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The dolphin proline-rich antimicrobial peptide Tur1A inhibits protein synthesis by targeting the bacterial ribosome

Mario Mardirossian¹, Natacha Pérébaskine², Monica Benincasa³, Stefano Gambato³, Sven Hofmann⁴, Paul Huter^{1,5}, Claudia Müller^{1,5}, Kai Hilpert⁴, C. Axel Innis², Alessandro Tossi³, Daniel N. Wilson^{1,5,6*}.

¹ Gene Center, Department for Biochemistry and Center for Integrated Protein Sciences, Munich (CiPSM), University of Munich, 81377 Munich, Germany.

² ARNA Laboratory, University of Bordeaux, Inserm U1212, CNRS UMR 5320, IECB, 33607 Pessac, France

³ Antimicrobial Peptides Laboratory, Department of Life Sciences, University of Trieste, 34127 Trieste, Italy

⁴ Institute of Infection and Immunity, St George's, University of London, SW17 0RE London, UK

⁵ Institute for Biochemistry and Molecular Biology, University of Hamburg, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany

⁶ Lead Contact

*Correspondence to: daniel.wilson@chemie.uni-hamburg.de

SUMMARY

1
2 Proline-rich antimicrobial peptides (PrAMPs) internalize into susceptible bacteria using specific
3 transporters and interfere with protein synthesis and folding. To date, mammalian PrAMPs have so
4 far only been identified in artiodactyls. Since cetaceans are co-phyletic with artiodactyls, we mined
5 the genome of the bottlenose dolphin *Tursiops truncatus*, leading to the identification of two
6 PrAMPs, Tur1A and Tur1B. Tur1A, which is orthologous to the bovine PrAMP Bac7, is
7 internalized into *E. coli* without damaging the membranes using the inner membrane transporters
8 SbmA and YjiL/MdM. Furthermore, like Bac7, Tur1A also inhibits bacterial protein synthesis by
9 binding to the ribosome and blocking the transition from the initiation to the elongation phase. By
10 contrast, Tur1B is a poor inhibitor of protein synthesis and may utilize another mechanism of
11 action. An X-ray structure of Tur1A bound within the ribosomal exit tunnel provides a basis to
12 develop these peptides as novel antimicrobial agents.
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25 **KEYWORDS:** antibiotic, Cathelicidin; Bac7, dolphin, PrAMP; ribosome; proline-rich
26 antimicrobial peptide, Tur1A, Tur1B.
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INTRODUCTION

1 Most antimicrobial peptides (AMPs), especially α -helical ones, mainly kill bacteria by disrupting
2 the bacterial cell membrane. This mechanism of action makes such AMPs potent broad-spectrum
3 antimicrobials, but with the drawback that they exhibit some toxicity towards host cells (Jenssen et
4 al., 2006). By contrast, proline-rich antimicrobial peptides (PrAMPs) prevalently kill some Gram-
5 negative bacteria without perturbing the cell membrane. PrAMPs utilize bacterial inner membrane
6 proteins to translocate into the cytoplasm where they inhibit intracellular targets, presumably
7 explaining their lower cytotoxicity (Scocchi et al., 2011). Until now, PrAMPs have been identified
8 in some arthropods and mammals, but as evolutionarily unrelated AMPs (Graf et al., 2017; Otvos,
9 2002; Scocchi et al., 2011). However, within a specific class of animals, it is possible to group the
10 PrAMPs into evolutionarily related families of peptides. In insects, for example, there are the
11 apidaecins, isolated from members of the *Apidea* and *Vespoidea* superfamilies (Casteels et al.,
12 1994). Similarly, pyrochoricin (Cociancich et al., 1994), metalnikowins (Chernysh et al., 1996) and
13 the oncocins (Knappe et al., 2010; Schneider and Dorn, 2001) were identified as members of
14 PrAMPs from the *Hemiptera* order. In mammals, PrAMPs discovered within different animals from
15 the *Artiodactyla* order can be grouped together as homologues of the same cathelicidin-derived
16 peptides. For example, the proline-rich Bac5 and Bac7 were isolated from distinct members of the
17 *Bovidae* family, such as *Bos taurus* (cow) (Gennaro et al., 1989), *Capra hircus* (goat) and *Ovis*
18 *aries* (sheep) ((Huttner et al., 1998; Shamova et al., 1999), reviewed by (Graf et al., 2017; Scocchi
19 et al., 2011)).

20 Distinct and unrelated PrAMPs do not necessarily display high sequence similarity, but
21 rather appear to be related to each other by a generally high content of proline and arginine residues
22 that are often arranged in short motifs repeated many times through-out the peptide sequence (*e.g.* -
23 PPXR- in Bac5 and -PRPX- in Bac7) (Graf et al., 2017; Scocchi et al., 2011). Additionally, most of
24 the PrAMPs characterized to date also display a similar uptake mechanism as well as inhibitory
25 properties on bacterial growth (Graf et al., 2017). The inner-membrane protein SbmA appears to be
26 the principle PrAMP transporter in *E. coli* (Mattiuzzo et al., 2007), while the MdtM/YjiL multi-
27 drug resistance transporter seems to play an accessory role (Krizsan et al., 2015). Once in the
28 bacterial cytosol, PrAMPs interfere with protein synthesis and folding (Graf et al., 2017; Scocchi et
29 al., 2011). Both mammalian and invertebrate PrAMPs have been shown to inhibit protein synthesis
30 by interacting with the ribosome (Krizsan et al., 2014; Mardirossian et al., 2014). Despite their
31 diverse sequences, all characterized PrAMPs bind to an overlapping site within the ribosomal exit
32 tunnel and inhibit translation by either blocking the transition from initiation to the elongation phase
33 (Gagnon et al., 2016; Roy et al., 2015; Seefeldt et al., 2016; Seefeldt et al., 2015) or preventing the
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1 dissociation of the release factors during translation termination (Florin et al., 2017). Although
2 PrAMPs also bind to and inhibit the activity of the bacterial chaperone DnaK (Otvos et al., 2000;
3 Scocchi et al., 2009), this is not sufficient, *per se*, to kill bacteria (Krizsan et al., 2014; Scocchi et
4 al., 2009). Inhibition of protein folding is therefore not the main mode of action of PrAMPs.
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8 In mammals, all described PrAMPs belong so far to the cathelicidin family (Scocchi et al.,
9 2011), one of the main families of vertebrate host defence peptides and a prime example of
10 diversity among AMPs. Cathelicidins are characterized by the presence of the cathelin-like domain
11 (CLD), a large, conserved pro-region of uncertain function (encoded by the first three exons), and a
12 highly variable AMP located at the C-terminus (encoded by the fourth exon) (**Figure 1**) (Zanetti,
13 2005). Following the secretion of the pro-peptide into the extracellular medium or into the
14 phagosomes of neutrophils, the active form of the AMP is produced upon proteolytic cleavage
15 (Tomasinsig and Zanetti, 2005). Most vertebrate animals express only one or few cathelicidins,
16 mostly comprising peptides that adopt helical conformations (Xhindoli et al., 2016). Artiodactyl
17 species are an exception since they express numerous cathelicidins, which are comprised of AMPs
18 with a diverse array of structures (α -helices, disulfide-stabilised β -hairpins or extended peptides
19 rich in particular residues, such as tryptophan or proline) (Tossi et al., 2017). The presence of
20 cathelicidin-derived PrAMPs in artiodactyls (Tossi et al., 2017) suggests that they may also be
21 present in *Cetacea* since they are co-phyletic within the unique order of *Cetartiodactyla* (O'Leary
22 and Gatesy, 2008; Spaulding et al., 2009). Here, we mined the available genome sequences of the
23 cetacean *Tursiops truncatus* (the bottlenose dolphin), as well as physically probing its gDNA for
24 homologues of known cathelicidin PrAMPs. This led to the identification of two previously
25 unknown PrAMPs, which we termed Tur1A and Tur1B. The Tur1A and Tur1B peptides were
26 synthesized and characterized for their antimicrobial activity, mode of entry into the bacterial cell
27 and capacity to inhibit protein synthesis on ribosomes.
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45 **RESULTS**

46 **Identification of potential PrAMPs in the bottlenose dolphin**

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48 To date, all mammalian PrAMPs identified in artiodactyls belong to the cathelicidin family. Since
49 cetaceans are co-phyletic with artiodactyls, we hypothesized that cathelicidin-related PrAMPs could
50 also exist in cetaceans. To investigate this, we searched the available genome sequence of the
51 cetacean *Tursiops truncatus* (bottlenose dolphin) using the bovine and pig cathelicidin PrAMP
52 sequences as queries. This led to the identification of a 32 residue proline-rich peptide with an
53 overall charge of +10, which we termed Tur1A (**Figure 1B**). Analogous with other mammalian
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1 cathelicidin, the Tur1A peptide was also encoded within the fourth exon of the gene and preceded
2 by three exons encoding the cathelin-like domain (**Figure 1A**). The sequence and conserved gene
3 organisation suggested that Tur1A was indeed a *bona fide* PrAMP, orthologous to the cathelicidin
4 Bac7.
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7 In an attempt to directly validate the sequence, specific primers were used to selectively
8 amplify the 4th exon of the gene from gDNA fragments extracted from *Tursiops truncatus* tissue.
9 Unexpectedly, when sequenced, the amplified DNA encoded a sequence of what was apparently a
10 paralogous PrAMP, which we termed Tur1B. Tur1B shares 53% identity with Tur1A, but has a
11 lower overall charge (+6) and contains a number of tryptophan (W) residues (**Figure 1B**). While the
12 EST database confirmed only the expression of Tur1B (GenBank: GT116023), the recently
13 available Sequence Read Archive (SRA) database [bioproject PRJNA313464; (Morey et al., 2016)]
14 validated the presence of both the *tur1A* and *tur1B* sequences in *Tursiops truncatus*. This bioproject
15 provides an RNA-Seq with the hit frequency suggesting that *tur1A* is more extensively expressed
16 than *tur1B*. These findings indicate that the bottlenose dolphin contains at least two distinct
17 PrAMPs, Tur1A and Tur1B. Tur1A has high sequence similarity (58-59%) and similar charge with
18 the fully active, 35-residue N-terminal regions of the bovine PrAMP Bac7 and porcine PR-39
19 (**Figure 1B**). The homology with PrAMPs such as Bac7 was lower for Tur1B, which instead shows
20 similarity in sequence and charge with the bovine Trp-rich Indolicidin (38% identity based on a 13
21 residue stretch). Indolicidin is reported to internalize into the bacterial cytoplasm via a self-
22 promoted uptake mechanism (Hsu et al., 2005; Shagaghi et al., 2016), where it is suspected to
23 selectively inhibit DNA synthesis (Ghosh et al., 2014).
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41 **Antimicrobial activity of Tur1A and Tur1B**

42 It has been previously shown that SbmA is the major PrAMP transporter, facilitating uptake of
43 Bac7 fragments into the bacterial cytoplasm (Guida et al., 2015; Mattiuzzo et al., 2007), but that at
44 higher concentrations of Bac7, the accessory transporter YjiL/MdtM also contributes to uptake
45 (Krizsan et al., 2015). The antimicrobial activity of Tur1A and Tur1B was therefore assessed
46 against *E. coli* strains lacking SbmA ($\Delta sbmA$), YjiL ($\Delta yjiL$) or lacking both SbmA and YjiL
47 ($\Delta sbmA \Delta yjiL$) (Krizsan et al., 2015), and compared with activity against the parental *E. coli* strain
48 BW25113 (**Table 1**). Tur1A had a comparable minimal inhibitory concentration (MIC) to Bac7(1-
49 35) against *E. coli* BW25113 (MICs of 1.2 μ M and 2.8 μ M, respectively), whereas Tur1B was less
50 active (MIC of 7 μ M). Furthermore, Tur1A seemed to be less dependent on the SbmA transporter
51 than Bac7(1-35) since the $\Delta sbmA$ mutant showed little change in the MIC, whereas the MIC
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1 increased more evidently for Bac7(1-35). Knocking-out the *yjiL* gene had little effect on the MIC
2 for either peptide, whereas the double *ΔsbmA/ΔyjiL* deletion mutant exhibited a marked increase (5-
3 fold) in MIC for both Tur1A and Bac7(1-35). The activity of Tur1B, while generally less potent,
4 did not seem to depend on the presence of either transporter. These data suggest that: (i) Tur1A uses
5 an assisted internalization mode similar to other PrAMPs, such as for example Bac7, (ii) Tur1A can
6 use either the SbmA or YjiL/MdtM transporters at concentrations close to the MIC (1-2 μM),
7 whereas Bac7(1-35) seems to use preferentially SbmA at lower concentrations and YjiL/MdtM only
8 at higher concentrations (8 μM), and (iii) Tur1B activity does not seem to depend on PrAMP
9 transporters, suggesting it may have a different mechanism of action and/or entry.
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19 **Internalization of Tur1A into *E. coli* cells**

