Internal targets and killing mechanism of the cathelicidin Bac7 in Gram-negative bacteria

Mario Mardirossian
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DOTTORANDO
Mario Mardirossian

COORDINATORE
Prof. Giannino Del Sal

SUPERVISORE DI TESI
Prof. Renato Gennaro

CO-SUPERVISORE DI TESI
Dr. Marco Scocchi

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RIASSUNTO


Abbiamo quindi misurato la concentrazione intrabatterica raggiunta da Bac7(1-35) in *E. coli* e abbiamo osservato che questa aumenta da valori micromolari nel terreno di coltura a millimolari nel citosol batterico, suggerendo un suo legame a strutture interne della cellula. Per questo abbiamo cercato possibili interazioni tra il peptide e macromolecole coinvolte in processi vitali del batterio. Con questi studi abbiamo appurato che Bac7(1-35) *in vitro* inibisce completamente il processo di trascrizione/traduzione a partire da una concentrazione di 50 μM. Successivamente abbiamo dimostrato che questa inibizione è una peculiarità di Bac7(1-35), in quanto altri AMP derivati da catelicidine ma non ricchi in prolina non hanno dimostrato un’attività comparabile. Inoltre, questa inibizione non è stereo-specifica, in quanto anche l’isomero D di Bac7(1-35) blocca tale processo esattamente come il suo isomero L.

Abbiamo inoltre dimostrato la capacità di Bac7(1-35) di legare *in vitro* il DNA, ma abbiamo escluso che tale legame rappresenti il meccanismo primario della sua attività battericida. Abbiamo anche dimostrato che il peptide non interferisce *in vitro* in maniera significativa con il processo di trascrizione, deducendo che l’effetto osservato sul processo di trascrizione/traduzione fosse da attribuirsi prevalentemente all’inibizione della traduzione.

Abbiamo verificato tali dati *in vivo* su cellule di *E. coli* misurando l’incorporazione di precursori radioattivi delle macromolecole batteriche. Abbiamo osservato che l’esposizione di batteri a Bac7(1-35) bloccava l’incorporazione di leucina radioattiva ma non di timidina ed uridina, indicando un blocco specifico della sintesi proteica ma non di quelle di DNA e RNA.

In futuro, una definizione ancora più chiara del target intrabatterico di Bac7(1-35) potrebbe portare alla sperimentazione di tale molecola o di suoi analoghi come farmaci antibiotici di nuova generazione.
ABSTRACT

Bac7(1-35) is the smallest fragment of the proline-rich cathelicidin Bac7 that shows the same antibacterial activity as the whole natural peptide of 60 residues. In this work, we remarked that the unique gene whose deletion can confer resistance to E. coli against Bac7(1-35) is sbmA, coding for an inner membrane protein involved in the penetration of this peptide into bacterial cells. Moreover, we provided evidence that SbmA is also involved in the transmembrane transport of a fragment of another proline-rich antimicrobial peptide, arasin1(1-23), isolated from the spider crab. These findings suggest a general role of this membrane protein in the uptake of proline-rich antimicrobial peptides (PR-AMPs) into Gram-negative bacteria.

We then measured the intrabacterial concentration reached by Bac7(1-35) in E. coli, and observed that this increases from micromolar in the medium to millimolar within the bacterial cell, suggesting that it may bind to cytosolic structures. For this reason, we looked for possible interactions between Bac7(1-35) and macromolecules involved in viable processes of bacteria. These studies showed that Bac7(1-35) completely inhibits in vitro the transcription/translation process starting from a concentration of 50 μM. Then we demonstrated that inhibition is: i) specific for Bac7(1-35), since it is not exerted by other cathelicidin-derived AMPs not belonging to the Pro-rich group, and ii) not stereo-specific, since it is exerted at the same level by the all-D isomer of Bac7(1-35).

We also demonstrated the ability of Bac7(1-35) to bind DNA in vitro, but we excluded that this binding may represent the primary mechanism of bactericidal action. We also showed that the peptide does not significantly affect in vitro the transcription process, deducing that the inhibition of the transcription/translation targets primarily the translation process.

We verified these data in vivo on E. coli cells measuring the incorporation of radioactive precursors of bacterial macromolecules. We observed that the exposure of bacteria to Bac7(1-35) inhibited the incorporation of radioactive leucine, but not of radioactive thymidine and uridine, indicating a specific block at the protein synthesis level and not of DNA and RNA synthesis.

