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The Host Antimicrobial Peptide $Bac7_{1-35}$ Binds to Bacterial Ribosomal Proteins and Inhibits Protein Synthesis

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SUMMARY

Antimicrobial peptides (AMPs) are molecules from innate immunity with high potential as novel anti-infective agents. Most of them inactivate bacteria through pore formation or membrane barrier disruption, but others cross the membrane without damages and act inside the cells, affecting vital processes. However, little is known about their intracellular bacterial targets. Here we report that $Bac7_{1-35}$, a proline-rich AMP belonging to the cathelicidin family, can reach high concentrations (up to 340 μM) inside the *E. coli* cytoplasm. The peptide specifically and completely inhibits in vitro translation in the micromolar concentration range. Experiments of incorporation of radioactive precursors in macromolecules with *E. coli* cells confirmed that $Bac7_{1-35}$ affects specifically protein synthesis. Ribosome coprecipitation and crosslinking assays showed that the peptide interacts with ribosomes, binding to a limited subset of ribosomal proteins. Overall, these results indicate that the killing mechanism of $Bac7_{1-35}$ is based on a specific block of protein synthesis.

INTRODUCTION

Antimicrobial peptides (AMPs) are ancient and widespread effectors of innate immunity in higher eukaryotes, including animals and plants, in which they act as a first line of defense against pathogen infection (Diamond et al., 2009; Lai and Gallo, 2009). AMPs are multifunctional molecules. Besides their capacity to directly kill microbes, they also exert immunomodulatory activity on the host (Lai and Gallo, 2009). Because of their broad activity spectrum and modest size, AMPs are currently being considered as potential alternatives or enhancers for antibiotics to help overcome the worsening problem of the emergence of multidrug-resistant bacteria (Brogden and Brogden, 2011; Nguyen et al., 2011). Despite their great molecular diversity, most AMPs show amphipathic α-helical or β sheet conformations suitable for a selective membrane-interacting activity, leading to a lethal disruption of the microbial envelope (Epand and Vogel, 1999). However, some AMPs affect microbial viability by mechanisms involving interaction with intracellular targets (Hale and Hancock, 2007; Nicolas, 2009). Several AMPs may recognize and inactivate cellular targets in vitro, such as nucleic acids, proteins, and enzymes (Nicolas, 2009), and their mode of action is assumed to be mediated by translocation across the plasma membrane in a nonlethal manner. However, a strict connection between binding to internal structures of the cells and antimicrobial activity has not yet been demonstrated. In addition, the precise concentration reached by the AMPs inside the bacterial cells remains elusive.

The group of proline-rich AMPs (PR-AMPs) is an example of nonmembranolytic peptides (Cudic and Otvos, 2002; Scocchi et al., 2011). PR-AMPs are present in many mammalian neutrophils (Scocchi et al., 2011) as well as in the hemolymph of several species of insects and crustaceans (Otvos, 2002). PR-AMPs share a positive charge mainly due to arginine residues, and they have a high content of proline residues, which confers an extended conformation (Scocchi et al., 2011). Insect and mammalian proline-rich peptides are most active against Gram-negative bacteria, show low cytotoxicity toward eukaryotic cells, and share also a similar mode of bacterial cell internalization. $Bac7_{1-35}$, a fully active N-terminal fragment of Bac7 (Benincasa et al., 2004) is actively transported into *E. coli* cells by a mechanism involving the inner membrane protein SbmA (Mattiuzzo et al., 2007). Mutants lacking this protein show decreased susceptibility to many mammalian and insect PR-AMPs (Mattiuzzo et al., 2007; Narayanan et al., 2014; Pränting et al., 2008). In addition, $Bac7_{1-35}$ also possesses the capacity to translocate into eukaryotic cells without cell damaging (Tomasinsig et al., 2006). The molecular chaperone DnaK has been proposed to be a cytoplasmic target for insect PR-AMPs (Kragol et al., 2001). However, the in vivo importance of DnaK inhibition remains controversial. Because *E. coli* ΔDnaK strains are still susceptible to these peptides (Czihal et al., 2012; Scocchi et al., 2009), this suggests that other intracellular targets of PR-AMPs are likely to exist. On the other hand, it has been shown that sublethal concentrations of $Bac7_{1-35}$ are able to profoundly modify the transcriptional profile of *E. coli* cells (Tomasinsig et al., 2004). A number of genes involved in transcription, translation, or both were among the upregulated genes, suggesting that the peptide could affect one of these processes.

In this study, we investigated the intracellular mechanism of action of the proline-rich peptide $Bac7_{1-35}$ and compared it

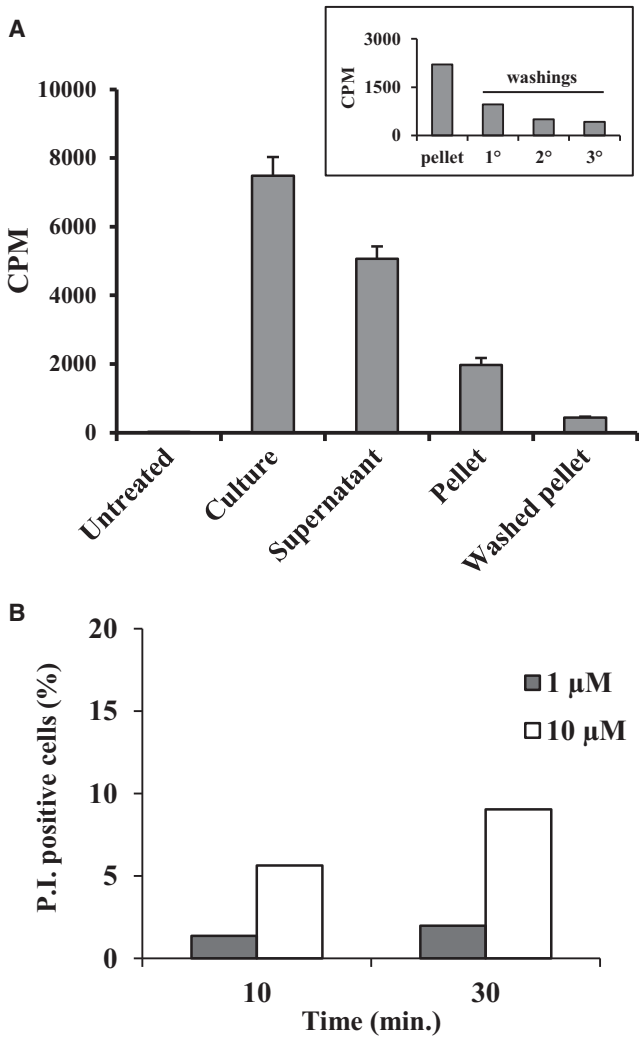

Figure 1. Intracellular Concentration of Radioactive Bac7$_{1-35}$Alch^{14}C in Intact *E. coli* Cells

(A) Radioactivity of different bacterial fractions treated with the peptide. BW25113 cells were exposed to 10 μM peptide for 10 min. The bacterial suspension was centrifuged, and the radioactivity of supernatant and pellet was measured before and after washings. A peptide-untreated culture was used for the blank. A representative result of radioactivity decrease due to pellet washing is shown in the inset. Error bars represent SD.