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21 In order to better correlate the uptake of Tur1A with the presence of a specific transport system,
22 internalization of boron-dipyrromethene (BODIPY)-labelled peptides into *E. coli* cells was
23 evaluated by cytofluorimetric analysis (**Figure 2A and B**). It should be noted that for these
24 experiments cells were treated with a quite low peptide concentration (0.1 μM). Cells were
25 extensively washed before the total fluorescence was determined (**Figure 2A**). Because the total
26 fluorescence comprises both tightly surface bound as well as internalized peptide, measurements
27 were also made after washing with trypan blue, which quenches the BODIPY (BY) fluorescence
28 derived by the surface bound peptide and thus allows the amount of internalized peptide to be
29 evaluated (**Figure 2B**). In the wildtype *E. coli* BW25113, the large difference between the
30 Tur1A(Cys³³)-BY fluorescence in the absence and presence of the trypan blue (**Figure 2A and 2B**)
31 suggests a strong surface binding capacity of this peptide (**Figure 2A**). This probably explains the
32 slower internalization of the peptide in comparison to the Bac7(1-35)(Cys³⁶)-BY (**Figure 2B**).
33 Nevertheless, the uptake of Tur1A(Cys³³)-BY into *E. coli* BW25113 was efficient, even at very low
34 concentrations. The absence of SbmA or YjiL did not significantly change the Tur1A(Cys³³)-BY
35 uptake, consistent with the unchanged MIC values (see **Table 1**). By contrast, uptake was
36 significantly affected in the *ΔsbmA/ΔyjiL* double knock-out (**Figure 2B**) where corresponding
37 increases in the MIC values were also observed (**Table 1**). Curiously, for both peptides, the
38 presence of the transporters correlates not only with higher levels of internalized fluorescent
39 peptide, but also with the increased amount of surface bound fluorescence. Propidium iodide (PI)-
40 uptake assays carried out in parallel indicated however that no membrane permeabilization occurred
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1 The effect of Tur1A on permeabilization of *E. coli* BW25113 membranes was characterized
2 by monitoring the PI-uptake after 15 min of incubation with Tur1A (**Figure 2C**) or Bac7(1-35)
3 (**Figure 2D**) at concentrations of 1 μM or 8 μM , respectively (*i.e.* the MIC values in the presence or
4 absence of transporters, as seen in **Table 1**). The membranolytic antibiotic polymyxin B (PxB) was
5 used at 0.25 μM as positive control for membrane permeabilization (>90% PI-positive cells). The
6 level of membrane permeabilization for both Tur1A and Bac7(1-35) at 1 μM was very low (<3% of
7 damaged cells). Since this is within the range of the MIC, this is consistent with the notion that
8 Tur1A, like Bac7(1-35), inhibits bacteria using an intracellular mechanism rather than via inducing
9 membrane damage. For comparison, almost all cells become permeabilized in the presence of
10 0.25 μM PxB. However, we note that by increasing the peptide concentration to 8 μM , deleterious
11 effects on membrane integrity were observed for Tur1A, as previously reported for Bac7(1-35) on
12 *Salmonella enterica typhimurium* (Podda et al., 2006). Specifically, at 8 μM , Bac7(1-35) damaged
13 the membranes of ~50% of cells, whereas Tur1A damaged ~70% cells. By prolonging the
14 incubation time (to 60 min), or by increasing Tur1A concentration (to 16 μM), permeabilization
15 could be increased (but never to 100%), suggesting a time and concentration dependency (data not
16 shown). However, for these longer times it is difficult to distinguish between a primary
17 membranolytic effect and the disruption that occurs following bacterial death. Taken together, these
18 results confirm that the antimicrobial activity of Tur1A against *E. coli* is principally correlated with
19 its internalization into the cells rather than via membrane lysis, but that nevertheless at higher
20 concentrations or incubation times, Tur1A, like Bac7(1-35), can induce membrane damage.
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39 **Inhibition of *in vitro* protein synthesis using *E. coli* lysates**

40 Since the PrAMP Bac7(1-35), which is homologous to the Tur1A and Tur1B peptides, is known to
41 inhibit bacterial protein synthesis (Mardirossian et al., 2014; Seefeldt et al., 2016), we investigated
42 whether the Tur1A and Tur1B peptides also have inhibitory activity in *E. coli* lysate-based *in vitro*
43 coupled transcription/translation assays (**Figure 3A**). The effect of the Tur1A and Tur1B peptides
44 was assessed by monitoring the luminescence resulting from translation of the firefly luciferase
45 reporter gene. As seen in **Figure 3A**, increasing concentrations of Tur1A efficiently reduced the
46 luminescence in a dose-dependent manner, with an estimated IC_{50} <5 μM . By contrast, Tur1B was
47 less effective with no influence on the luminescence observed at concentrations up to 10 μM , and a
48 partial (70%) inhibition of protein synthesis observed only at 100 μM . This suggests that Tur1B is a
49 worse translation inhibitor than Tur1A, and that transcription-translation may not be the main target
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1 To distinguish between effects on transcription and translation, we also assessed the direct effect on
2 the bacterial translation machinery (**Figure 3B**). To do this, the *in vitro* translation assays were
3 repeated but using transcribed mRNA template encoding the firefly luciferase, rather than DNA
4 template as was used for the coupled assays. As seen in **Figure 3B**, similar results were obtained
5 when mRNA template replaced the DNA template, namely, that Tur1A was an excellent inhibitor at
6 concentrations over 10 μ M and that the inhibition of luminescence was only observed for Tur1B at
7 100 μ M. These findings led us to conclude that Tur1A acts directly on the translation apparatus,
8 rather than on transcription, as was observed previously for Bac7 (Mardirossian et al., 2014),
9 whereas Tur1B only effects translation at high concentrations and is therefore likely to have another
10 target and/or mechanism of action.
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20 **Tur1A prevents the transition from initiation to elongation of translation**

21 To understand which step of the protein synthesis was inhibited by Tur1A, toeprinting assays were
22 performed using a fully reconstituted *E. coli in vitro* translation system (Shimizu et al., 2001), as
23 described previously for other PrAMPs (Gagnon et al., 2016; Seefeldt et al., 2016; Seefeldt et al.,
24 2015). The toeprinting assay uses reverse transcription to monitor the position of ribosomes on a
25 reporter mRNA (Hartz et al., 1988). As shown in **Figure 4**, in the absence of antibiotic or peptide,
26 ribosomes initiate and translate the mRNA until becoming stalled on three consecutive prolines
27 (PPP) due to the absence of elongation factor EF-P in the system (Starosta et al., 2014). In the
28 presence of the antibiotic thiostrepton (ThS), ribosomes can initiate at the AUG start codon but
29 cannot translate further due to the inhibitory effect of the drug on elongation factors (Wilson, 2009).
30 This is seen in the toeprint gel by an increase in the band corresponding to ribosomes stuck at the
31 AUG codon and the loss of the band corresponding to ribosomes stalled at the PPP-motif. By
32 contrast, the antibiotic edeine (Ede) prevents initiation complex formation by blocking fMet-tRNA
33 binding to the 30S subunit (Dinos et al., 2004) and therefore represents a control for background
34 reverse transcription stops. The reverse transcription stops, presumably due to secondary structure,
35 are observed between the AUG and PPP codons in all reactions (**Figure 4**). For comparison, the
36 PrAMP Myticalin A5 (MytA5) was also tested in the assay since this peptide inhibits T7 RNA
37 polymerase but does not significantly affect bacterial protein synthesis (Leoni et al., 2017). As
38 expected, increasing concentrations of MytA5 lead to a reduction and, at higher concentrations a
39 complete loss, of all toeprint bands including the full-length mRNA. Like ThS, increasing
40 concentrations of Tur1A led to the decrease in ribosomes stalled at the downstream polyproline
41 sequence (PPP), as well as a concomitant appearance of toeprint signal corresponding to ribosomes
42 stalled at the AUG start codon. At very high concentrations (100 μ M), a reduction in the AUG
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toeprint signal was observed, which may result from drop-off of the fMet-tRNA and disassembly of the ribosome initiation complex (**Figure 4**), as was observed previously for high concentrations of the PrAMPs Bac7(1-16) and Bac7(1-35) (Seefeldt et al., 2016). In contrast to Tur1A, concentrations up to 100 μ M of Tur1B did not lead to a complete loss of the toeprint signal at the PPP-motif, indicating that ribosomes can still elongate in the presence of the Tur1B peptide. However, a slight reduction in the signal intensity of the PPP-band, coupled with the appearance of weak toeprints at the AUG start codon at 10 μ M and 100 μ M suggest that Tur1B can inhibit translation at higher concentrations, consistent with the results obtained using the *in vitro* translation assays (**Figure 3**).

Tur1A inhibits protein synthesis on *Thermus thermophilus* ribosomes

Previous structural studies investigating the ribosome binding site of PrAMPs, such as oncocin and Bac7, utilized ribosome crystals from the thermophilic bacteria *T. thermophilus* (Gagnon et al., 2016; Roy et al., 2015; Seefeldt et al., 2016; Seefeldt et al., 2015), however, it was not demonstrated that such PrAMPs actually display inhibitory activity against *T. thermophilus* translation. To address this, we established a *T. thermophilus* lysate-based *in vitro* translation assay based on an S12 lysate protocol that has been successfully used in the past for *E. coli* (Kim et al., 2006) Huter et al., 2017) and *Bacillus subtilis* (Sohmen et al., 2015). Using the *T. thermophilus* lysate-based *in vitro* translation with firefly luciferase as a reporter it was possible to assess whether Tur1A can inhibit translation on *T. thermophilus* ribosomes. The Tur1B was not further tested since its inhibiting activity towards translation was low (see above **Figure 3**). As shown in **Figure 5**, the Tur1A peptide inhibited translation on *T. thermophilus* ribosomes very efficiently, with 80% inhibition observed even at 1 μ M concentration of Tur1A. This indicates that structural studies using *T. thermophilus* ribosomes represent a valid model system for investigating the binding site of PrAMPs, such as Tur1A, on other bacterial ribosomes.

The binding site of Tur1A on *T. thermophilus* ribosome

We determined the structure of Tur1A bound to the *T. thermophilus* 70S ribosome at 3.3 Å resolution from X-ray diffraction data collected using a single co-crystal of a ternary complex between *T. thermophilus* 70S, Tur1A and YfiA, a protein that is used to lock the head of the 30S subunit in order to improve crystal-to-crystal reproducibility (**Table S1**) (Polikanov et al., 2014). A minimally biased F_o-F_c difference map calculated after refinement of a model of an empty *T. thermophilus* 70S ribosome showed clear density for residues 1-16 of Tur1A, with weaker density

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visible for residues 17-22. This made it possible to build a complete model of Tur1A(1-16) and to trace the backbone for the remainder of the peptide. As in earlier structures of insect and mammalian PrAMPs bound to *T. thermophilus* 70S (Gagnon et al., 2016; Roy et al., 2015; Seefeldt et al., 2016; Seefeldt et al., 2015), Tur1A(1-16) binds to the exit tunnel in a reversed orientation relative to a nascent polypeptide chain (**Figure 6A**), blocking the binding site for the A-site tRNA and a significant portion of the ribosomal exit tunnel. Although its structure is very similar to that of the mammalian Bac7(1-16), the side chains of Arg1 and Arg4 of Tur1A showed no noticeable density, suggesting that these residues are more flexible in the case of Tur1A. Due to their strong structural resemblance to Bac7(1-16), the first 16 residues of Tur1A make similar contacts with the ribosome. A few differences are (i) in Tur1A, Phe5 stacks upon Pro7 (**Figure 6B**), whereas this is not possible in Bac7 because residue 5 is a proline, (ii) in Tur1A, Tyr9 stacks upon the basepair formed by nucleotides C2452 and U2504 of the 23S rRNA that comprise the ribosomal tunnel (**Figure 6C**). In Bac7, Arg9 makes an analogous stacking interaction with the C2452-U2504 base pair (Gagnon et al., 2016; Seefeldt et al., 2016), (iii) in Tur1A, Arg15 stacks upon basepair C2586-C1782 of the 23S rRNA (**Figure 6D**), whereas in Bac7, residue 15 is a proline, and (iv) in Tur1A, Arg16 stacks against the side chain of residue His69 of ribosomal protein L4 at the tunnel constriction (**Figure 6D**), whereas in Bac7, Arg16 stacks against A2062 (Gagnon et al., 2016; Seefeldt et al., 2016). Residues 17-22 of Tur1A extend towards the exit of the nascent polypeptide tunnel, but do not appear to make any specific contacts with ribosomal components, in agreement with their weaker electron density. As with other known PrAMPs, the structural data therefore indicate that Tur1A is likely to inhibit translation by interfering with the binding of aminoacyl-tRNA to the A-site.

The N-terminal region is critical for the inhibitory activity of Tur1A

The structural data suggest that the N-terminal region (residues 1-16) is critical for binding of Tur1A to the ribosome. To evaluate this, we synthesized three overlapping 16-residue Tur1A fragments, Tur1A(1-16), Tur1A(8-24) and Tur1A(16-32), and analysed their activity on living bacteria (**Table 1**). The MIC assays were performed against *E. coli* BW25113 and revealed that Tur1A(1-16) retained activity (MIC = 4 μ M), indicating that the C-terminal region is indeed dispensable for Tur1A antimicrobial activity. By contrast, both the Tur1A(8-24) and Tur1A(16-32) exhibited no antimicrobial activity, confirming that the N-terminal region is critical for the inhibitory activity of Tur1A. These findings are consistent with previous analysis of the related Bac7 orthologue, where the Bac7(1-16) fragment was also identified as being the shortest active

1 fragment (Benincasa et al., 2004). Nevertheless, the absence of antimicrobial activity of the
2 Tur1A(8-24) and Tur1A(16-32) fragments may be due to lack of uptake, rather than inability to
3 bind to ribosomes. To distinguish between these scenarios, the three Tur1A fragments were
4 analysed for their inhibitory activity in an *E. coli in vitro* coupled transcription/translation assay
5 (Figure 6E). Similar to the MIC results using whole cells, only the Tur1A(1-16) fragment inhibited
6 *in vitro* protein synthesis to any extent, whereas the Tur1A(8-24) and Tur1A(16-32) fragments were
7 completely inactive, even at concentrations as high as 100 μ M (Figure 6E). This indicates that the
8 inactivity of the N-terminally deleted Tur1A peptides is mainly due to their inability to bind to the
9 ribosome. Deletion of the N-terminus removes the RRIR sequence of Tur1A (Figure 1), which is
10 also conserved and essential in the Bac7 PrAMP (Benincasa et al., 2004).
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21 **Effect of ribosomal RNA and protein mutations on PrAMP resistance**

