the degradation of penicillin G (a) and the appearance of benzylpenilloic acid, the
466 metabolite resulting from the enzymatic hydrolysis of penicillin G (b), at times (T) T0 (0
467 hour), T4 (4 hours), T24 (24 hours), and T48 (48 hours).

468 PBS, Phosphate-Buffered Saline; TupBlac, tupanvirus expressed protein

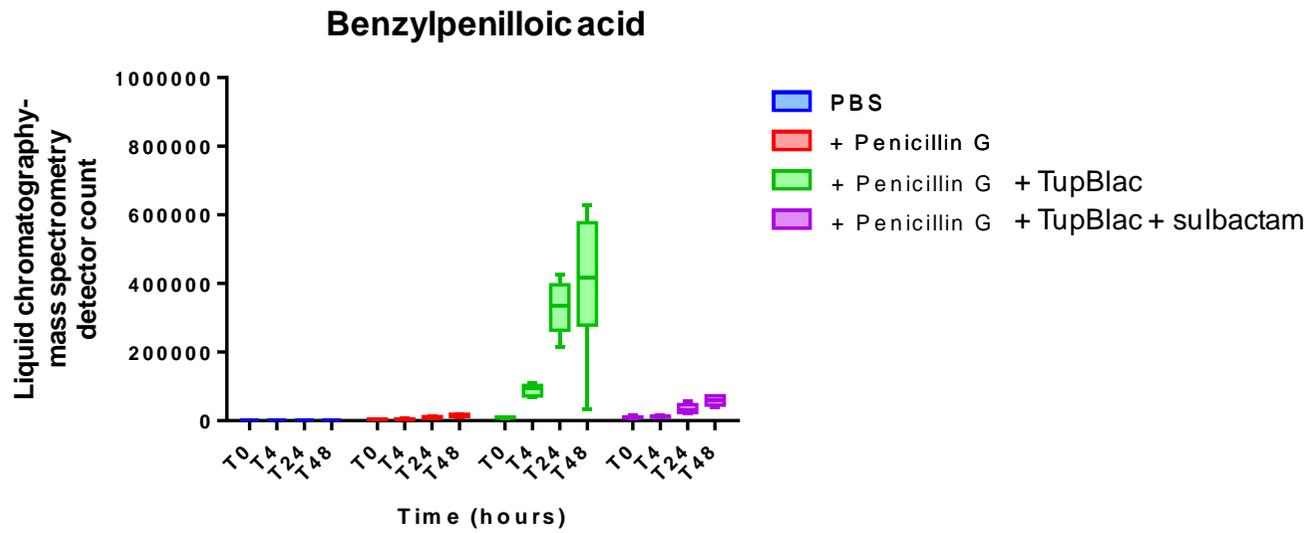
469

470

a.



b.



472 **Figure 4 | Nuclease activity on various types of nucleic acids of expressed Tupanvirus**
473 **protein (TupBlac) as assessed by dPAGE.**

474 Denaturant polyacrylamide gel electrophoresis (12% dPAGE) of nuclease activity on
475 synthetic (+) and (-) single-stranded DNAs (130 nucleotide-long) (a), synthetic double-
476 stranded DNA (b) (see also *Supplementary Table S3*), or *Escherichia coli* RNA (b).
477 No treatment (A); buffer (B); succinate dehydrogenase enzyme produced and purified by the
478 same process and collected in the same fractions as Tupanvirus beta-lactamase TupBlac, used
479 as negative control (C); Tupanvirus beta-lactamase TupBlac (D).

480

481

483 **Figure 5 | Digital gel images of RNase activity of expressed Tupanvirus protein**

484 **TupBlac on *E. coli* RNA.**

485 RNA samples (1 µg) were incubated with 15 µg of TupBlac at 30°C in the absence or
486 presence of 10 µg/mL of sulbactam or 200 µM of ceftriaxone. Nuclease activity was
487 visualized as digital gel images performed using the Agilent Bioanalyzer 2100 with the RNA
488 6000 Pico LabChip (Agilent Technologies, Palo Alto, CA).

489 a: RNA used as substrate was from *Escherichia coli*; no treatment (a); buffer (b); sulbactam
490 (c); TupBlac in the absence (d) or presence (e) of sulbactam.

491 b: RNA used as substrate was from *Escherichia coli*; reactions were stopped at different times
492 (5 min, 10 min, 30 min, 1 h and 2 h) by the addition of proteinase K (10 µg) and incubation
493 for 1h at 37°C. The first lane corresponds to no treatment; lanes 2 to 6 to RNA treatment with
494 TupBlac in the absence of ceftriaxone; lanes 7 to 11 to RNA treatment with TupBlac in the
495 presence of ceftriaxone.