(B) Permeabilization assay on BW25113 *E. coli* cells. Bacteria were exposed in MH broth to 1 and 10 μM Bac7$_{1-35}$ for 10 and 30 min in the presence of propidium iodide and analyzed by flow cytometry.

with other cathelicidins that are not rich in proline residues. We determined the effective concentrations reached by Bac7$_{1-35}$ inside *E. coli* cells. By using in vitro transcription and transcription/translation systems and by evaluating the cellular incorporation of macromolecule precursors, we identified protein synthesis as a major target of the peptide. In line with that, we showed that the peptide alone is able to bind to 70S ribosomes in vitro. We thus propose a model in which AMPs act inside the cell by interacting with ribosomes, leading to protein synthesis inhibition. These results are of interest to design new drugs with antibiotic activity based on the mechanism of action of this proline-rich peptide.

RESULTS

Bac7$_{1-35}$ Is Concentrated inside the *E. coli* Cell Cytoplasm

We calculated the intracellular concentration reached by Bac7$_{1-35}$ inside *E. coli* cells (Figure 1). The radioactive derivative of Bac7, Bac7$_{1-35}$Alch^{14}C, was incubated with bacteria at 10 μM, a concentration that does not permeabilize the cell membranes (Figure 1B). The radioactivity of the supernatant and the bacterial pellet was measured after extensive washing with high-salt buffer. We found that a significant amount (~6%) of the radioactivity was strongly associated with the washed bacterial pellet (Figure 1A). We assumed that this pellet-associated radioactivity is localized in the cytosol because we previously demonstrated by immunogold electron microscopy that Bac7$_{1-35}$ is nearly exclusively accumulating in the *E. coli* cytosol (Podda et al., 2006). Taking into account the ratio between the cell volume and the total culture volume, we estimated that the peptide reached an intracellular concentration of ~340 μM inside the *E. coli* cells. This result is in agreement with the energy-driven translocation of Bac7$_{1-35}$ through the inner membrane SbmA protein (Runti et al., 2013) and is likely sufficient to interact with intracellular structures.

Bac7$_{1-35}$ Inhibits the Transcription/Translation Process

In vitro transcription/translation assays were performed to evaluate any inhibitory activity exerted by Bac7$_{1-35}$ on one or both of these processes, as previously suggested (Tomasinsig et al., 2004). Increasing amounts of Bac7$_{1-35}$ were added to reactions in an in vitro transcription/translation assay, and the inhibitory effect of the peptide was evaluated by the level of a luciferase reporter estimated by SDS-PAGE protein bands and by the luminescence intensity resulting from luciferase activity. At 1 μM, Bac7$_{1-35}$ already exerted a strong inhibition of the luciferase activity (80%), which was completed at 50 μM peptide (Figures 2A and 2B). The inhibition thus takes place in a concentration-dependent manner and at concentrations much lower than those that can be reached by the peptide inside the bacterial cytoplasm.

We repeated the assay with different types of AMPs to assess the specificity of the inhibition. The cathelicidins LL-37 and BMAP-27 were chosen because their size, net charge, and antimicrobial potency (minimal inhibitory concentration [MIC] = 1–5 μM) are similar to those of the proline-rich peptide (Podda et al., 2006; Skerlavaj et al., 1996; Zelezetsky et al., 2006), while on the other hand they have unrelated structure and a different mechanism of action that is based on membrane damage (Zanetti et al., 2000).We found that both LL-37 and BMAP-27 at 50 μM only slightly inhibited the synthesis of luciferase, to less than 50% reduction compared with the untreated control (Figures 2C and 2D). In contrast, the protein synthesis inhibitor kanamycin at 50 μM completely inhibited the activity of luciferase (Figures 2C and 2D).

Interaction of Bac7$_{1-35}$ with DNA Cannot Explain Its Specific Inhibition on Transcription/Translation

To determine whether the inhibitory effect of Bac7$_{1-35}$ on transcription/translation was due to its electrostatic interaction with nucleic acids, we evaluated the in vitro capability of Bac7$_{1-35}$

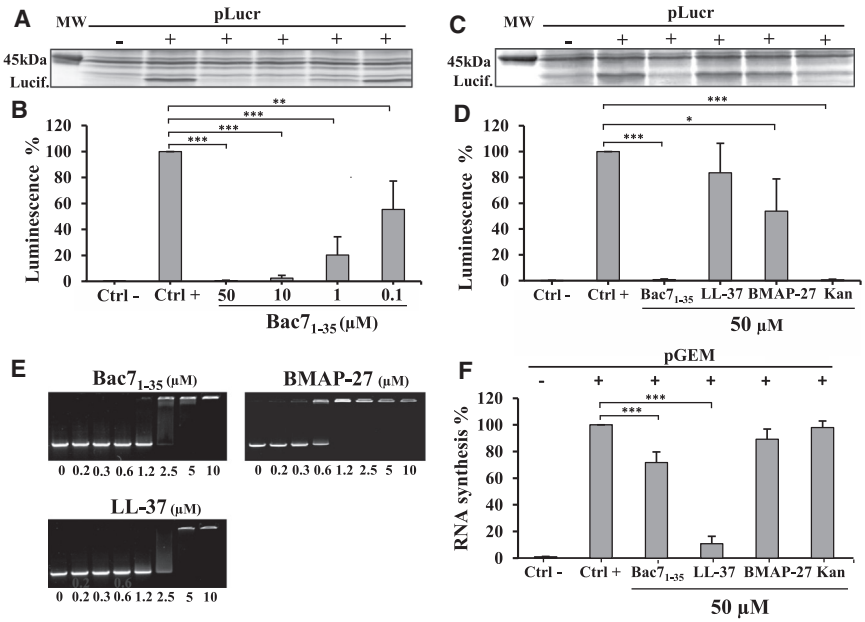

Figure 2. In Vitro Transcription/Translation Assay in the Presence of Bac7$_{1-35}$

(A and B) Synthesis of the reporter luciferase incubated without (Ctrl+) or with Bac7$_{1-35}$ was evaluated by SDS-PAGE (A) and by luminescence intensity, shown as percentage of the positive control (100%) (B).