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23 Tur1A binds the ribosome inside the exit-tunnel, which is also the ribosomal binding site of the
24 antibiotic erythromycin. Previously, it was demonstrated that mutations in the ribosome that confer
25 resistance to erythromycin also confer cross-resistance to PrAMPs, such as oncocin and apidaecin
26 (Gagnon et al., 2016, Florin et al., 2017). Therefore, we determined the MIC of Tur1A against *E.*
27 *coli* erythromycin-resistant strains bearing mutations within the ribosomal exit tunnel, namely,
28 A2503C, A2059G or A2059C in the 23S rRNA and alterations in ribosomal proteins L4 (K63E)
29 and L22 (Δ_{82} MRK₈₄) (Table S2). As controls, we also determined the MIC in the presence of
30 Bac7(1-35), the apidaecin derivative Api137 and erythromycin. As expected, all the strains bearing
31 rRNA mutations or ribosomal protein alterations had increased MIC against erythromycin
32 compared to the wildtype strains (Table S2). Ribosomal protein alterations in L4 and L22 led to a
33 modest increase in MIC for Api137, as reported previously (Florin et al., 2017). By contrast, there
34 was no significant change in the MIC in the presence of Bac7(1-35), Tur1A or Tur1B (Table S2).
35 While mutations of A2503 and A2059 have been reported to confer resistance to oncocin derivative
36 Onc112 as well as Api137 (Gagnon et al. 2016; Florin et al., 2017), no resistance was observed with
37 Bac7(1-35) (Gagnon et al., 2016). Curiously, we observed that while A2059C mutations confer
38 resistance to Api137, A2059G did not, whereas the A2503G tested previously (Florin et al., 2017)
39 and the A2503C tested here (Table S2), both conferred resistance to Api137. By contrast, we
40 observed no increase in the MIC in the presence of Tur1A and Tur1B (Table S2), consistent with
41 their high sequence similarity with Bac7 (Figure 1).
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61 **DISCUSSION**

1 Here we have identified two PrAMPs from the bottlenose dolphin *Tursiops truncatus*, which we
2 termed Tur1A and Tur1B. Tur1A is highly similar to the PrAMPs from *Bos taurus* Bac7 and from
3 *Sus scrofa* PR-39, sharing 58% and 59% sequence identity, respectively (**Figure 1**). According to
4 our findings, Tur1A is the only PrAMP that is conserved across the order of *Cetartiodactyla*. This
5 suggests that the Tur1A/Bac7/PR-39 family could be the most ancient family of mammalian
6 PrAMPs, dating back approximately 60 million years ago, as calculated by TimeTree (Hedges et al.,
7 2015), to before the evolutionary split between dolphins, cows and pigs. Consistent with their high
8 sequence identity, the mechanism of action and uptake of Tur1A is also similar to Bac7 and PR-39.
9 Like Bac7, Tur1A is also taken up by the bacterial cell using the SbmA and YjiL/MdtM
10 transporters and mainly inhibits bacterial growth by targeting the ribosome, rather than *via*
11 membrane permeabilization. However, unlike Bac7, the isolated role of both these transporter does
12 not affect significantly the uptake of the peptide, indicating that Tur1A exploits the combined effect
13 of these transporters to enter the bacterial cell.
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24 The inhibition of protein synthesis *in vitro* by Tur1A has been demonstrated not only in *E.*
25 *coli*, but also in *T. thermophilus* lysates, thereby confirming the validity of using *T. thermophilus*
26 70S ribosomes for the structural analysis. The binding site of Tur1A overlaps significantly with that
27 observed previously for Bac7 (Gagnon et al., 2016; Seefeldt et al., 2016) (**Figure 6**), and Tur1A
28 exhibits the same mechanism of action as Bac7 to prevent the transition from the initiation to the
29 elongation phase of translation (**Figure 4**). However, slight sequence deviations between Tur1A and
30 Bac7 result in subtle differences in the binding mode and interaction with the components of the
31 ribosomal peptide exit tunnel (**Figure 6**). Such detailed insights will be important for understanding
32 the sensitivity of particular regions of the PrAMPs to sequence variation. Sequence variations are
33 also likely to explain why Tur1A was slightly more permeabilizing towards bacterial membranes
34 than Bac7 (**Figure 2**). A detailed mutagenesis analysis exchanging residues between Tur1A and
35 Bac7 could be used to identify which sequence determinants are critical for uptake and/or ribosome
36 interaction, and thus provide a structure-activity relationship upon which to base further rational
37 design of synthetic PrAMPs for clinical applications.
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50 Although the partial sequence homology suggests that Tur1B may be evolutionarily related
51 to the Tur1A/Bac7/PR-39 family of PrAMPs, Tur1B is significantly divergent so as to have only a
52 modest inhibitory effect on translation (**Figures 3 and 4**). Instead, the presence of four tryptophan
53 residues within Tur1B and its lower charge, imparts characteristics on this peptide that are similar to
54 indolicidin, a short bovine proline-tryptophan rich AMP (Selsted et al., 1992). Tur1B almost
55 appears to be intermediate between the proline-arginine rich AMPs and the proline-tryptophan rich
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1 AMPs, therefore further studies into Tur1B could provide additional evolutionary insight into this
2 latter group of peptides. Indeed, as observed for indolicidin (Mattiuzzo et al., 2007), the transporter
3 SbmA does not play a significant role in the mode of action of Tur1B. While the details of the mode
4 of action of indolicidin are still under discussion, permeabilization of bacterial membrane appears to
5 be an important part of its moderate antimicrobial activity (Falla et al., 1996). Therefore, we think it
6 is likely that Tur1B also acts mainly on the bacterial membrane. However, further studies will be
7 necessary to address this directly.
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13 In conclusion, our study has extended our knowledge on the distribution among animals of
14 PrAMPs and cathelicidins, providing insight into their evolution. We demonstrate that sequence
15 variations within the cathelicidin family of PrAMPs, as evident by comparing Tur1A with Bac7 and
16 PR-39 sequences, leads to subtle alterations in the binding mode and interaction with the ribosome
17 but retains the potent activity and distinct mechanism of action. In contrast, further sequence
18 variation as observed in the Tur1B peptide leads to a peptide with a completely unrelated properties
19 and mechanism of action. We believe such structure-activity relationships will be critical for
20 development of this class of AMPs as valid lead compounds to combat the ever-increasing
21 emergence of multi-drug resistant bacteria.
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32 **SIGNIFICANCE**

33 The discovery of the two proline-rich antimicrobial peptides (PrAMPs) Tur1A and Tur1B in
34 dolphins indicates that these peptides also exist amongst cetaceans. Previously, evidence of
35 PrAMPs was reported only in some terrestrial mammals. Tur1A was shown to kill bacteria by
36 inhibiting protein synthesis, specifically preventing the transition from initiation to elongation. This
37 reiterates the mode of action described for other PrAMPs, such as Bac7 and oncocin, previously
38 identified in cows and insects. This also provides information on the evolutionary convergence of
39 Tur1A with some insect PrAMPs, and on its evolutionary relationship with some bovine PrAMPs.
40 By contrast, Tur1B displays a low inhibitory effect on protein synthesis. Instead, Tur1B appears to
41 be an intermediate between a ribosome-targeting PrAMP and other proline-rich peptides that act
42 mainly by permeabilizing the bacterial membrane, thus offering interesting hints on the relationship
43 between these two groups of PrAMPs. Lastly, both the Tur peptides, but especially Tur1A, have a
44 potent antibacterial activity to be exploited in the fight against the increasing prevalence of
45 antibiotic-resistant pathogens.
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23 **AUTHORS CONTRIBUTION**

24 M.M., N.P., M.B., S.G., S.H., P.H., C.M. performed the experiments. M.M., D.N.W., N.P., C.A.I.,
25 M.B., S.G., A.T. designed the experiments and analysed data. M.M., D.N.W., A.T., K.H. wrote the
26 paper. D.N.W. supervised the whole project.
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32 **DECLARATION OF INTEREST**

33 Kai Hilpert is also founder and director of TiKa diagnostics Ltd and Sven Hofmann is an employee
34 of this company.
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Table 1. Minimal inhibitory concentrations (MIC) for Tur1A, Tur1B, Bac7(1-35) and Tur1A fragments against *E. coli* BW25113 strains.

MIC (μM) on <i>E. coli</i> BW25113 strains				
Peptide	wild type	$\Delta sbmA$	$\Delta yjiL$	$\Delta sbmA / \Delta yjiL$
Tur1A	1.2 (± 0.4)	2.3 (± 1.3)	1.9 (± 1.1)	6.4 (± 2.2)
Tur1B	7 (± 1.9)	4	8	8
Bac7(1-35)	2.8 (± 1.0)	6.3 (± 2.6)	1.8 (± 0.5)	14 (± 3.7)
Tur1A(1-16)	4	nd	nd	nd
Tur1A(8-24)	>64	nd	nd	nd
Tur1A(16-32)	>64	nd	nd	nd

Data represent the average and standard deviation (\pm) calculated after three independent experiments performed as internal duplicates (n=6). nd, not determined.

FIGURE LEGENDS

Figure 1. Gene structure and peptide sequences of dolphin PrAMPs Tur1A and Tur1B. (A) Cathelicidin gene structure with PrAMP encoded in Exon 4. (B) Peptide sequences of dolphin Tur1A and Tur1B compared with orthologues from bovine Bac7(1-35) and porcine PR-39, as well as bovine indolicidin. ^a %Id indicates identity relative to the Tur1A or Tur1B sequences as determined using Clustal Omega. Conserved residues among PrAMPs are shaded grey. Conserved residues between Tur1B and indolicidin are underlined.

Figure 2. Flow-cytometry evaluation of Tur1A internalization into *E. coli* cells and membrane interaction/permeabilization. The fluorescence intensity (MFI) of wildtype BW25113 (wt) and mutant $\Delta sbmA$, $\Delta yjiL$ and $\Delta sbmA/\Delta yjiL$ *E. coli* cells exposed to fluorescent derivatives of Tur1A or Bac7(1-35) is shown. Bacterial cells (1×10^6 CFU/ml) were incubated with 0.1 μ M of each peptide for 10 min, extensively washed, and analyzed by flow cytometry (A) without or (B) with incubation with 1 mg/ml of the trypan-blue quencher for 10 min at 37°C. Data are expressed as the average MFI with a standard deviation for three independent experiments. Flow cytometric analysis of propidium iodide-uptake in *E. coli* BW25113 cells after 15 min treatment with (C) 1 μ M and 8 μ M Tur1A or (D) 1 μ M and 8 μ M Bac7(1-35) or 0.25 μ M PolymixinB (PxB). The fluorescence of the untreated cells is indicated by the grey histogram. The reported data are representative of at least three independent experiments with comparable results (see **Figure S1**). PxB was used only once, as a comparison. 10^4 cells (events) were read for each measurement. Statistical significance: Student-Newman-Keuls Multiple Comparisons Test, ANOVA (*= $p < 0.05$; **= $p < 0.01$).

Figure 3. Effect of Tur1A and Tur1B on *in vitro* translation reactions. (A) Effect of Tur1A and Tur1B on *E. coli in vitro* coupled transcription/translation assays and (B) on *E. coli in vitro* translation assay using mRNA template. Assays were performed in the absence (-) or presence of increasing concentrations of peptides (1 μ M, 10 μ M or 100 μ M). The luminescence resulting from reactions performed in the absence of peptide was normalized to 100%. Reactions lacking DNA (No DNA) or RNA (No RNA) template were performed as negative controls. The error bars represent the standard deviation of the mean from three independent experiments.

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Figure 4. Toe-print analysis of Tur1A and Tur1B effect on translation. Fluorescence scan of a polyacrylamide gel analysis of toeprinting reactions performed in the absence (-) or presence of 100 μ M thiostrepton (ThS), 1-100 μ M Tur1A, Tur1B, MytA5 (MytA5) and 50 μ M edeine (Ede). Toeprint signals corresponding to ribosomes stalled at the AUG start codon or at the polyproline stretch (PPP) are indicated with arrows, as is the reverse transcription product of the full-length mRNA (FL) and bands resulting from reverse transcription stops at secondary structure (SS). Sequencing lanes (C and A) are included for reference with corresponding nucleotide and peptide sequences for these regions. This gel is representative of three independent experiments (see **Figure S2**).

Figure 5. Effect of Tur1A on *in vitro* translation assays using *T. thermophilus* extracts. Translation reactions were performed in the absence (-) or presence of increasing concentrations of Tur1A. The luminescence resulting from reactions performed in the absence of peptides was normalized to 100%. Reactions lacking RNA (No RNA) template were performed as negative controls. The error bars represent the standard deviation of the mean from three independent experiments.

Figure 6. Binding site of Tur1A on the ribosome. (A) The structure of Tur1A(1-22) (blue) is superimposed with that of Bac7(1-16) (green) (Seefeldt et al., 2016) and density contoured at $+2.5 \sigma$ of a minimally-biased *Fo-Fc* map shows the location of the Tur1A peptide (blue mesh). The density was trimmed using the carve function in Pymol, with a 3 Å cutoff. The various sections of the nascent polypeptide exit tunnel are labelled, as well as ribosomal proteins L4 and L22 (white). (B-D) detailed view of (B) Phe5 stacking on Pro7 of Tur1A, (C) Tyr9 of Tur1A stacking on the C2452-U2504 basepair of the 23S rRNA, and (D) Arg15 and Arg16 of Tur1A stacking on basepair C2586-C1782 of the 23S rRNA and His69 of L4, respectively. (E) Luciferase activity after *in vitro* *E. coli* coupled transcription/translation assays performed in presence of Tur1A(1-16), Tur1A(8-24) or Tur1A(16-32). Reactions were performed in the absence (-) or presence of increasing concentrations of peptides. The luminescence resulting of reactions performed in the absence of peptides was normalized to 100%. Reactions lacking DNA template (No DNA) were performed as negative controls. The error bars represent the standard deviation of the mean from two independent experiments.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
 - Bacterial strains and growth conditions
- METHOD DETAILS
 - Peptide identification
 - Peptide synthesis
 - Minimum inhibitory concentration determination
 - Flow cytometry
 - *In vitro* transcription and translation in *E. coli*
 - *In vitro* translation in *T. thermophilus*
 - Toe-printing assays
 - Purification of *T. thermophilus* 70S ribosomes
 - Purification of YfiA
 - *T. thermophilus* 70S-YfiA-Tur1A complex formation
 - Crystallization of *T. thermophilus* 70S-YfiA-Tur1A
 - Data collection and processing
 - Model building and refinement
- QUANTIFICATION AND STATISTICAL ANALYSIS
 - *In vitro* data analysis
- DATA AND SOFTWARE AVAILABILITY
 - Accession numbers

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Daniel N. Wilson (daniel.wilson@chemie.uni-hamburg.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

E. coli strain and growth conditions

The *E. coli* strain BL21STAR was grown at 37°C in Luria-Bertani broth with shaking (200 rpm). All the other *E. coli* strains were grown at 37°C in Müller-Hinton broth with shaking (140 rpm). The *E. coli* strains BW25113, N281, N282 and AB301 required no antibiotics. The *E. coli* strains BW25113 Δ *sbmA* and BW25113 Δ *yjiL* required 50 µg/ml kanamycin, BW25113 Δ *yjiL*/ Δ *sbmA* required 50 µg/ml kanamycin and 15 µg/ml tetracycline. The *E. coli* strains SQ110 Δ *tolC*, SQ110 Δ *tolC* A2059C, SQ110 Δ *tolC* A2059G, SQ110 Δ *tolC* A2305C required 25 µg/ml kanamycin and 50 µg/ml spectinomycin. The *T. thermophilus* strain HB8 was grown in 1×YT medium without antibiotics at 70°C.