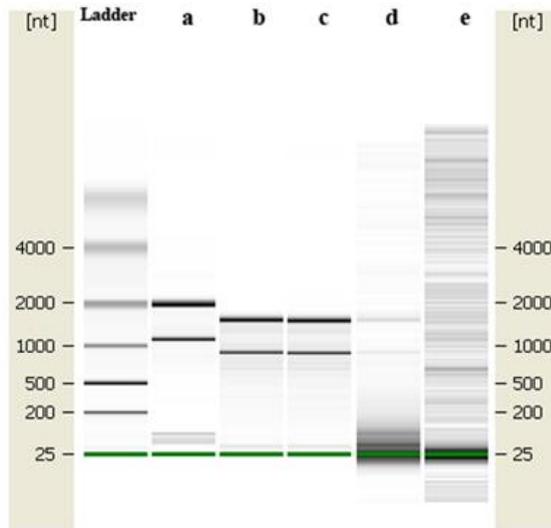
496 c: nuclease activity on RNAs originating from *Acanthamoeba castellanii*; no treatment (a);
497 buffer (b); TupBlac in the absence (c, d) or presence (e) of sulbactam.

498 d: nuclease activity on RNAs originating from bacteria that differ by the G+C-content of their
499 genome, as indicated at the top of the digital gel image. For each RNA, three samples were
500 analyzed: no treatment (1); treatment with TupBlac in the absence of sulbactam (2); and
501 treatment with TupBlac in the presence of sulbactam (3).

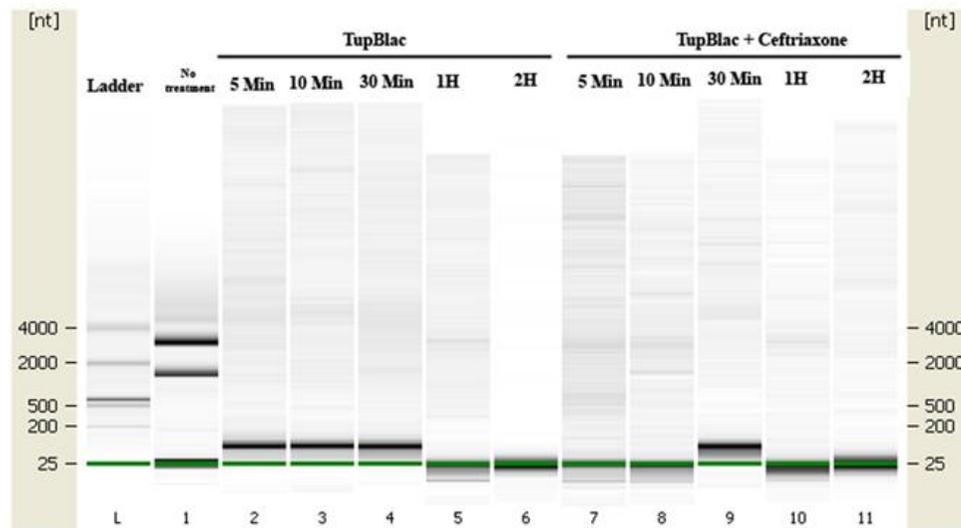
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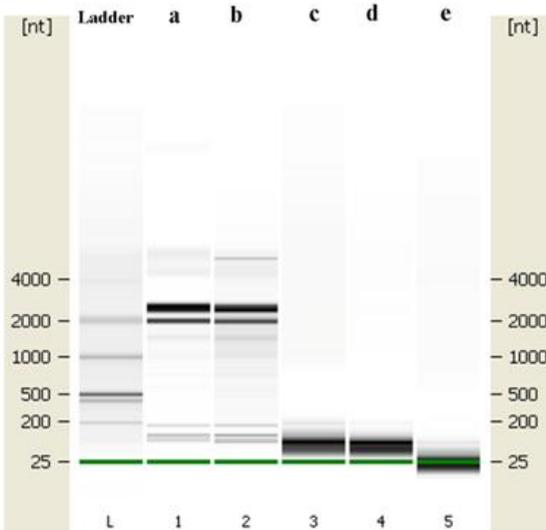
a. Substrate : RNA from *Escherichia coli*



b. Substrate : RNA from *Escherichia coli*



c. Substrate : RNA from *Acanthamoeba castellanii*



d. Substrate : RNA from bacteria with various G+C contents