(C and D) Synthesis of the reporter luciferase incubated without (Ctrl+) or with 50 μM LL-37, BMAP-27, and kanamycin (Kan) was evaluated by SDS-PAGE (C) and by luminescence intensity (D). As negative control, reactions without the luciferase-encoding DNA (Crtl-) were used.

(E) DNA-AMP interaction assay. Increasing amounts of Bac7$_{1-35}$, LL-37, and BMAP-27 were coincubated with linearized pBluescript SK+ plasmid and separated on agarose gel.

(F) In vitro transcription assay in the presence of Bac7$_{1-35}$. RNA synthesis in absence (Ctrl+) or presence of 50 μM Bac7$_{1-35}$, LL-37, BMAP-27, or kanamycin (Kan) shown as percentage of a positive control sample (100%). A reaction without DNA template (pGEM) was used as a negative control (Ctrl-). Error bars represent SD.

to bind DNA. An electrophoretic mobility shift assay was performed on plasmid DNA in the presence of the Bac7$_{1-35}$, LL-37, and BMAP-27 peptides. Each peptide was incubated with the same amount of linearized plasmid using a buffer approximating the salt and protein concentration of the bacterial cytosol (Park et al., 1998). Over a range of peptide concentrations of 0.75 to 2.5 μM, all the AMPs used retained DNA in the agarose wells, consistent with a peptide-induced precipitation of the nucleic acid. Thus, a similar plasmid retardation pattern was observed with all the peptides at high concentration, and no specific effect was detected using Bac7$_{1-35}$ (Figure 2E). This behavior suggests that unspecific electrostatic interactions occurred between the negatively charged DNA and each of the cationic AMPs, regardless of their identity. Thus the specific inhibition of transcription/translation by Bac7$_{1-35}$ and not by LL-37 and BMAP-27 cannot be explained only by its DNA-binding activity.

Bac7$_{1-35}$ Does Not Affect Transcription

To understand whether Bac7$_{1-35}$ inhibits transcription, translation, or both, we performed an in vitro transcription assay in the presence of the peptide or the two other cathelicidin AMPs used above. After the in vitro transcription reaction, the newly synthesized RNA was purified and quantified with a Bioanalyzer. No dramatic reduction in the amount of RNA (<30%) was observed in the presence of 50 μM Bac7$_{1-35}$, indicating that the peptide did not strongly interfere with transcription even at high concentrations (Figure 2F). Unexpectedly, the LL-37 peptide at 50 μM inhibited transcription in a remarkable manner, while BMAP-27 did not show any appreciable effect.

Bac7$_{1-35}$ Specifically Inhibits Protein Synthesis in *E. coli* Cells

In order to verify the in vivo effects of Bac7$_{1-35}$ on protein synthesis and/or RNA transcription and DNA replication, we measured the incorporation of radioactive leucine, uridine, and thymidine, respectively, in protein, RNA, and DNA within bacterial cells treated with the peptide. BW25113 *E. coli* cells were incubated with 1 μM Bac7$_{1-35}$, a concentration that is in the range of the MIC of Bac7$_{1-35}$ against this strain. After exposure to the peptide, bacteria showed an important decrease only in the incorporation of ^{3}H-leucine (Figure 3A). This effect was indistinguishable from that shown by kanamycin, an aminoglycoside known to bind the 30S subunit of ribosomes and to inhibit protein synthesis. Conversely, the incorporation of radioactive ^{3}H-thymidine and ^{3}H-uridine did not show any notable decrease compared with the untreated controls, while the antibiotics nalidixic acid and rifampicin, respectively, inhibited DNA and RNA synthesis, in agreement with their known inhibitory activities (Figures 3B and 3C). These results strongly indicate that only protein synthesis is affected by Bac7$_{1-35}$, confirming the specific inhibition of translation observed in vitro.

The SbmA protein is an inner membrane transporter involved in the uptake of Bac7$_{1-35}$ into bacterial cells. *sbmA* null mutant strains showed a reduced internalization and decreased susceptibility to this peptide (Mattiuzzo et al., 2007). We found that the *E. coli* BW25113 Δ*sbmA* strain treated with 1 μM Bac7$_{1-35}$ showed a higher level of ^{3}H-leucine incorporation compared with the similarly treated wild-type strain, indicating that the inhibition of protein synthesis by Bac7$_{1-35}$ is dependent on its internalization (Figure 3D).

Bac7$_{1-35}$ Cosediments with Purified Ribosomes

The previous results suggested translation as Bac7$_{1-35}$ target in bacteria, so we hypothesized that ribosomes could be involved in its mechanism of action. Therefore, we checked whether Bac7$_{1-35}$ can bind directly to them. We incubated purified 70S *E. coli* ribosomes with increasing amounts of purified Bac7$_{1-35}$ and separated ribosome-bound Bac7$_{1-35}$ by pelleting ribosomes through ultracentrifugation. The presence of Bac7$_{1-35}$ in the ribosomal pellet fraction was analyzed by immunoblots using specific antiserum against the peptide. After centrifugation, we

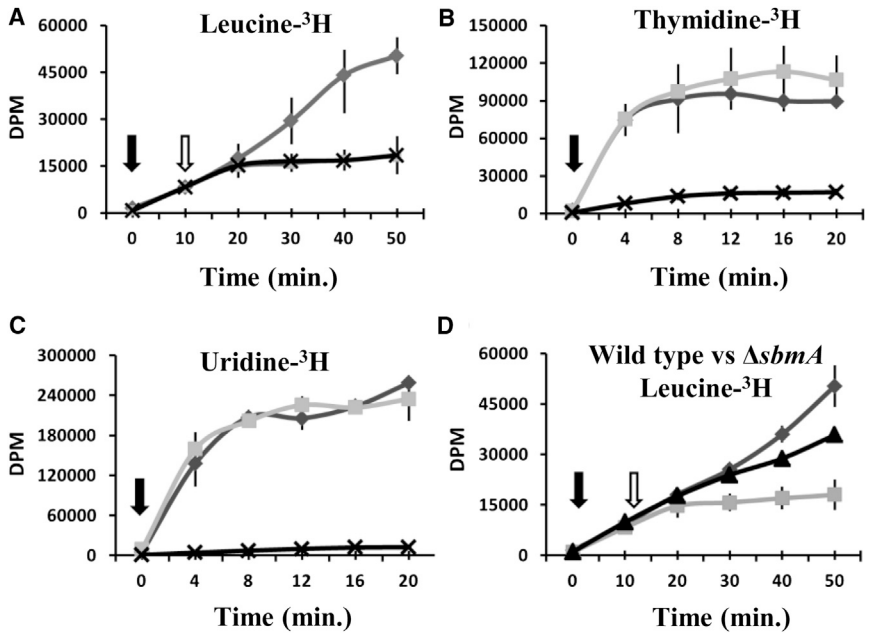

Figure 3. Incorporation of Radioactive Precursors in Macromolecules in Bac7$_{1-35}$-Treated *E. coli* BW25113