The *E. coli* strains BW25113, BW25113 Δ *sbmA::Kmr* and BW25113 Δ *yjiL::Kmr* are part of the KEIO collection (Baba et al., 2006). The double mutant *E. coli* BW25113 Δ *sbmA*/ Δ *yjiL::KmrTetr* (Krizsan et al., 2015) was generously provided by Prof. Ralf Hoffmann, University of Leipzig, Germany. The *E. coli* strains AB301 with its mutants N281(mutation on L22) and N282(mutation on L4), the SQ110 Δ *tolC::Kmr* and its mutants SQ110 Δ *tolC::KmrSprA2059C*, SQ110 Δ *tolC::KmrSprA2059G*, SQ110 Δ *tolC::KmrSprA2305C* were generously provided by Profs Alexander Mankin and Nora Vasquez-Laslop, University of Illinois, Chicago, USA.

METHODS DETAILS

Peptide identification

The *Tursiops truncatus* genome sequence is available as part of the *Mammalian Genome Project* (Lindblad-Toh et al., 2011), however, it was determined by first generation Sanger sequencing and has a relatively low coverage (2.6-fold), so it is only considered partial. Blasting the bovine and porcine PrAMP sequences led to the identification of a possible orthologue in both the Ensemble turTru1 scaffold 36647 and GeneScaffold 2343, which we termed Tur1A. The *tur1A* gene sequence encoding Tur1A was not present in Genebank, and the low coverage of turTru1 raised the question of the certainty of the identification. Thus, to validate the identification, an attempt was made to selectively amplify and sequence the corresponding gene fragment from gDNA purified from bottlenose dolphin tissue, at the Sequencing Facility of the Applied and Comparative Genomics group (University of Trieste). Frozen muscle tissue samples were obtained from the Mediterranean Marine Mammal Tissue Bank, at the Dept. Veterinary Experimental Sciences, Univ. of Padova, and DNA was extracted using the E.Z.N.A.® Mollusc DNA Kit (Omega Bio-Tek), following the

1 provided protocol. Amplification primers were designed using Genomics Workbench 5.1 (CLCbio),
2 based on the DNA sequence 5' and 3' to the 4th exon encoding Tur1A. The forward primer, 5'-
3 CTTGTGACCCTGGGA-3', was based on a sequence in the intron preceding the fourth exon, the
4 reverse primer 5'-ATAACTTCCTCCAGGCTTCA-3' was based on a sequence from the 3'-UTR.
5 The PCR product was purified on a 2% agarose gel showing a major band of the expected size (470
6 bp), which was excised and extracted using the Perfectprep® Gel Clean-up kit (Eppendorf). Sanger
7 sequencing was carried out on a Hitachi 3130 Genetic Analyzer (Applied Biosystems) using the
8 same forward and reverse primers, but resulted in a different sequence than expected and was
9 therefore named *tur1B*, encoding the Tur1B peptide. Since the direct sequencing had not confirmed
10 the presence of *tur1A*, suggesting instead the presence of a paralogous sequence, the EST database
11 was blasted with both sequences to determine if they were expressed. The EST database confirmed
12 the expression of Tur1B only (GenBank: GT116023). On the other hand, the more recent Sequence
13 Read Archive database bioproject PRJNA313464 (Morey et al., 2016) confirmed the presence of
14 both the *tur1A* and *tur1B* sequences in *Tursiops truncatus*.
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28 Peptide synthesis

29 Tur1A, Tur1B, the orthologous bovine peptide fragment Bac7(1-35) and the mussel peptide
30 Myticalin A5 were synthesised on a Biotage Initiator+ Alstra automated microwave synthesizer
31 using Fmoc chemistry. All peptides were synthesized on a 0.1 mmole scale using Fmoc-Pro-TGA
32 or Fmoc-Arg(Pbf)-TGA resins (Novabiochem/Merck) as appropriate, and single couplings with a 6-
33 fold amino acid excess at 75°C. Peptides were cleaved with a version of Reagent K (85% TFA, 6%
34 DODT, 3% thioanisole, 2% phenol, 2% triisopropylsilane, 2% water), subsequently purified by
35 preparative RP-HPLC (Phenomenex Kinetex, C18, 2.6 µm, 100 Å, 50x4,6 mm) using a 15-45%
36 H₂O to CH₃CN gradient (0.05% trifluoroacetate (TFA)) and confirmed by ESI-MS (Bruker Esquire
37 4000) [Tur1A calculated MW=3972.9, measured MW=3972.3; Tur1B calculated MW=4032.8,
38 measured MW=4033.2; Bac7(1-35) calculated MW=4201.2, measured MW=4207.1; MW
39 calculated with Peptide Companion, Coshi Soft]). Tur1A and Tur1B stock solutions were prepared
40 in deionized water from purified peptides that had been lyophilized three times from a 10 mM HCl
41 solution to remove TFA. The concentrations were estimated by using a Nanodrop 2000 based on the
42 extinction coefficients at 214 nm as described by (Kuipers and Gruppen, 2007), and based on the
43 absorption of Tyr at 280 nm for Tur1A ($\epsilon = 1450 \text{ M}^{-1}\text{cm}^{-1}$) or of Trp at 280 nm for Tur1B ($\epsilon = 5500$
44 $\text{M}^{-1}\text{cm}^{-1}$).
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Fluorescently labelled Tur1A(Cys³³)-BY and Bac7(1-35)(Cys³⁶)-BY were prepared in the same manner, but adding a C-terminal Cys residue to each sequence by using Fmoc-Cys(Trt)-2-chlorotrityl chloride resin (Novabiochem/Merck). After cleavage, peptides were reacted with BODIPY-FL [N-(2-aminoethyl)maleimide] (1 eq. peptide/10 eq. dye) in 30% CH₃CN, 10 mM sodium phosphate buffer at pH 7.4, as described previously (Mattiuzzo et al., 2007). The labelled peptides were purified by reverse-phase high performance liquid chromatography (RP-HPLC) on a Phenomenex semi-preparative column (Jupiter™, C18, 5 μm, 300 Å, 100x10 mm) with a linear gradient from 10-30% of CH₃CN in 40 min, and the correct sequence verified by ESI-MS [Tur1A(Cys³³)-BY calculated MW=4489.8, measured MW=4489.9; Bac7(1-35)(Cys³⁶)-BY calculated MW=4310.3, measured MW=4310.7]. After lyophilisation from 10 mM HCl, the concentration of labelled peptide stock solution was determined by spectrophotometric determination of BODIPY ($\epsilon_{504} = 79000 \text{ M}^{-1} \text{ cm}^{-1}$ in MeOH) (Invitrogen Molecular Probes Handbook, section 2.2).

Tur1A fragments were synthesized by automated solid-phase peptide synthesis (SPPS) on a Whatman 50 cellulose membrane using a MultiPep RSI peptide synthesizer/pipetting robot (Intavis), the manual synthesis protocol was described in (Hilpert et al., 2007). Briefly, before robot synthesis, functionalisation of the cellulose membrane (10 cm x 15 cm) was carried out by overnight incubation in 0.2 M Fmoc-Gly-OH (Aldrich), 0.24 M N,N'-diisopropylcarbodiimide (DIC, Fluka) and 0.4 M N-methylimidazole (NMI, Aldrich) in dimethylformamide (DMF, VWR). Functionalisation was followed by Glycine deprotection in 20% piperidine (v/v, Acros Organics) in DMF (20 min + 10 min). Peptide synthesis at discrete spots addressed by the robot was performed using 9-fluorenyl-methoxycarbonyl/tert-butyl (Fmoc/*t*Bu) strategy. Fmoc amino acids [Bachem, 0.5 M stock solutions in N-methyl-2-pyrrolidone (NMP, VWR)] were pre-activated with equimolar quantities of 1-hydroxybenzotriazole hydrate (HOBt, Aldrich) and DIC (both 1.1 M stock solutions in NMP) and assembled in double coupling procedure (2x10 min) per cycle to ensure higher coupling efficiency at each amino acid position. Spotting volumes of 0.8 μl for the first cycle and 0.9 μl for the following cycles were used. After amino acid coupling cycle, unreacted residues were capped applying a 5 min acetic anhydride treatment (5% v/v in DMF, Fluka). Subsequent Fmoc cleavage was achieved using 20% (v/v) piperidine in DMF (2x 5 min). Final cleavage of amino acid side-chain protecting groups was carried out with 25 ml of 90% TFA (Acros Organics), 3% triisopropylsilane (TIPS, Acros Organics) and 2% water in dichloromethane (DCM, Acros Organics) for 30 min followed by a 120 min treatment with 25 ml of 50% TFA, 3% TIPS and 2% water in DCM. Peptide amides were cleaved from the solid support by incubating the membrane in a saturated ammonia gas atmosphere overnight. An internally standardised control peptide, and

1 individually chosen peptides from that synthesis, were used to determine SPOT synthesis yield and
2 quality. Individual SPOTs were punched-out with a one-hole-puncher, transferred into a sterile 96-
3 well round-bottomed polypropylene non-treated microtiter plate and dissolved overnight in 200 μ l
4 of autoclaved water. Peptide solution were quantified with a NanoDrop ND1000 spectrophotometry
5 at 280 nm. Dissolved peptides were further analysed by analytical RP-HPLC on a Shim-pack VP-
6 ODS column (120 Å, 150x4.6 mm, Shimadzu) using a LC2010AHT system (Shimadzu). The
7 binary solvent system contained 0.1% (v/v) TFA in H₂O (HPLC-grade, VWR, solvent A) and 0.1%
8 (v/v) TFA in acetonitrile (HPLC-grade, VWR, solvent B). A linear gradient of 5% to 70% solv B in
9 32.5 min with an initial 3 min isocratic equilibration was used at a flow rate of 1 ml/min. The purity
10 of the crude control peptides were between 37% and 68%. The remaining spots were then excised
11 from the membrane, placed in microtiter plates and the cleaved peptides resuspended overnight in
12 200 μ l sterile water, lyophilised and re-lyophilized from 200 μ l 10 mM HCl solution to remove
13 TFA. The final stock solutions, obtained by resuspending the pellets in 50 μ l of sterile water, were
14 quantified spectrophotometrically using a Nanodrop 2000 as described above.
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28 **Minimum inhibitory concentration determination**

29 Bacterial cultures were grown overnight to the stationary phase, diluted in fresh Mueller-
30 Hinton broth (MHB) and incubated to an OD₆₀₀ \approx 0.3 at 37°C under agitation (if required, in the
31 presence of antibiotic) and diluted to 5×10^5 colony forming units (CFU)/ml in medium.
32 Antimicrobial agents, diluted in MHB to a concentration of 128 μ M, were added to the first
33 wells of a round-bottom microtiter plate and then serially two-fold diluted with MHB into
34 successive wells in a final volume of 50 μ l. Subsequently, 50 μ l of the bacterial suspension was
35 added to each well, to a final load of 2.5×10^4 CFU/well and reducing by half the antibacterial
36 compound concentration in each well. The plate was sealed to minimise evaporation and
37 incubated overnight at 37°C. The MIC was calculated as the lowest compound concentration
38 inhibiting visible bacterial growth.
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51 **Flow cytometry**

52 The integrity of bacterial cell membranes was assessed by measuring the Propidium iodide (PI)
53 uptake *via* flow cytometric assays, performed with a Cytomics FC 500 (Beckman-Coulter),
54 acquiring 10^4 bacterial cells for each measurement. as described previously (Benincasa et al., 2009;
55 Guida et al., 2015). Briefly, mid-log phase bacterial cultures, diluted to 1×10^6 CFU/ml in MHB,
56 were incubated at 37°C for different times with increasing concentrations of peptides, in the
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1 presence of propidium iodide (PI) at a final concentration of 10 µg/ml. Membrane damage was
2 assessed in terms of the % of PI positive cells. For evaluation of peptide uptake, bacterial cultures in
3 mid-log phase were diluted to 1×10^6 CFU/ml in MHB and incubated at 37°C for different times
4 with different concentrations of BOPIDY (BY) fluorescently marked peptides [Tur1A(Cys)³³-BY or
5 Bac7(1-35)(Cys)³⁶-BY] and analysed as described previously (Benincasa et al., 2009; Guida et al.,
6 2015)). Analyses were carried out after extensive washing only, or after washing and addition of
7 Trypan Blue (TB) quenching the fluorescence of the peptide bound to the bacterial surface
8 (Benincasa et al., 2009). Data analysis was performed with the FCS Express3 software (De Novo
9 Software). Data are expressed as the mean \pm standard deviation (S.D.).
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19 ***In vitro* transcription/translation and translation in *E. coli***