In the near future, a clearer definition of the intrabacterial target(s) of Bac7(1-35) would hopefully lead to the experimentation of this molecule or of its derivatives as a new generation antibiotic drug.
INTRODUCTION

Antimicrobial peptides (AMPs) are small molecules of endogenous origin endowed with antimicrobial activity. They are ancient and widely diffused weapons of innate immunity, representing a first line of defence conserved in evolutionarily distant multicellular organisms, AMPs act by taking advantage of the differences between eukaryotic and microbial cells, ensuring a wide antimicrobial activity and, at the same time, conserving a high specificity for the surface of microbes and low toxicity for the cells of the host. More than two thousands AMPs are known to date, and they can be classified following different criteria according to their structure, amino acid composition or mode of action (Hancock et al., 2006).

Most AMPs show an amphipathic helical or β-sheet conformation, suitable for a lytic activity on microbial membranes, so that the most common mechanism of action is a broad spectrum recognition and subsequent disruption of the microbial envelope, a process that leads to death of the target microorganisms (Zasloff, 2002). It is well known that most AMPs heavily damage the bacterial membranes at lethal concentrations. However, more recent evidence also suggests a different inhibiting activity exerted by AMPs on bacterial cells at sub-lethal concentrations, before a massive membrane-permeabilization event takes place. Under these conditions, some AMPs of different groups and structures are internalized into bacterial cells without membrane disruption. These AMPs interact with some intracellular structures, even though the link between binding to these interactors and bactericidal activity has not been completely understood and clarified (Nicolas, 2009). For example, it has been suggested that buforin II, an antimicrobial peptide with an α-helical structure, binds to DNA and RNA after a non-lytic penetration into the bacterial cell. The Authors suggest that binding to nucleic acids is part of the bactericidal mechanism for this peptide (Park et al. 1998, 2000). Pleurocidin, an α-helical cationic peptide, inhibited RNA synthesis at the MIC, followed after a while by a less specific inhibition of DNA and protein synthesis in E. coli and then by a complete permeabilization of the membranes (Patrzykat et al., 2002). Polyphemusin I, an amphiphilic antiparallel β-hairpin peptide, can enter bacterial cells at concentrations that do not significantly permeabilize the membrane. It has been proposed that this peptide can interact with DNA (Powers et al., 2006). Some evidence indicates that the short Trp-rich peptide indolicidin also inhibits quite specifically the synthesis of DNA (Subbalakshmi and Sitaram, 1998) and binds to this nucleic acid (Hsu et al., 2005).
An important group of antimicrobial peptides is represented by the proline-rich AMPs (PR-AMPs). As most other antimicrobial peptides, PR-AMPs are cationic with a positive charge mainly conferred by arginine. These peptides share a high content of proline residues ranging from 25% to 50%, which are frequently arranged in a repeated pattern and that confer an extended conformation. It is well known that proline residues disturb the folding of a polypeptide into α-helix or β-sheet conformations and that contiguous prolines tend to structure in a particular spatial conformation, known as polyproline (PP-II) helix that has been proposed for some PR-AMPs (Raj and Edgerton, 1995). For this reason and unlike other AMPs, PR-AMPs are mainly extended coils and conserve this conformation even in presence of an environment mimicking biological membranes (i.e., SDS-micelles or liposomes) (Cabiaux et al., 1994), or in solvents that usually force peptide structuring (i.e., trifluoroethanol).

It is worth noting that these characteristics are shared by PR-AMPs isolated from evolutionary unrelated organisms. In fact, proline-rich peptides have been isolated both in vertebrates and invertebrates, ranging from mammals (e.g., cow, pig, dolphin, etc.) to insect (e.g., honeybee, moth, fly, etc.) to aquatic invertebrates (e.g., crab, shrimp and mussel). The evolutionary distance separating these species suggests that the proline-rich peptides also derive from convergent evolution (Scocchi et al., 2011).
The random coiled structure of PR-AMPs, maintained even in the presence of biological membranes, is an indication that their mechanism of action differs from that of most other antimicrobial peptides that undergo a structure transition on interaction with membranes. A non-lytic bactericidal mechanism of action is in fact the prevalent mode of action of PR-AMPs. Even though proline-rich peptides can permeabilize bacteria at concentrations several fold above the MIC, at the MIC they exert their killing activity via internalization into bacteria without significant membrane disruption (Podda et al., 2006). After internalization in bacteria, the proline-rich AMPs kill microbes by targeting some intracellular structures not yet clearly identified. For example, it has been reported that pyrrhocoricin, drosocin and apidaecin, PR-AMPs isolated from insects, target DnaK, an important bacterial chaperone protein. So it has been suggested that these antimicrobial peptides can lead to bacterial inactivation by impairing the crucial role that DnaK plays in the folding of newly synthesized proteins (Kragol et al., 2001).