(A) Incorporation of ^{3}H-leucine in an untreated culture (diamonds), in the presence of 1 μM Bac7$_{1-35}$ (squares), or 200 μM kanamycin (crosses).

(B) Incorporation of ^{3}H-thymidine in an untreated culture (diamonds), in the presence of 1 μM Bac7$_{1-35}$ (squares), or 100 μM nalidixic acid (crosses).

(C) Incorporation of ^{3}H-uridine in an untreated culture (diamonds), in the presence of 1 μM Bac7$_{1-35}$ (squares), or 100 μM rifampicin (crosses).

(D) Incorporation of ^{3}H-leucine in BW25113 Δ*sbmA* untreated cells (diamonds) and in the presence of 1 μM Bac7$_{1-35}$ (triangles), or in wild-type BW25113 in the presence of 1 μM Bac7$_{1-35}$ (squares).

Black arrows indicate the time of addition of the radioactive precursor; white arrows indicate the addition of the antimicrobial peptide (where the arrow is not shown, the antimicrobial compound was added 20 min before the labeled precursor). Differences in timing were due to the different physiologic incorporation rate of the precursors. Error bars represent SD.

found Bac7$_{1-35}$ in the pellet fraction only in the presence of ribosomes (Figure 4A), which suggests a direct interaction between Bac7$_{1-35}$ and the ribosome. This interaction was not disrupted after washing the pellet fraction with salt-solution, indicating that this association is not only dependent on charge interactions. In addition, increasing concentration of Bac7$_{1-35}$ in the binding reaction resulted in a complete saturation of the binding when 20-fold molar excess of Bac7$_{1-35}$ was used (Figure 4B), which suggests that there is one or multiple specific Bac7$_{1-35}$-binding sites on the ribosome. We calculated that the apparent dissociation constant for the Bac7$_{1-35}$-ribosome complex is in the micromolar range (~6 μM), a value of the same order as other ribosomal binding factors (Kudva et al., 2013).

To evaluate the specificity of the peptide-ribosome interaction, purified, nontranslating ribosomes were incubated in the presence of Bac7$_{1-35}$BPA20, a photocrosslinkable derivative of Bac7$_{1-35}$. Western blot analysis of the peptide-treated ribosomes using an anti-Bac7 antibody showed two major high-molecular weight bands crosslinked to Bac7$_{1-35}$ (Figure 4C) among the whole ribosomal protein set. These bands have apparent molecular weights of ~20 and ~30 kDa, indicating that a specific binding occurred with ribosomal proteins of ~16 and ~26 KDa.

Taken together, our findings show that Bac7$_{1-35}$ associates with ribosomes by binding specific ribosomal proteins, and we propose that this association is responsible for the reduced protein synthesis induced by the peptide in vitro and in living bacteria.

DISCUSSION

Data concerning the activity of AMPs on molecular targets other than the cytoplasmic membrane are scarce and incomplete, and the killing mechanisms of non-membrane-disrupting peptides remain largely unknown. In this study, we demonstrate that the AMP Bac7$_{1-35}$ is able to accumulate at high concentrations inside the target bacteria and to specifically inhibit protein synthesis in the micromolar concentration range, probably by binding to ribosomes. This mode of action has previously been postulated for other AMPs (Hale and Hancock, 2007; Nicolas, 2009), but this study clearly demonstrated that inhibition of protein synthesis is the killing mechanism for an AMP.

In contrast to most AMPs that have the membrane as main target, PR-AMPs and some other AMPs (Nicolas, 2009) penetrate into the Gram-negative bacteria without damaging the membrane. For example, it has been shown by immunogold electron microscopy that the Bac7$_{1-35}$ peptide is present in the bacterial cytosol without causing notable membrane damage (Podda et al., 2006). Despite that, the concentration reached intracellularly by these peptides has remained undetermined so far. Here we show that Bac7$_{1-35}$ added to a *E. coli* culture at a concentration that does not perturb the membrane (10 μM) (Benincasa et al., 2009; Podda et al., 2006) is more than 30-fold enriched in the cells, reaching a concentration close to 0.34 mM, which represents thus the effective amount of peptide acting on its bacterial internal target(s). The extent of accumulation is in agreement with an active import mechanism already described for some PR-AMPs dependent on the SbmA protein (Mattiuzzo et al., 2007; Runti et al., 2013). In addition, other factors may contribute to increase the intracellular peptide concentration. The peptide, once in the cytoplasm, is likely sequestered by macromolecular cell components such as nucleic acids or protein complexes (Scocchi et al., 2009), and this can drive the further uptake of the peptide.

The processes of transcription, translation, or both were considered plausible candidates as possible intracellular targets of Bac7$_{1-35}$. In addition to the fact that several genes linked to these processes were found upregulated after exposure of *E. coli* cells to sublethal amounts of Bac7$_{1-35}$ (Tomasinsig et al., 2004), it seemed reasonable that a highly cationic peptide could bind the negatively charged nucleic acids or that it could be electrostatically attracted by the nucleoid, where the transcription and the translation take place. The in vitro experiments of coupled transcription/translation indicated that the peptide