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21 For *in vitro* transcription/translation assays, the RTSTM 100 *Escherichia coli* HY (Biotech Rabbit)
22 was used for testing all samples. 0.1 µl of RNase inhibitor (RNasin®, 20-40 U/µl, Promega) and
23 1 µl of peptide solution were added to 5 µl of RTS reaction mix (containing either mRNA or DNA
24 template encoding the *Photinus pyralis* luciferase protein), with a final peptide concentration of
25 1 µM, 10 µM or 100 µM as required. After incubation for 1 h at 30°C with shaking (750 rpm), 2 µl
26 of each reaction were mixed with 8 µl kanamycin (50 mg/ml) to stop the process and 40 µl of
27 Luciferase assay substrate solution (Promega) and then transferred into a white 96-well, flat bottom
28 microtiter plate (Greiner). In positive controls, nuclease-free water was added instead of peptide
29 solution. Negative controls contained nuclease-free water instead of both the peptide solution and
30 mRNA/DNA template. The activity of the reporter protein was assessed and quantified using a
31 Tecan Infinite M1000 plate reader. Relative values were calculated as a percentage of the positive
32 control. The *in vitro* translation assay was performed using the *PURExpress* System (NEB).
33 Reactions were performed according to the manual at 37°C for up to half an hour. Luminescence
34 was determined as described above for the *in vitro* transcription/translation assay
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50 ***In vitro* translation on *Thermus thermophilus* ribosomes**

51 The *in vitro* translation on *T. thermophilus* ribosomes was performed using an S12 lysate, prepared
52 based on protocol described for *E. coli* (Kim et al., 2006) Huter et al., 2017) with minor variations.
53 *T. thermophilus* strain HB8 (DSM-579) were grown in 1 x YT medium to an OD₆₀₀ \approx 0.6 at 70°C,
54 centrifuged at 5000 x g at 4°C for 15' and washed three times with Buffer A (10 mM Tris-acetate
55 buffer (pH 8.2), 14 mM MgOAc, 60 mM KOAc, 1 mM DTT, 6 mM β -mercaptoethanol). The cell
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1 pellet was flash-frozen in liquid nitrogen and stored at -80°C until use. The pellet was thawed,
2 resuspended in Buffer A and cells were lysed by three passages through an M-110L Microfluidizer
3 Processor (Microfluidics) at >15000 psi. Cell debris was removed by centrifuging at 12000 x g for
4 10 min at 4°C. The cleared supernatant was aliquoted, flash frozen in liquid nitrogen and stored at -
5 80°C. To perform the *in vitro* translation, 6.75 µL of cell lysate and 1 µL of peptide solution (to a
6 final concentration of 1 µM, 10 µM or 100 µM) were added to a reaction mixture consisting of 240
7 mM HEPES-KOH, pH 8.0, 0.6 mM PEG8000, 60 mM glucose, 4.4 µg tRNA mix, 1.2 mM ATP,
8 1.2 mM GTP, 0.85 µg folinic acid, 1 mM DTT, 90 mM potassium glutamate; 80 mM ammonium
9 acetate, 20 mM K₂HPO₄, 1.8 mM of each amino acid, 12.6 mM magnesium acetate and 200 ng of
10 custom made firefly luciferase mRNA, to a final volume of 25 µL. Samples were incubated at 30°C
11 (due to heat sensitivity of the reporter Fluc protein) for 1 h with shaking (550 rpm) and 8 µL from
12 each sample were then mixed with 2 µL kanamycin (50 mg/ml) to block further translation, and 40
13 µL of Luciferase assay substrate (Promega) in the wells of a white 96-well flat bottom microtiter
14 plate (Greiner). The luminescence was measured using a Tecan Infinite M1000 plate reader.
15 Relative values were determined with respect to positive control in the absence of peptide and
16 defined as 100%.
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32 **Toe-printing assay**

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34 For toe-printing assays, the PURExpress *in vitro* transcription/translation kit (NEB) was used with
35 an H-ns-PPP template, comprising the N-terminal 1-36 nucleotides of *E. coli hns* (Uniprot-
36 P0ACF8) gene but modified such that residues 20-22 are replaced by three proline residues (5'-
37 ATTAATTACGACTCACTATAGGGATATAAGGAGGAAAACAT**ATGAGCGAAGCACTTAA**
38 AATTCTGAACAACCTGCGTACTCTTCGTGCGCAGGCAATT**CCGCCGCCG**CTTGAAACG
39 CTGGAAGAAATGCTGGAAAAATTAGAAGTTGTTGTTTAAAGTGATAGAATTCTATCGTT
40 **AA**TAAGCAAATTCATTATAACC-3', start-, PRO₃- and stop-codons respectively are in
41 underlined bold). Reactions were set up by mixing 2 µL Solution A, 1.5 µL Solution B, 1 µL (0.5
42 pmol) of H-ns-PPP template 0.1 µL of RNasin® (20-40 U/µl, Promega), 1 µL of peptide (to a final
43 concentration of 1 µM, 10 µM or 100 µM) or 1 µL of antibiotic (to a final concentration of 100 µM
44 for thiostrepton or 50 µM for edeine) in PCR tubes. The control contained only nuclease-free water
45 instead of peptide or antibiotic. Samples were incubated for 15 min at 37°C for
46 transcription/translation, under agitation (550 rpm), cooled on ice for 5 min and then equilibrated at
47 RT for 2 min. 1 µL (2 pmol) of Alexa647-labelled NV-1 toe-print primer (5'-
48 GGTTATAATGAATTTTGCTTATTAAC-3') was then added to each reaction and samples were
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1 incubated for 5 min at 37°C. For the reverse transcription, 0.5 µl of AMV reverse transcriptase
2 (NEB), 0.1 µl dNTP mix (10 mM) and 0.4 µl Pure System Buffer were added to each reaction and
3 samples were incubated for 20 min at 37°C. To stop the reaction and degrade RNA, 1 µl of 5M
4 NaOH was added to each reaction and samples were incubated for 15 min at 37°C. After
5 neutralizing with 0.7 µl HCl 25% (v/v) and 20 µl of toe-print resuspension buffer, samples were
6 purified using the QIAquick Nucleotide Removal Kit (Qiagen), adding to samples 200 µl of PN1
7 buffer and following the supplier's instructions. DNA was eluted using 80 µl of RNase-free water,
8 dried in a vacuum centrifuge and re-suspended in 4 µl of formamide-loading dye. Samples were
9 heated for 5 min at 95°C, then separated by electrophoresis on a 6% polyacrylamide gel (19:1
10 acrylamide: bisacrylamide) containing 7 M urea, at 2000 V. Gels were scanned using a Typhoon
11 FLA9500 imaging system (GE Healthcare). Sequencing was carried out by mixing 1 µL (0.5-0.8
12 pmol) of DNA template, 5 µL of Sequencing buffer, 9 µL of nuclease-free water, 1 µL (10 pmol) of
13 Alexa647-labelled NV-1 toe-print primer and 1 µL of Hemo Klen Taq polymerase. To 4 µL
14 aliquots of this mix were then respectively added 2 µL of ddATP, ddTTP, ddGTP or ddCTP, and
15 the mixtures incubated in a thermocycler [2 sec 95°C, 30 x (30 sec 95°C, 30 sec 42°C, 1 min 70°C),
16 1 min 70°C, 8°C storage]. Samples were then heated and loaded on a gel as indicated for the toe-
17 print assay.
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31 **Purification of *T. thermophilus* 70S ribosomes**

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36 *T. thermophilus* 70S ribosomes were purified as described previously (Selmer et al., 2006) and
37 resuspended in buffer containing 5 mM HEPES-KOH, pH 7.5, 50 mM KCl, 10 mM NH₄Cl, and 10
38 mM Mg(CH₃COO)₂ to yield a final concentration of 26-32 mg/mL. For storage, *T. thermophilus*
39 70S ribosomes were flash frozen in liquid nitrogen and kept at -80 °C.
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46 **Purification of YfiA**

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48 YfiA was expressed from a pGS21A expression vector in BL21 Star RARE cells as described
49 previously (Polikanov et al., 2014). The culture was grown at 37°C (220 rpm) to an OD₆₀₀ of 0.6
50 and induced with 1 mM IPTG for 4 hours. Cells were harvested and lysed with a French press three
51 times at 15000 psi in 20 mM Tris-HCl pH 7.6, 100 mM NH₄Cl, 10 mM MgCl₂ and 1 mM β-
52 mercaptoethanol. The lysate was centrifuged at 50000 x g for 1 hour and the YfiA protein was
53 purified on a Ni-NTA matrix using a linear gradient from 0-1 M imidazole in 20 mM Tris-HCl pH
54 7.6, 100 mM NH₄Cl, 10 mM MgCl₂ and 1 mM β-mercaptoethanol. Following size exclusion
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1 chromatography using a Superdex 75 column (GE healthcare) in 20 mM Tris-HCl pH 7.6, 100 mM
2 NH₄Cl, 10 mM MgCl₂, 1 mM β-mercaptoethanol, YfiA was concentrated to 87 mg/mL and flash
3 frozen in liquid nitrogen.
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6 7 8 ***T. thermophilus* 70S-YfiA-Tur1A Complex formation** 9

10 A ternary complex was formed by first incubating a mixture of 5 μM *T. thermophilus* 70S
11 ribosomes and 50 μM YfiA at 37°C for 10 min, followed by the addition of 50 μM Tur1A and
12 further incubation at room temperature for at least 15 min. The complex was then centrifuged
13 briefly before use for crystallization. The final sample buffer prior to crystallization contained 5
14 mM HEPES-KOH, pH 7.6, 50 mM KCl, 10 mM NH₄Cl and 10 mM Mg(CH₃COO)₂.
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21 22 **Crystallization of the *T. thermophilus* 70S-YfiA-Tur1A complex** 23

24 Published conditions were used as a starting point for screening crystallization conditions by vapor
25 diffusion in sitting-drop trays at 20°C (Polikanov et al., 2014; Selmer et al., 2006). Crystallization
26 drops consisted of 3 μl of ternary *T. thermophilus* 70S-YfiA-Tur1A complex and 3-4 μl of reservoir
27 solution containing 100 mM Tris-HCl, pH 7.6, 2.9% (v/v) PEG 20,000, 7-10% (v/v) 2-methyl-2,4-
28 pentanediol (MPD) and 175 mM L-arginine. Crystals appeared within 2-3 days and grew to approx.
29 1000 × 100 × 100 μm within 7-8 days. For cryoprotection, the concentration of MPD was increased
30 in a stepwise manner to yield a final concentration of 40% (v/v). The ionic composition during
31 cryoprotection was 100 mM Tris-HCl, pH 7.6, 2.9% (v/v) PEG 20000, 50 mM KCl, 10 mM NH₄Cl
32 and 10 mM Mg(CH₃COO)₂. Back soaking of the peptide was prevented by including 50 μM Tur1A
33 in the final cryoprotection solution. Following overnight incubation at 20°C, crystals were flash
34 frozen in a nitrogen cryostream at 90 K for subsequent data collection.
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47 48 **Data collection and processing** 49

50 Diffraction data were collected at beamline ID23-1 of the European Synchrotron Radiation Facility
51 (ESRF) in Grenoble, France. A complete dataset was obtained by merging 0.1° oscillation data
52 collected at 100 K with a wavelength of 0.97625 Å from multiple regions of the same crystal. Initial
53 data processing, including integration and scaling, was performed with XDS (Kabsch, 2010). The
54 data collected could be indexed in the *P*2₁2₁2₁ space group, with unit-cell dimensions around 210
55 Å × 450 Å × 625 Å and an asymmetric unit containing two copies of the *T. thermophilus* 70S
56 ribosome.
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1 2 **Model building and refinement**

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4 A high-resolution structure of the apo *T. thermophilus* 70S ribosome crystallized in the same space
5 group and similar unit cell as the crystal used in this study (PDB 4Y4O) was used directly for rigid
6 body refinement in *Phenix* (Adams et al., 2010). Rigid bodies comprised four domains from the
7 small 30S subunit (head, body, spur and helix h44) and three domains from the large 50S subunit
8 (body, L1 stalk and the N-terminus of ribosomal protein L9). Restrained crystallographic
9 refinement consisting of multiple cycles of positional and individual *B*-factor refinement was then
10 carried out using the *Phenix* package. Non-crystallographic symmetry restraints between the two
11 copies of the *T. thermophilus* 70S ribosome in the asymmetric unit were also applied during
12 refinement. After confirming that density corresponding to the Tur1A peptide was visible inside the
13 exit tunnel in a minimally biased F_o-F_c map, a model of Tur1A was built manually in *Coot* (Emsley
14 and Cowtan, 2004). The sidechains of Arg1 and Arg4 of Tur1A showed no noticeable density in the
15 original F_o-F_c map or in the $2F_o-F_c$ map obtained after complete refinement of the structure and
16 therefore were not included in the final model. Further refinement and model validation were
17 carried out in *Phenix* and on the MolProbity server (Chen et al., 2010), respectively.
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31 32 33 **QUANTIFICATION AND STATISTICAL ANALYSIS**