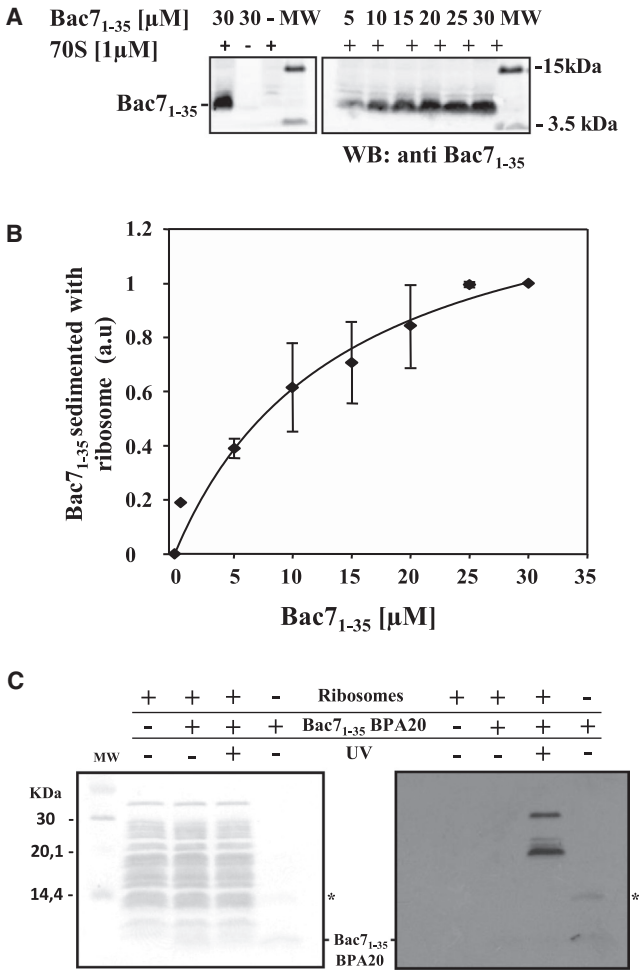

Figure 4. The Bac7$_{1-35}$ Peptide Binds Bacterial Ribosomes

Binding of Bac7$_{1-35}$ peptide to *E. coli* 70S ribosomes was analyzed by cosedimentation assays using increasing concentrations of peptide and 1 μM ribosomes.

(A) Ribosomal pellets were analyzed by immune blotting using an anti-Bac7 antiserum. Representative immunoblots are shown.

(B) Signals from immunoblot as in (A) were quantified. The solid line is a fit of the theoretical Michaelis-Menten equation to the data set. a.u., arbitrary units.

(C) Ponceau red staining (left) and western blot using an α-Bac7$_{1-35}$ antibody (right) after SDS-PAGE separation of 1 μM ribosomes incubated with 10 μM Bac7$_{1-35}$BPA20 and UV crosslinked. An additional band (asterisk) is visible in the control with Bac7$_{1-35}$BPA20 only, because in the absence of the reducing agent TCEP, the peptide forms dimers (C). Error bars represent SD.

strongly inhibits the whole process at considerably lower concentrations than those intracellularly reached by Bac7$_{1-35}$, (80% inhibition at 1 μM). Most important, the inhibition of transcription/translation is a specific effect of Bac7$_{1-35}$. The α-helical cationic cathelicidins, LL-37 and BMAP-27, resembling Bac7$_{1-35}$ respectively in the number of amino acid residues, positive charges, and antimicrobial activity did not affect the transcription/translation processes in a comparable manner, indicating that a specific effect of the peptide occurs, not simply an unspecific "interference" due to cationic nature of the peptide.

Cationic AMPs could have an unspecific bactericidal effect due to their ability to interact with nucleic acids. Although we found that Bac7$_{1-35}$ binds to DNA, LL-37 and BMAP-27 also do so and to a comparable extent. The same binding property has been previously observed with several different AMPs, such as indolicidin, tachyplesin, and buforin II (Hsu et al., 2005; Park et al., 1998), but a connection between DNA binding and inhibition of vital processes has not yet been demonstrated for these peptides (Nicolas, 2009). It seems likely that interactions between AMPs and DNA are due to electrostatic attraction between the positively charged peptide and the phosphate groups of the nucleic acid. In the DNA-peptide interaction experiments, we observed the precipitation of DNA at peptide concentrations corresponding to a 2:1 peptide/DNA ratio (w/w). This result suggests that Bac7$_{1-35}$ can bind and aggregate DNA when its amount is twofold than that of the nucleic acid. Because of the amount of nucleic acids in a single bacterial cell, estimated at ≈64 fg (Neidhardt, 1996), and the amount of Bac7$_{1-35}$ calculated as 9 fg for a single bacterium in our experiments, the peptide concentration is far too low to reach the peptide/DNA ratio required for DNA aggregation. In contrast, transcription/translation inhibition occurs with amounts of peptide much lower than those needed to effectively interact with DNA. Taken together, these results indicate that the inhibiting activity of Bac7$_{1-35}$ on the transcription/translation processes cannot be simply explained as a "disturbing activity" caused by binding of the peptide to DNA, eventually followed by DNA aggregation. Results of transcription inhibition assays and in vivo incorporation of radioactive precursors are in agreement with a specific action on protein synthesis only, while DNA or RNA synthesis is not affected. Moreover, the dependence of in vivo protein synthesis inhibition by Bac7$_{1-35}$ on the presence of SbmA transporter, and thus peptide import, is consistent with one or several intracellular targets of the peptide. In line with that, it was recently shown that nodule-specific cysteine-rich (NCR) peptides, produced in rhizobium-infected plant cells and having antimicrobial activity, penetrate bacteria and form complexes with many bacterial proteins (Farkas et al., 2014). Moreover, the LL-37 and BMAP-27 did not block protein synthesis in the coupled transcription/translation assay. Surprisingly, the LL-37 peptide showed a strong inhibitory activity on in vitro transcription. We hypothesize that, because this peptide is known to form aggregates and to be "sticky" (Zelezetsky et al., 2006), it could bind to RNA polymerase, reducing its activity. Synthesis of RNA in the presence of LL-37 was reduced but not completely blocked. It is thus possible that a sufficient amount of RNA is produced in the presence of LL-37 to fully support protein synthesis in the in vitro transcription/translation assay.

Our data on the incorporation of precursors in viable bacterial cells differ for some aspects from those reported previously by Skerlavaj et al. (1990). In that study, it was observed that the native Bac7 peptide (60 residues in length) inhibited the synthesis of both RNA and protein, while at the same time causing membrane permeabilization. The authors proposed a lytic mechanism of action for Bac7, which indirectly affected protein and RNA synthesis. Similarly Boman et al. (1993) proposed that PR-39, a different PR-AMP, stopped protein and DNA synthesis. However, both studies used the native complete form of the peptides at concentrations much higher than those corresponding to the respective MIC values. It is known that these PR-AMP peptides, when applied at high concentrations and especially in their

longer native form, can also exert a concurrent membrane permeabilizing effect (Podda et al., 2006). Under these conditions, inhibition of protein and RNA synthesis results from an unspecific and indirect effect due to a remarkable membrane damage.

The cosedimentation of Bac7$_{1-35}$ with ribosomes shown here and the crosslinking of the peptide to specific ribosomal proteins suggest that the AMP inhibits specific subunits of the ribosome or ribosome-associated proteins. The molecular chaperone DnaK has been proposed as a primary molecular target for several PR-AMPs, such as the insect apidaecins and pyrrhocoricin (Kragol et al., 2001). Also, Bac7$_{1-35}$ was shown to directly interact with and to inhibit in vitro the activity of this chaperone protein. However, *E. coli* ΔDnaK strains are still susceptible to the peptide (Scocchi et al., 2009) and to the apidaecin derivative Api88 (Czihal et al., 2012), suggesting that other crucial intracellular targets of Bac7$_{1-35}$ and of other PR-AMPs should exist. DnaK, the major bacterial Hsp70, functions as a central hub in the *E.coli* chaperone network (Castanié-Cornet et al., 2014). Interestingly, DnaK and its DnaJ cochaperone cooperate with the upstream ribosome-associated trigger factor (TF) and the downstream GroEL to assist folding of newly synthesized polypeptides. Upon deletion of TF, a significant increase of the number of newly synthesized polypeptides interacting with DnaK has been shown in vivo, including ribosomal and small basic proteins (Calloni et al., 2012), supporting a functional redundancy between these two chaperones. The discovery that Bac7$_{1-35}$ specifically inhibits protein synthesis in viable *E. coli* cells and that this peptide is able to interact in vitro with purified nontranslating ribosomes with an affinity comparable to that of other cotranslational factors leads us to propose a model for the intracellular molecular mechanism by which Bac7$_{1-35}$ acts on bacteria. We suggest that it could block protein synthesis by targeting the ribosomes, binding specific ribosomal proteins, and thus possibly preventing further cotranslational events (e.g., interaction of cotranslational chaperones with ribosomes) necessary to ensure translation of any polypeptide chain. It will be interesting in the future to identify the molecular identity of the ribosomal proteins bound by the peptide and so to exactly determine its ribosomal docking region. This information will be important to shed light on the possible interplay of the peptides with other essential cotranslation factors and will have implications in the field of biomedicine. Indeed, protein synthesis and the ribosomes are the targets of many conventional antibiotics. Thus, one of the possible outcomes of understanding the details of interaction between Bac7$_{1-35}$ and ribosomes will be a unique opportunity to use this peptide as a starting point to design new molecules that inhibit bacterial ribosomes, leading to the development of novel and effective antibiotic drugs. Alternative antibiotics to those currently available are urgently required because multidrug resistance in pathogenic bacteria has been rising enormously in recent decades.

SIGNIFICANCE

The emergence and spread of antibiotic-resistant pathogens and the urgent need for new antimicrobial drugs have revived interest in molecules with alternative modes of combating them. This work clarifies the molecular mechanism of action of Bac7, a PR-AMP of natural origin, showing that it kills bacteria by selectively inhibiting protein synthesis, likely interacting with specific ribosomal proteins. A vast number of antimicrobial peptides have been described and their antibacterial properties reported, but information on their modes of action often remains fragmentary. Bac7 is a rare example for which the killing mechanism is described in detail, including the previously described protein transporter required for its translocation into the cytoplasm and now intracellular concentrations reached, as well as the internal targets it interacts with. These results represent a significant advance in the understanding of the functioning of this type of AMPs and represent a promising starting point for the design of new antibiotics.

EXPERIMENTAL PROCEDURES

Peptides

The Bac7 N-terminal fragments Bac7$_{1-35}$ and Bac7$_{1-35}$ with an additional C-terminal cysteine (Bac7$_{1-35}$Cys) were synthesized as previously described and their concentrations determined as previously reported (Benincasa et al., 2004). All peptides, with a purity of at least 95%, were stored in Milli-Q water at −20°C until use. The radioactive derivative Bac7$_{1-35}$Alch^{14}C was prepared by alkylating a Bac7$_{1-35}$Cys peptide with ^{14}C-iodocetamide. A modification of a preexisting protocol was used (Creighton, 1997) (see below). Bac7$_{1-35}$BPA20 was synthesized as an ordinary Bac7$_{1-35}$Cys but substituting the phenylalanine in position 20 with a 4-benzoyl-L-phenylalanine (Fmoc-4-benzoyl-L-phenylalanine, Sigma). LL-37 and BMAP-27 were generously provided by Prof. A. Tossi (University of Trieste).

Bac7$_{1-35}$ Radioactive Labeling

Two milligrams of Bac7$_{1-35}$Cys was dissolved in 300 μl of 10 mM HCl (Carlo Erba) (prebubbled with N_2), divided into five aliquots of 60 μl, and immediately frozen at −20°C until use. All the following procedures were done in the dark. Twenty-five microliters of ^{14}C-iodoacetamide (IAA) (stock 2 mM^{14}C-IAA in ethanol, 50–60 mCi/mmol, Biotrend) was diluted in 175 μl of 0.5 M Tris-acetate (Sigma) and 2 mM Na_2EDTA (Sigma) (pH 8), named Tris8 (prebubbled with N_2). Then 5 μl of 0.1 mM ascorbic acid (Sigma) was added, immediately followed by the first 60 μl aliquot of Bac7$_{1-35}$Cys. The mixture was left at room temperature under gentle agitation for 30 min in a N_2-enriched atmosphere. Another three Bac7$_{1-35}$Cys aliquots were added every 30 min under the same conditions, and the third one was immediately followed by 8 μl of nonradioactive 10 mM IAA (Fluka) dissolved in Tris8 and 2 μl of 1 mM ascorbic acid (Sigma). An additional aliquot of Bac7$_{1-35}$Cys, followed by 8 μl of nonradioactive 100 mM IAA dissolved in Tris8 and 2 μl of 1 mM ascorbic acid was then added after 15 min followed by 100 μl of nonradioactive 100 mM IAA in Tris8 and 2 μl of 10 mM ascorbic acid. After 15 min, the mixture was vigorously vortexed for 60 s and then 8 μl of 0.5 M citric acid was added and the mixture was vigorously vortexed for 20 s. The whole reaction mixture was loaded on a desalting column (Hi-trap desalting, GE Healthcare) and separated by high-performance liquid chromatography using 100 mM NaCl in Milli-Q water and at 1 ml/min. Fractions were analyzed by mass spectrometry and the fraction having the highest content of labeled peptide (Bac7$_{1-35}$Alch^{14}C) was frozen and stored for quantification and subsequent uses.