34 35 ***In vitro* data analysis**

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37 Data are presented as mean values +/- the standard deviation (SD) calculated from independent
38 experiments using the software Excel (Microsoft). The number of experimental and technical
39 replicates for each experiment is also described in each individual figure legend. The statistical
40 significance has been calculated using the Student-Newman-Keuls Multiple Comparisons Test,
41 ANOVA (*= $p < 0.05$; **= $p < 0.01$), with the software GraphPad InStat 3. For flow-cytometry
42 experiments, data analysis was performed with the FCS Express3 software (De Novo Software).
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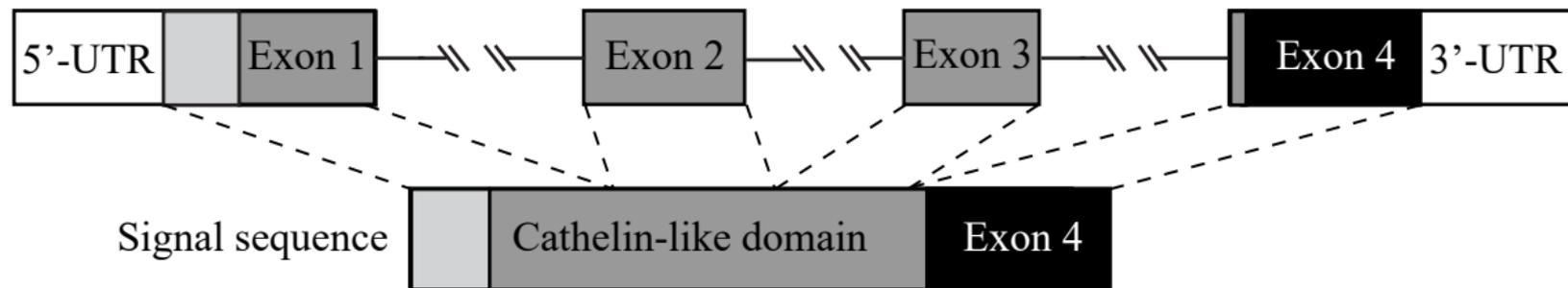
51 **DATA AND SOFTWARE AVAILABILITY**

52 53 **Accession numbers**

54
55 The atomic coordinates for the Tur1A-70S complex have been deposited in the PDB with the
56 accession number 6FKR.
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Figure 1

A



B

Peptide	Organism	Sequence alignment	%Id ^a	Charge
PR-39	<i>S. scrofa</i>	RR-RPRPPYLPRPRPPFFFPRL---PPRIPPGFPPRFPPRFPP	58 (32)	+10
Bac7(1-35)	<i>B. taurus</i>	RRIRPRPPRLPRPRPRPLPFFPRPGRRPIPRPLPFP-----	59 (40)	+10
Tur1A	<i>T. truncatus</i>	RRIRFRPPYLPRPGRRPRFPFPF---PIPRIPRIP-----	100 (53)	+10
Tur1B	<i>T. truncatus</i>	RRIPFWPPNWPGEWLPWSPPDF---RIPRILRKR-----	53 (100)	+6
Indolicidin	<i>B. taurus</i>	-----ILPWKWPWWPWRR-----	23 (38)	+3

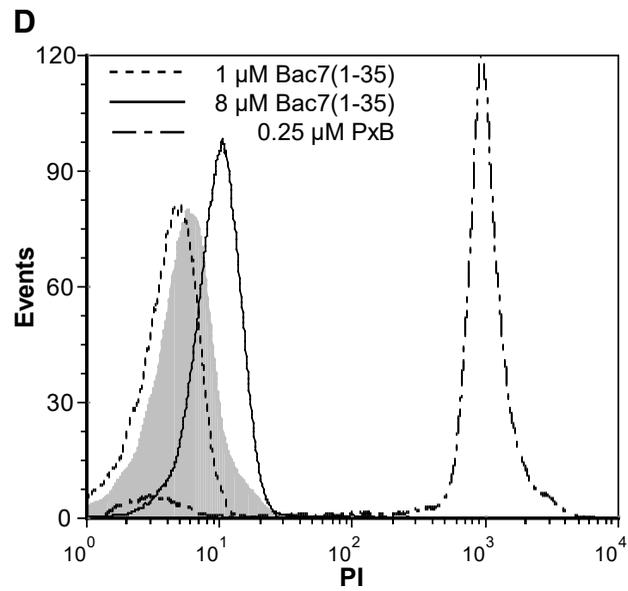
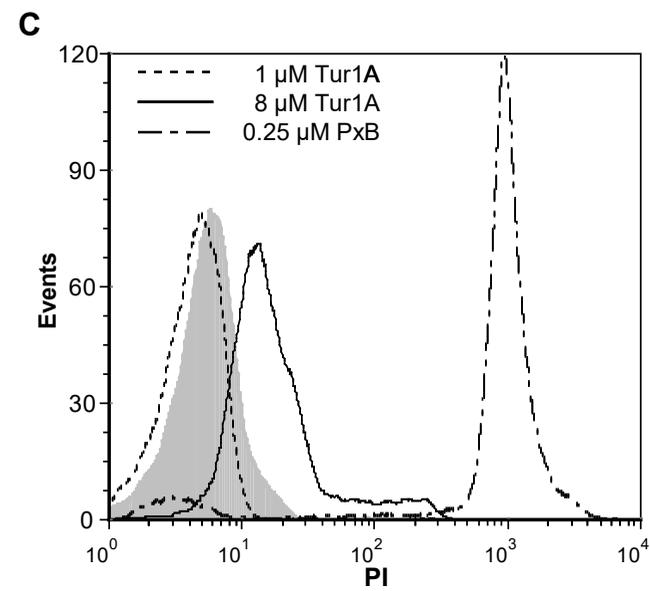
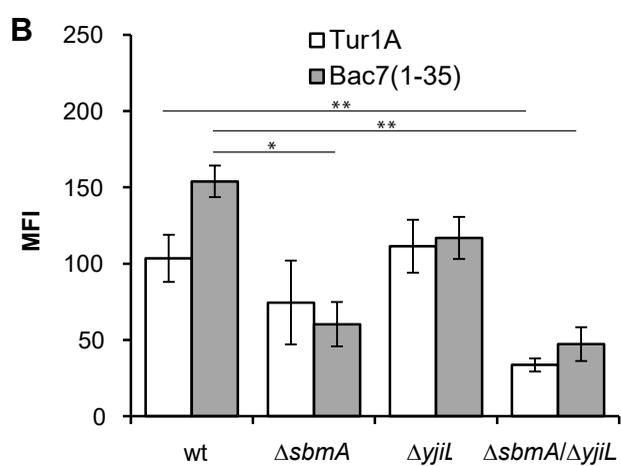
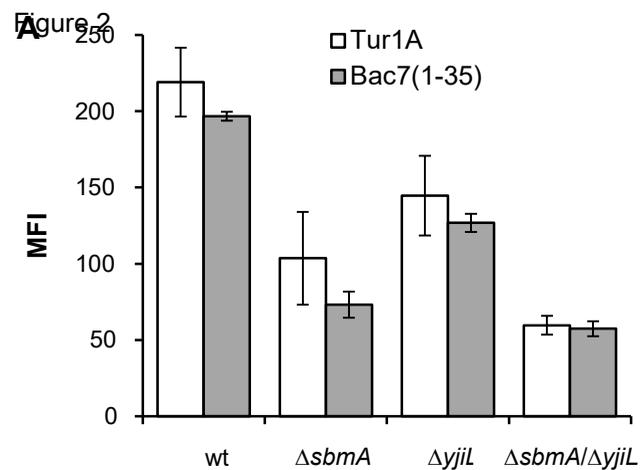


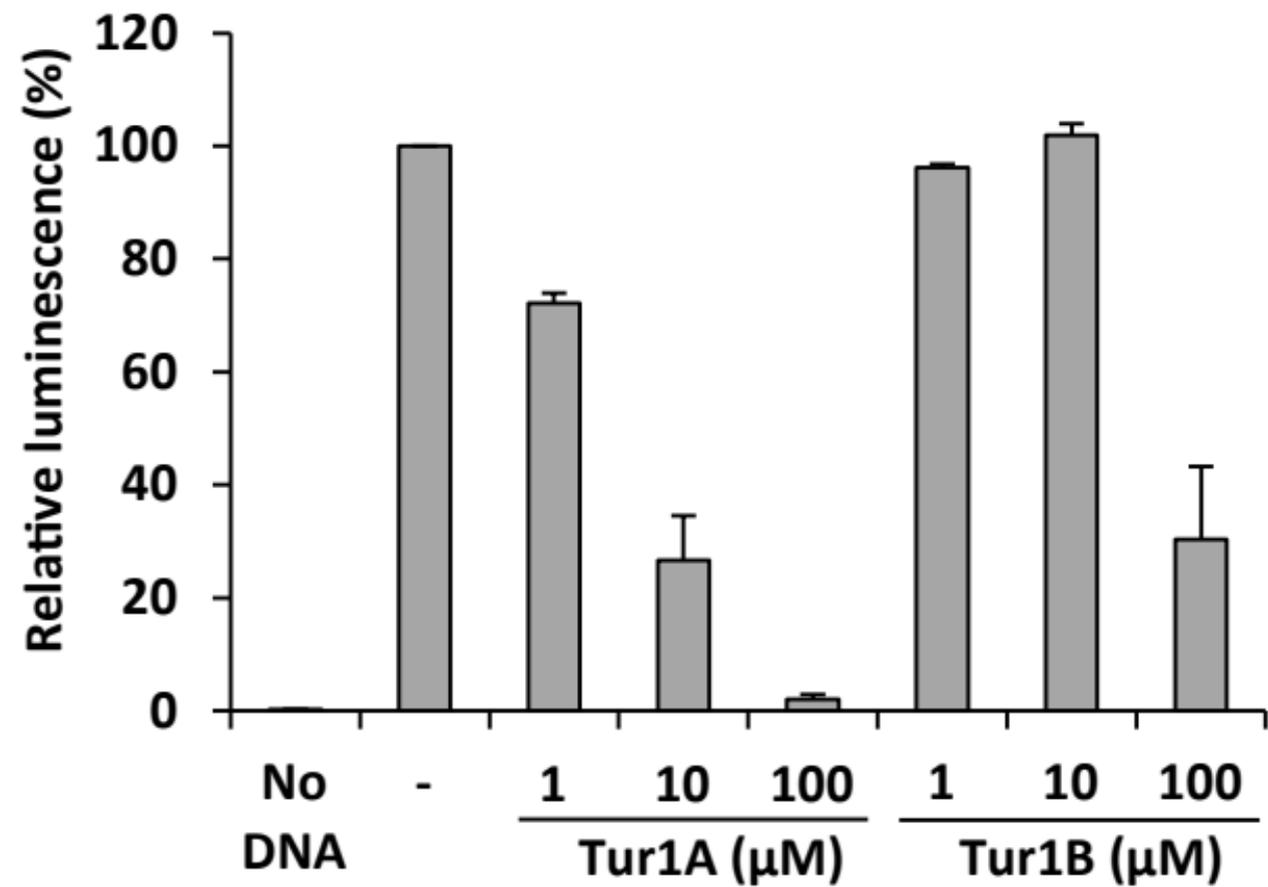
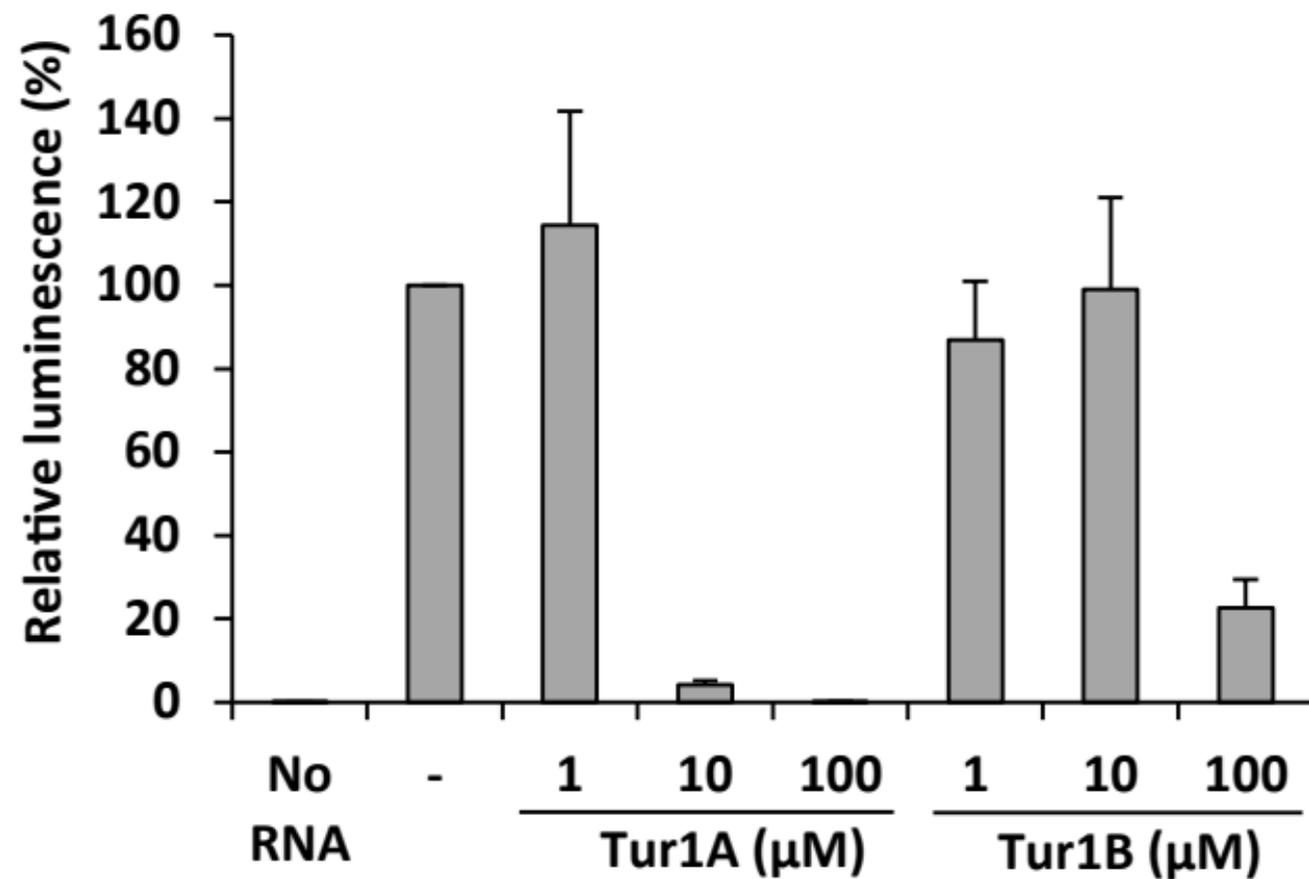
Figure 3**B**

Figure 4

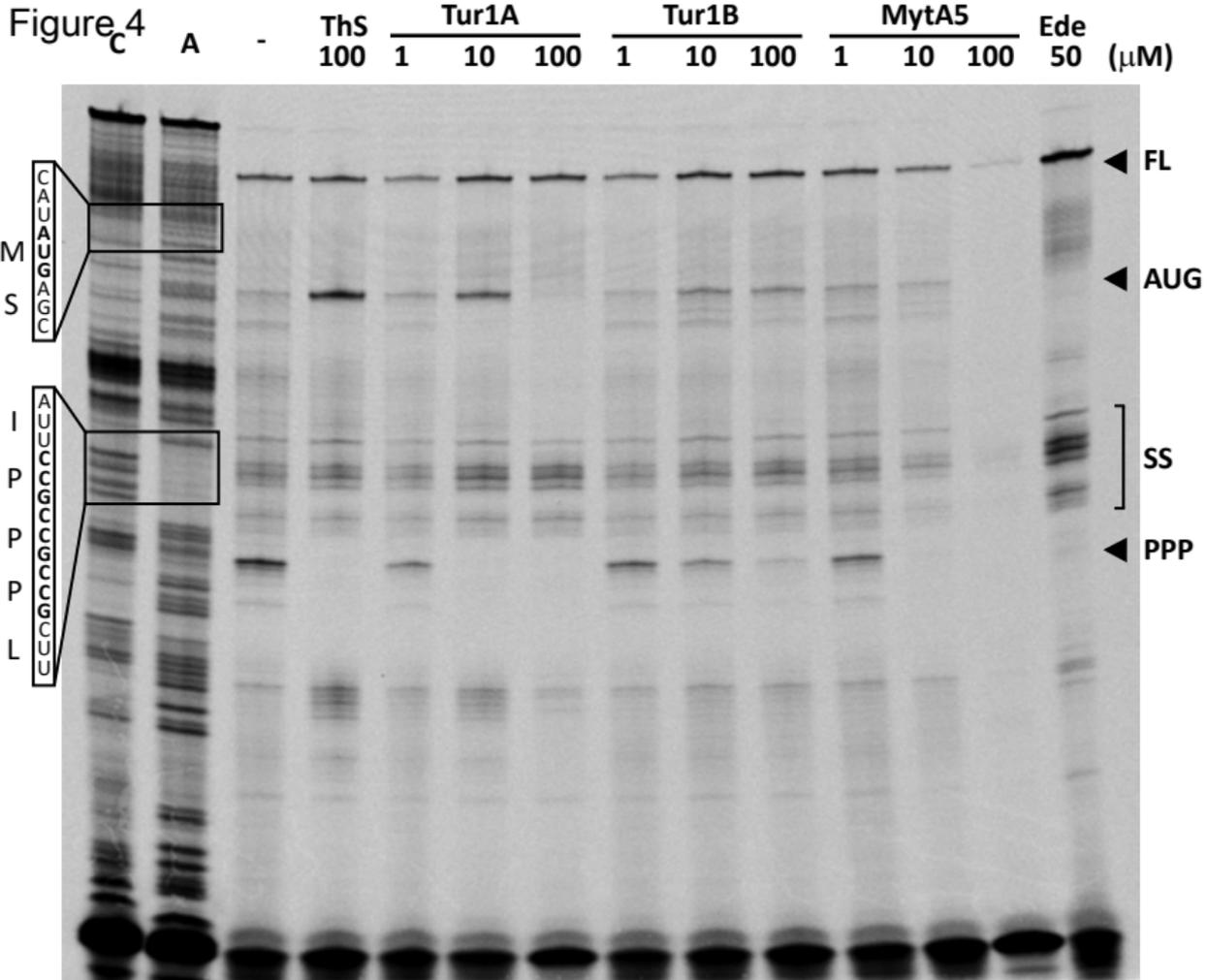
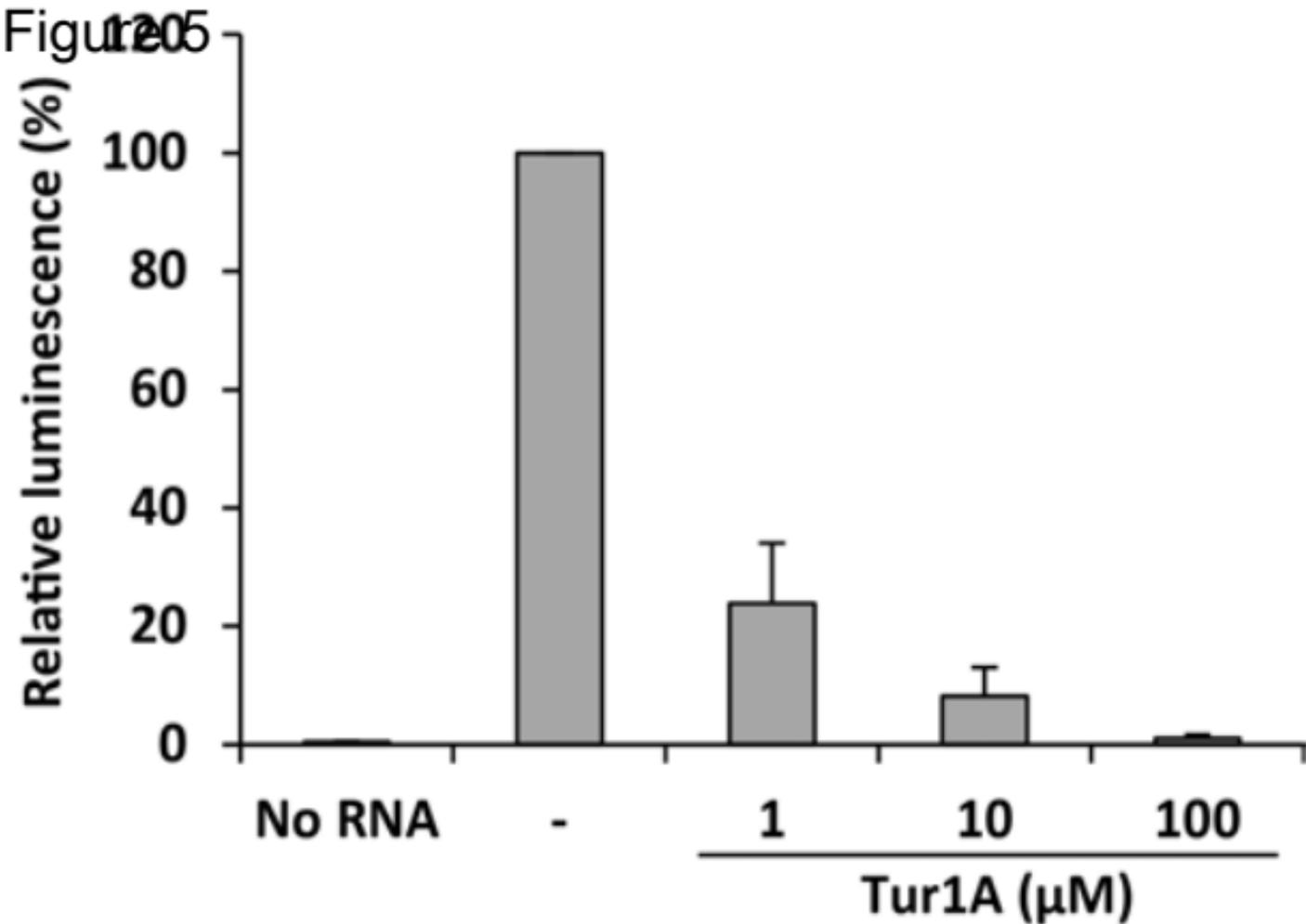
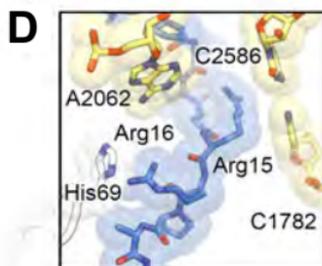
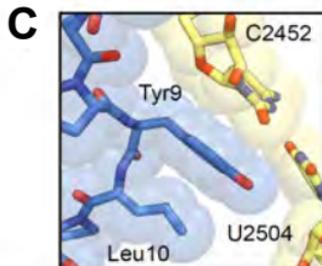
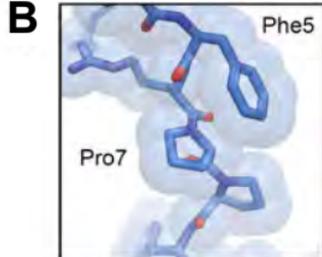
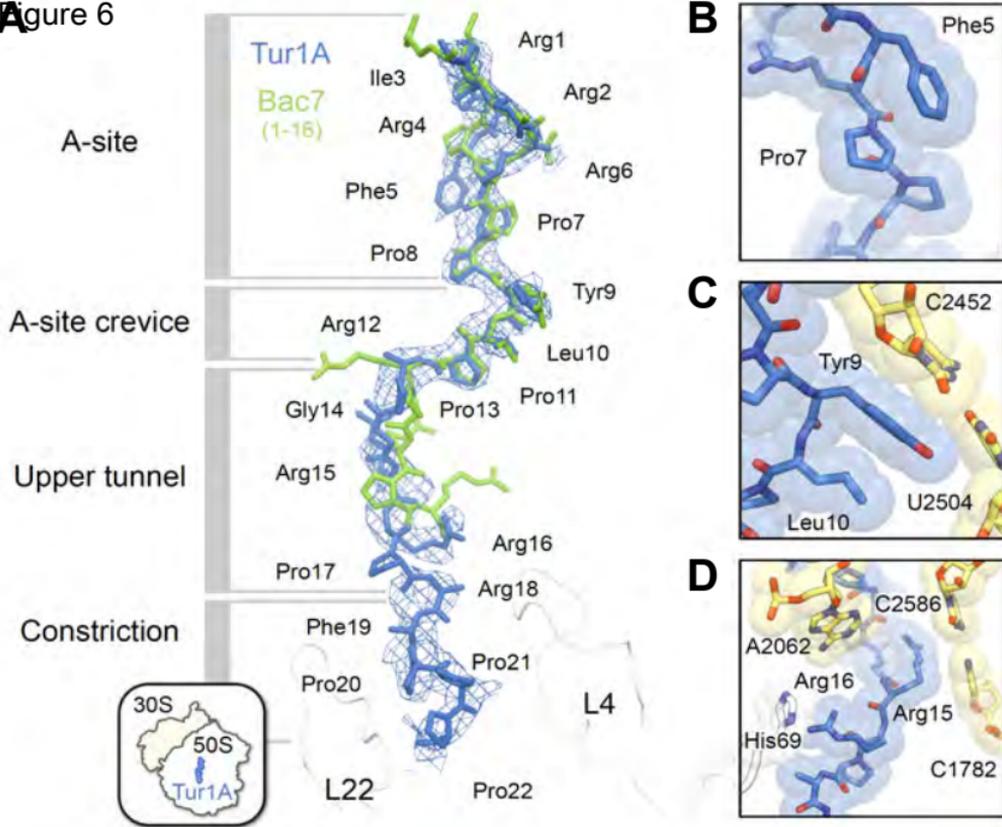
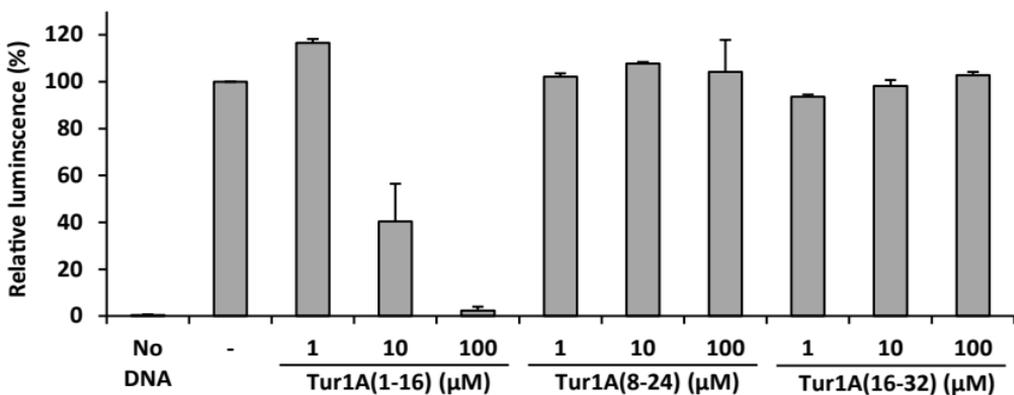