Bacterial Cultures

E. coli BW25113 cells were grown overnight in Mueller-Hinton (MH) broth (DIFCO) at 37°C under agitation. Two hundred microliters of overnight culture was put in 10 ml of MH broth and incubated at 37°C under agitation until an optical density at 600 nm (OD_{600}) of approximately 0.3 was attained. The bacterial suspension was centrifuged (2,000 × g, 20 min) and the pellet resuspended in adequate volumes of MH broth or M9-salt minimal medium with 5 mM glucose and 1% v/v MH broth.

Determination of the Intracellular Concentration of Bac7$_{1-35}$

Midlog phase *E. coli* BW25113 cells were incubated in MH broth with 10 μM Bac7$_{1-35}$Alch^{14}C for 10 min at 37°C under agitation. Then, 1 ml of culture

was collected and stored, another milliliter of culture was centrifuged (6,200 × g, 10 min), the medium was collected, and the pellet was resuspended in 1 ml of fresh medium. In parallel, a third milliliter of the peptide-treated culture was centrifuged (6,200 × g, 10 min), the medium was discarded, and the pellet was resuspended and washed three times in 1 ml of high-salt phosphate buffer (10 mM Na phosphate buffer, 10 mM $MgCl_2$, 400 mM NaCl), to remove the peptide from the bacterial external surface. After the third washing, the sample was centrifuged (6,200 × g, 10 min), the high-salt phosphate buffer was discarded, and the pellet was resuspended in 1 ml of fresh MH medium. The β counter MicroBeta Trilux (Wallac) was used for radioactivity evaluation, normalizing the result with the OD_{600} of the samples. Measurements were performed in triplicate for each experiment, mixing 200 μl of each sample with 500 μl of Opti Phase Supermix (PerkinElmer). Calculation of the intracellular amount of Bac7$_{1-35}$Alch^{14}C was carried out by taking into account the volume of a single BW25113 cell (~4.0 fL) (Volkmer and Heinemann, 2011), the number of bacteria (8 × 10^7 colony forming units [cfu]), and the volume of the sample (200 μl) used for each measurement. The volume of the pellet was therefore calculated and used to normalize its radioactivity value. Data were derived from three repetitions of independent experiments. Membrane integrity was evaluated by flow cytometric analysis and indicated as percentage of propidium iodide-positive cells (Benincasa et al., 2009). *E. coli* BW25113 cells (4 × 10^8 cfu/ml) after treating with the peptide in MH broth at 37°C were diluted to 4 × 10^6 cfu/ml immediately before measurements.

DNA-Peptide Interaction Assays

The reaction, as modified from Park et al. (1998), was set up by incubating at 37°C for 1 hr 100 ng of linearized pBluescript II KS(+) and different amounts of each AMP in the presence of 5% glycerol, 10 mM Tris-HCl (pH 7), 1 mM EDTA, 1 mM dithiothreitol, 20 mM KCl, and 50 μg/ml BSA. Samples of the reaction mixtures were then loaded onto a 1% agarose gel in TAE buffer including 1:10000 Gel red (Biotium) and separated by electrophoresis (90 min, 65 V).

In Vitro Transcription Assay

The commercial kit Riboprobe System–T7 (Promega) was used according to the instructions for the positive-control samples, but adding each antimicrobial agent (50 μM) to the reactions. As a negative control, the DNA template was not added to a reaction. After 60 min of incubation at 37°C, RNA was purified from all samples using TRIzol (Life Technologies). Size and amount of the transcribed RNA were evaluated using a Bioanalyzer 2100 (Agilent) with the kit Agilent RNA 6000 nano kit (Agilent). Results are the average of three independent experiments.

In Vitro Transcription/Translation Assay

The commercial kit S30 T7 High-Yield Protein Expression System (Promega) was used according to the protocol for the positive-control samples. The provided plasmid carrying the *Renilla* luciferase gene is indicated as pLucr. Forty units of RNase inhibitor (RNase Inhibitor Murine, New England Biolabs) was added to each reaction, followed by the antimicrobial agent as the last component. Samples were incubated at 37°C for 1 hr at 1,200 rpm. Then, the presence of the luciferase was assessed and quantified using both the commercial kit *Renilla* Luciferase Assay System (Promega) and 12.5% SDS-PAGE with Coomassie blue staining. Using the *Renilla* Luciferase Assay System (Promega), luminescence was measured by using a Chameleon multitechnology plate reader (Hidex) equipped with Mikrowin 2000 software. Results are the average of three independent experiments.

Incorporation of Radioactive Macromolecular Precursors In Vivo

Midlog phase BW25113 *E. coli* cells (1.5 × 10^7 cfu/ml) in M9-salt minimal medium containing 5 mM glucose and 1% v/v MH broth were incubated at 37°C. For leucine incorporation, 7.5 μl of ^{3}H-leucine (1 mCi/ml, PerkinElmer) was added to 650 μl of bacterial suspension. Eleven minutes after the addition of the ^{3}H-leucine, Bac7$_{1-35}$ was added to a final concentration of 1 μM. For uridine incorporation, Bac7$_{1-35}$ was added to a final concentration of 1 μM and 20 min after the addition of Bac7$_{1-35}$, 4 μl of ^{3}H-uridine (1 mCi/ml, PerkinElmer) was added to the culture. For thymidine incorporation, Bac7$_{1-35}$ was added to 650 μl of bacteria to a final concentration of 1 μM. Twenty minutes after the addition of the Bac7$_{1-35}$, 4 μl of ^{3}H-thymidine (1 mCi/ml, PerkinElmer) was added to the culture. After sampling at different times, bacteria were lysed and macromolecules precipitated by incubation on ice in 10% TCA (with 0.2 mg/ml BSA for protein assay, 0.2 mg/ml salmon sperm DNA for nucleic acid assay) for at least 60 min, then filtered on 0.22 μm filters (GSTF, Millipore). Radioactivity was measured by scintillation counting: the filters were dried for 40 min on paper, put into 4 ml of scintillation fluid (Ecolite[+] liquid scintillation cocktail, MP Biomedicals), and stored for 60 min at room temperature in the dark. The β counter 2200 CA TRI-CARB liquid scintillation analyzer (Packard) was used, measuring each sample for 10 min. Kanamycin (200 μM), rifampicin (100 μM), and nalidixic acid (100 μM) were used with identical protocols, respectively, as positive control for protein, RNA, and DNA synthesis inhibition. Sterile Milli-Q water was used as negative control for inhibition. Results are the average of three independent experiments.