Figure 1205



A Figure 6**E**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial Strains		
<i>Escherichia coli</i> K12 BW25115	Keio collection (Baba et al., 2006)	N/A
<i>Escherichia coli</i> K12 BW25113 Δ <i>sbmA</i>	Keio collection (Baba et al., 2006)	Strain number JW0368-1
<i>Escherichia coli</i> K12 BW25113 Δ <i>yjiL</i>	Keio collection (Baba et al., 2006)	Strain number JW5785-1
<i>Escherichia coli</i> K12 BW25113 Δ <i>yjiL</i> / Δ <i>sbmA</i>	(Krizsan et al., 2015)	N/A
<i>Escherichia coli</i> N281	(Wittmann et al., 1973)	N/A
<i>Escherichia coli</i> N282	(Wittmann et al., 1973)	N/A
<i>Escherichia coli</i> AB301	(Bouck and Adelberg, 1970)	N/A
<i>Escherichia coli</i> SQ110 Δ <i>tolC</i>	(Florin et al., 2017)	N/A
<i>Escherichia coli</i> SQ110 Δ <i>tolC</i> A2059C	(Florin et al., 2017)	N/A
<i>Escherichia coli</i> SQ110 Δ <i>tolC</i> A2059G	(Florin et al., 2017)	N/A
<i>Escherichia coli</i> SQ110 Δ <i>tolC</i> A2305C	(Florin et al., 2017)	N/A
<i>Thermus thermophilus</i> HB8	DSMZ	Catalog # DSM-579
BL21 Star	ThermoFisher	Catalog # C601003
Biological Samples		
<i>Tursiops truncatus</i> muscle tissue	Mediterranean Marine Mammal Tissue Bank	N/A
tRNA from <i>Escherichia coli</i> MRE600	Roche	Catalog # 10109550001
<i>Thermus thermophilus</i> 70S ribosomes	This study	N/A
Chemicals, Peptides, and Recombinant Proteins		
YfiA protein	This study	N/A
Tur1A	This study	N/A
Tur1B	This study	N/A
Tur1A(Cys ³³)-BY	This study	N/A
Bac7(Cys ³⁶)-BY	This study	N/A
Myticalin A5	(Leoni et al., 2017)	N/A
Tur1A(1-16)	This study	N/A
Tur1A(8-24)	This study	N/A
Tur1A(16-32)	This study	N/A
Kanamycin	Sigma	Catalog # 60615
Erythromycin	Sigma	Catalog # E6376
Spectinomycin	Sigma	Catalog # S4014
Thiostrepton	Sigma	Catalog # T8902
Edeine	N/A	N/A
Fmoc-Arg(Pbf)-NovaSyn® TGA	Novabiochem	Catalog # 856042
Trifluoroacetic acid (TFA)	Sigma	Catalog # 91700
3,6-dioxa-1,8-octanedithiol (DODT)	Sigma	Catalog # 465178
Thioanisole	Sigma	Catalog # 88470
Triisopropylsilane (TIPS)	Sigma	Catalog # 233781
N,N'-diisopropylcarbodiimide (DIC)	Fluca	N/A
N-methylimidazole	VWR	Catalog # AAA12575-22
Dimethylformamide (DMF)	VWR	Catalog # BDH1117-4LG
Piperidine	Acros Organics	Catalog # P/3520
BDP FL maleimide	Lumiprobe	Catalog # 21480
N-Methyl-2-Pyrrolidinone	VWR	Catalog # CA71007-814

1-hydroxybenzotriazole hydrate (hOBt)	Sigma	Catalog # 54802
Prpidium iodide	Sigma	Catalog # P4170
RNasin	Promega	Catalog # N2511
rNTPs	Sigma	Catalog # 27-2025-01
PEG-8000	Sigma	Catalog # 1546605
AMV reverse transcriptase	NEB	Catalog # M0277
Hemo Klen Taq	NEB	Catalog # M0332
ddNTPs	Sigma	Catalog # GE27-2045-01
2-methyl-2,4-pentanediol (MPD)	Sigma	Catalog # 000000008208191000
Critical Commercial Assays		
E.Z.N.A.® Mollusc DNA Kit	Omega Bio-Tek	Catalog # D3373
Perfectprep® Gel Clean-up Kit	Eppendorf	N/A
RTS™ 100 <i>Escherichia coli</i> HY	Biotech Rabbit	Catalog # BR1400101
Luciferase Assay System	Promega	Catalog # E1500
PURExpress <i>in vitro</i> transcription/translation kit	NEB	Catalog # E6800S
QIAquick Nucleotide Removal Kit	Qiagen	Catalog # 28304
Deposited Data		
The crystal structure of the Tur1A-70S complex	This study	PDB ID 6FKR (https://www.rcsb.org/structure/6fkr)
Oligonucleotides		
Tur1A FW 5'-CTTGTGACCCTGGGA-3'	Eurofins Genomics	N/A
Tur1A RV 5'-ATAACTTCCTCCAGGCTTCA-3'	Eurofins Genomics	N/A
NV-1 5'GGTTATAATGAATTTTGCTTATTAAC-3'	Thermo-Fisher	N/A
Recombinant DNA		
H-ns-PPP	This study	N/A
pGS21A	(Polikanov et al., 2014)	N/A
2 <i>XermCL_S10K</i>	(Arenz et al., 2016)	N/A
Software and Algorithms		
Genomics Workbench 5.1 (CLCbio)	QIAGEN bioinformatics	https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/
FCS Express3 software	De Novo Software	https://www.denovosoftware.com/site/DownloadLanding.shtml
XDS	(Kabsch, 2010)	http://xds.mpimf-heidelberg.mpg.de/html_doc/downloading.html
Phenix	(Adams et al., 2010)	https://www.phenix-online.org/download/

Coot	(Emsley and Cowtan, 2004)	https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/
Other		
Hitachi 3130 Genetic Analyzer	Applied Biosystems	N/A
Biotage Initiator+ Alstra	Biotage	Catalog # 356017
Kinetex, C18, 2.6 μm , 100 \AA , 50x4,6 mm	Phenomenex	Catalog # 00A-4462-E0 00
Esquire 4000	Bruker Daltonics	N/A
NanoDrop2000	Thermo Fisher Scientific	Catalog # ND-2000
Jupiter™, C18, 5 μm , 300 \AA , 100x10 mm	Phenomenex	Catalog # 00G-4053-E0
MultiPep RSi	Intavis	N/A
Shim-pack VP-ODS column (120 \AA , 150x4.6 mm.)	Shimadzu	N/A
Cytomics FC 500	Beckman-Coulter	N/A
Tecan Infinite M1000	Tecan	N/A
M-110L Microfluidizer Processor	Microfluidics	N/A
Typhoon FLA9500	GE Healthcare	Catalog # 29187191
Protino Ni-NTA agarose beads	Macherey-Nagel	Catalog # 745400
Superdex HiLoad S75 16/600	GE Healthcare	Catalog # 28989333

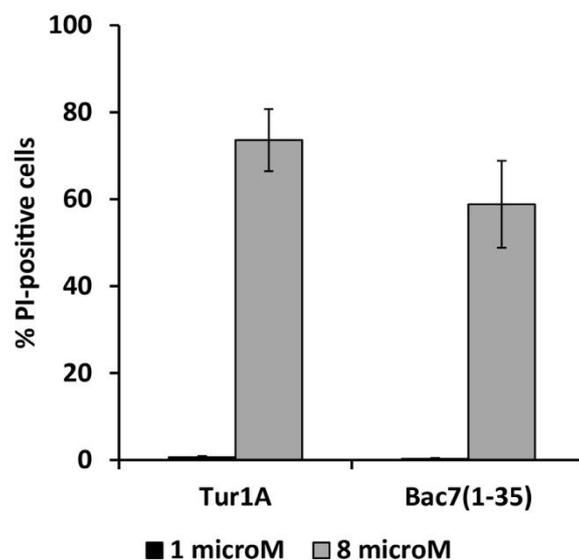
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Figure S1, related to Figure 2. Flow-cytometry evaluation of Tur1A membrane permeabilization on BW25113 *E. coli* cells. Propidium iodide-uptake in *E. coli* BW25113 cells after 15 min treatment with 1 μ M and 8 μ M Tur1A or 1 μ M and 8 μ M μ M Bac7(1-35). Error bars represent the standard deviation calculated on three and five independent experiments performed for both peptides at 1 μ M and 8 μ M, respectively. 10^4 cells (events) were read for each measurement.

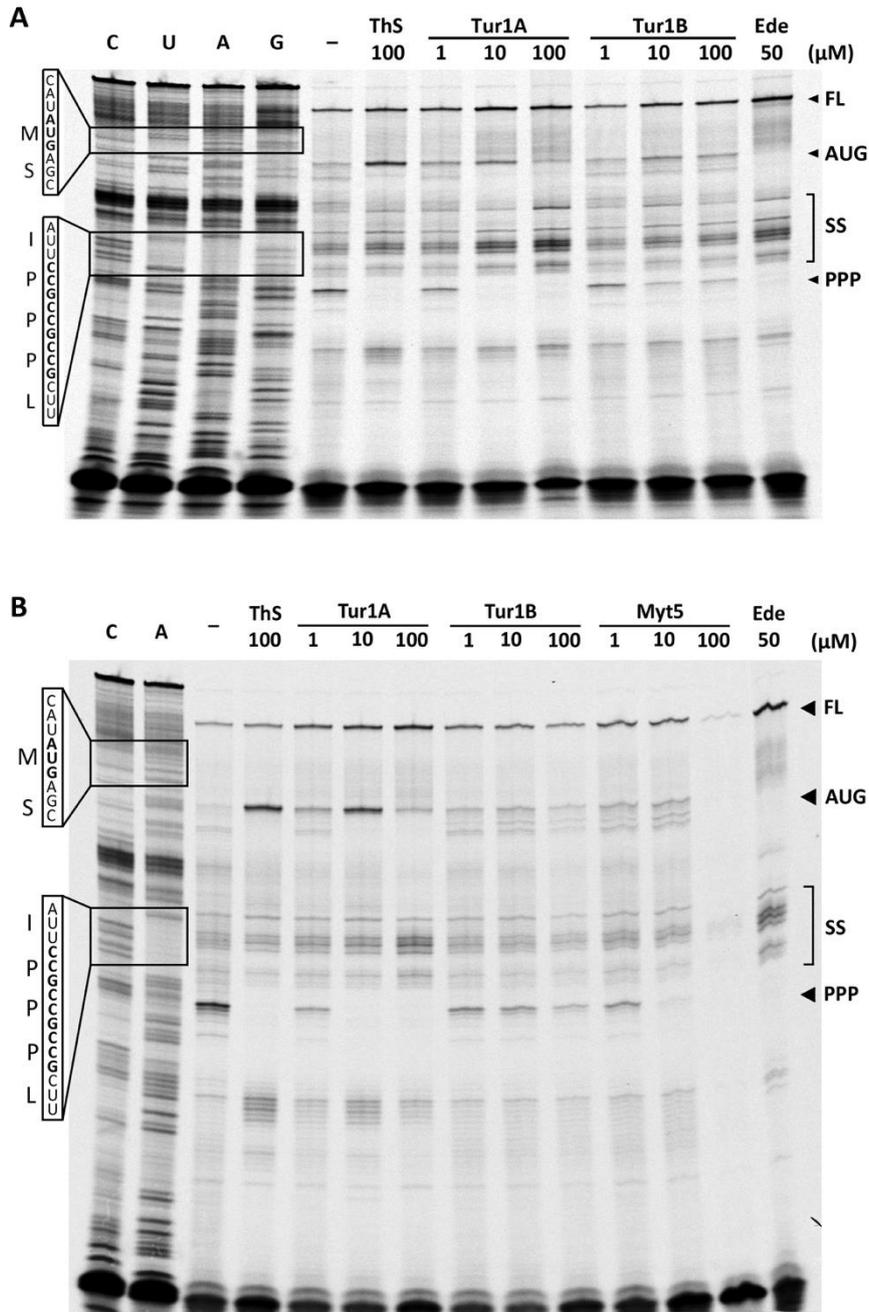


Figure S2, related to Figure 4. Toe-print evaluation of Tur1A and Tur1B effect on ribosomal function, at increasing concentrations. (A,B) Fluorescence scan of a polyacrylamide gel analysis of toe-printing reactions performed in the absence (-) or presence of 100 μM thiostrepton (ThS), 1-100 μM Tur1A and Tur1B, 50 μM edeine (Ede) and in (B) also of 1-100 μM Myticalin A5. Toe-print signals corresponding to ribosomes stalled at the AUG start codon or at the polyproline stretch (PPP) are indicated with arrows, as is the reverse transcription product of the full-length mRNA (FL). Sequencing lanes (C, U, A and G in (A) and C, A in (B)) are included for reference.

Table S1, related to Figure 6. Data collection and refinement statistics

Tur1A-70S complex

Data collection

Space group	$P2_12_12_1$
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	209.68, 449.24, 621.91
α , β γ (°)	90.00, 90.00, 90.00
Resolution (Å)	49.8(3.3)
R_{sym} or R_{merge}	67.8(206.1)
$I / \sigma I$	5.21(1.01)
Completeness (%)	98.5(98.9)
Redundancy	7.0(7.2)

Refinement

Resolution (Å)	3.3
No. reflections	858,052
$R_{\text{work}} / R_{\text{free}}$	0.19 / 0.25
No. atoms	
Protein/nucleic acid	289,856
Ligand/ion	2,702
Water	0
<i>B</i> -factors	
Protein/nucleic acid	42.5
R.m.s. deviations	
Bond lengths (Å)	0.03
Bond angles (°)	2.05

Table S2, related to Figure 6. Sensitivity of erythromycin resistant *E. coli* strains to PrAMPs.

	MIC (μ M) on <i>E. coli</i> strains						
	N281 (L4)	N282 (L22)	AB301 (wt)	SQ110 Δ tolC (wt)	SQ110 Δ tolC A2059C	SQ110 Δ tolC A2059G	SQ110 Δ tolC A2503C
Tur1A	1	1	1	0.5	0.5	0.5	0.5
Tur1B	8	10.7 (\pm 4.6)	8	8	8	8	6.7 (\pm 2.3)
Bac7 (1-35)	2.7 (\pm 1.1)	2.7 (\pm 1.3)	4	1	1	1	1
Api137	8.0	>32	2.3 (\pm 1.3)	0.8 (\pm 0.3)	>32	2	>32
Ery*	1024	1024	112 (\pm 32)	2,0	2048	2048	2048

Data represent the average and the standard deviation (\pm) calculated on three independent experiments (n=3). *Ery stands for erythromycin.

Supplementary Information consists of Figures S1-S2 and Tables S1-S2

Figure S1 Flow-cytometry evaluation of Tur1A membrane permeabilization on BW25113 *E. coli* cells.

Figure S2 Toe-print evaluation of Tur1A and Tur1B effect on ribosomal function, at increasing concentrations.

Table S1 Data collection and refinement statistics

Table S2 Sensitivity of erythromycin resistant *E. coli* strains to PrAMPs