Purification of 70S Ribosomes

E. coli strain MRE600 was grown at 37°C to 1.8 OD_{600} in 3 l of Luria-Bertani medium. The cells were then cooled on ice, harvested by 20 min of 4,000 × g centrifugation, and washed with 50 ml of PBS (Biosolve). The cell pellet was resuspended in 50 ml of 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NH_4Cl, 7 mM β-mercaptoethanol, 8.25% of sucrose supplemented with one tablet of Complete cocktail protein inhibitors EDTA-free (Roche) and 0.5 mg/mL lysozyme. After cell lysis by sonication, cell debris was removed by centrifugation at 28,000 × g for 30 min in a Beckman centrifuge with a JA20 rotor. Cleared extract was treated with DNase I (1 mg/l) and centrifuged for 3 h 30 min at 184,000 × g, 4°C, in a 70 Ti rotor (Beckman). The ribosomal pellet was resuspended overnight at 4°C in 20 ml of 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NH_4Cl, and 7 mM β-mercaptoethanol (RSP) supplemented with Complete cocktail protein inhibitors EDTA-free. After centrifugation for 20 min at 23,000 × g and 4°C, the supernatant was layered onto a 18% sucrose cushion in RSP containing 0.5 M NH_4Cl and centrifuged at 184,000 × g for 3 h at 4°C (ultracentrifuge Beckman Optima). Washing was repeated with 18% sucrose cushion in RSP containing 1 M NH_4Cl. Finally, the ribosomal subunits were dissociated at low Mg^{2+} (1 mM $MgCl_2$) and centrifuged over a two-sucrose cushion, the upper cushion containing 10 ml of 20% sucrose in 50 mM Tris-HCl (pH 7.5), 30 mM KCl, 30 mM NH_4Cl, and 0.5 mM $MgCl_2$ and the lower one 5 ml of 30% sucrose in 50 mM Tris-HCl (pH 7.5), 30 mM KCl, 30 mM NH_4Cl, and 13 mM $MgCl_2$. After 6 h 30 min of ultracentrifugation at 118,000 × g and 4°C, the reassociated 70S ribosomal pellet was resuspended in 2 ml RSP and dialyzed into RSP containing 50% glycerol. The ribosome concentration was determined by UV absorbance at 260 nm (1 A_{260} unit = 25 pmol/ml). The quality of the ribosomes was controlled (1) by SDS-PAGE, (2) in the poly(U)-dependent poly(Phe) synthesis assay, and (3) by centrifugation in the 15% to 35% sucrose gradient, as previously described (Graham and Rickwood, 1997).

Ribosome-Bac7$_{1-35}$ Cosedimentation Assay

One micromolar purified 70S ribosomes were incubated at 4°C for 30 min with increasing concentrations of Bac7 peptide (0.5–30 μM) in a total volume of 150 μl binding buffer (20 mM HEPES-KOH, 100 mM NH_4OAc, 20 mM $MgCl_2$, 1 mM TCEP [pH 7.4]). Samples were cleared by centrifugation (16,100 × g, 10 min, 4°C). Supernatants were transferred into 1.5 ml Eppendorf tubes and centrifuged in a Beckman TL100 ultracentrifuge using a TLA100.3 rotor (8,6000 × g, 128 min, 4°C). Ribosomal pellets were washed twice with 200 μl of ice-cold binding buffer and dissolved in 20 μl of 2× Laemmli buffer. Proteins from each sample were separated by using 12% NuPAGE Bis-Tris Precast Gels in MES buffer (Life Technologies). After electrophoresis, the proteins were transferred to a low-fluorescent polyvinylidenedifluoride membrane (GE Healthcare) and treated according to the manufacturer's instructions. Blots were blocked immediately after transfer in 5% (w/v) ECL Advance blocking reagent (GE Healthcare) in 1× PBS with 0.3% (v/v) Tween-20 for 2 hr at room temperature with agitation or overnight at 4°C. Bac7$_{1-35}$ peptide was detected with anti-Bac7 primary serum used at dilution 1:200 (1 h, RT). The blots were washed several times before incubation with the secondary antibody (ECL Plex goat antirabbit IgG-Cys5; GE Healthcare) at a dilution of 1:2,000 for 1 h. The membranes were washed before drying and scanned for Cy5 on the Pharos FX Molecular Imager System (Bio-Rad). Imaging was performed using a 635-nm laser with a 695-nm band-pass filter. The images were then

analyzed and the intensity of bands was quantified using Quantity One software (Bio-Rad) according to the manufacturer's instructions. To quantify and normalize each band (Adam et al., 2011), we used the relative volume intensity of the band that corresponded to the total signal intensity within a defined area. Background was subtracted from obtained values. Relative volume intensities of immunoblot bands are shown as arbitrary units. The quantity of loaded ribosome was controlled by western blot membrane staining with Ponceau red.

UV Crosslinking

One micromolar ribosomes were incubated with 10 μM Bac7$_{1-35}$ BPA20 for 30 min at 8°C with gentle agitation in the presence of 20 mM HEPES-KOH, 100 mM CH_3COONH_4, 20 mM $MgCl_2$, and 1 mM TCEP (pH 7.4). The reaction mix was subsequently exposed to UV light (λ = 365 nm) for 5 min at 8°C in a quartz cuvette (Amersham, *l* = 0.2 cm). The crosslinked proteins were revealed by 16% tricine SDS-PAGE and western blot using an anti-Bac7 antibody.

Statistical Analysis

Data obtained were subjected to computer-assisted analysis using Graph Pad Instat 3 and statistical significance was assumed at $p < 0.05$ (ANOVA, Tukey-Kramer posttest). Error bars represent SD.

AUTHOR CONTRIBUTIONS

M.M. designed and performed most of the experiments and wrote the initial draft of the manuscript. R. Grzela provided technical support and performed experiments with purified ribosomes. C.G. and T.M. contributed to the data analysis and offered conceptual advice. P.M. provided conceptual and practical advice on in vivo protein synthesis inhibition. R. Gennaro offered conceptual advice and contributed to the writing of the manuscript. M.S. supervised the experiments and edited the manuscript with contributions from all other authors.

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