Due to their non-lytic mode of action, the bactericidal activity of PR-AMPs is slower and their spectrum of activity is narrower than that of other lytic AMPs. Nevertheless, proline-rich peptides exhibit antimicrobial activity at μM concentrations, mostly against Gram-negative bacteria (Casteels and Tempst, 1994) (Benincasa et al., 2004) (Otvos et al., 2000) (Scocchi et al., 2011).

In mammals, all the known PR-AMPs are members of the cathelicidin family. This family groups many antimicrobial peptides characterized by a conserved pro-region, the cathelin-like domain, which confers the name to the group. Even though cathelicidins show strong similarity in the conserved N-terminal pro-region, they are highly variable in the C-terminal part of the sequence. This region of the cathelicidin is cleaved off by the enzymes elastase or protease 3, and represents the final active form of the antimicrobial peptide (Zanetti, 2005). The pro-region is thought to have an inhibiting activity on the C-terminal part of the cathelicidin before the activation proteolytic process takes place. It has been proposed that the negative charge of the pro-sequence could allow its interaction with the positively charged C-terminus, thus inhibiting the activity of the latter. A small antimicrobial activity has been reported also for the pre-proregion itself, after the release of the C-terminal AMP, but it is not yet clear whether there are specific functions associated with this part of the molecule (Zaiou et al., 2003).

This work will focus its attention on the bovine proline-rich cathelicidin Bac7. Bac7 is a proline-rich cathelicidin isolated from granules of cow neutrophils (Gennaro et al., 1989), but its orthologs and also paralogs have been later isolated in other mammals such buffalo, sheep.
and goat (Scocchi et al., 2011). As with other cathelicidins, Bac7 is synthesized as an inactive immature precursor, in order to be safely produced and managed by the cell. Bac7 is synthesized as a pre-proform of 23.5 kDa by bovine myeloid cells in the bone marrow. The production of the pre-proform seems to be switched off during the myeloid differentiation process, because in mature peripheral neutrophils no evidences of pre-proform synthesis was found. In mature neutrophils Bac7 can be found stored into the large granules as an inactive proform of 20 kDa (Zanetti et al., 1990). This precursor can be processed to the active Bac7 after the activation of the neutrophil, during the process of degranulation. The exposure of neutrophils to opsonised bacteria, leads to the release of neutrophil granule contents. So the Bac7 precursor, released by the large granules, becomes accessible to the elastase, a protease released by azurophil granules, and the pre-propeptide can be cleaved to the active antimicrobial peptide of 7 kDa (Zanetti et al., 1991) (Scocchi et al., 1992).

The antimicrobial activity of mature Bac7 was tested against different bacterial species. The peptide showed a preferential activity against Gram-negative bacteria. Indeed the peptide inhibited the growth of selected collection strains of *E. coli*, *S. typhimurium*, *K. pneumonia* and *E. cloacae* (MIC: 12-25 μg/ml). A lower effect was observed against *P. aeruginosa* (MIC: 50 μg/ml) and no significant effect was observed against *P. vulgaris* (MIC: 200 μg/ml). The same lack of antimicrobial activity was observed against Gram-positive bacteria selected among collection strains of *S. epidermidis*, *S. aureus* and *S. agalactiae* (MIC: >200 μg/ml) (Gennaro et al., 1989).

After the sequencing of Bac7 (Frank et al., 1990), it was discovered that Bac7 presents an high content of proline and arginine residues: respectively 28 and 17 out of a total number of 60 residues.

RRIRPRPPRLPRPRPRPLPFPFRPGPRPDIRPRPRLPFPRPGPRPDIRPRPRLPFPRPGPRPDIRPRPRL

Figure 2. Amino acid sequence of the bovine PR-AMP Bac7.

Sequence analysis shows that the motif PRPX is repeated 11 times along the sequence. The significant presence of arginine confers a net charge of +17 (Frank et al., 1990) to the peptide and the high number of proline residues forces the peptide to a linear structure. Concerning the mechanism of action, when used at 10-50 μg/ml (1.5-.5 μM), Bac7 was shown to kill *E. coli* cells by permeabilizing their membranes and causing also a rapid drop of the bacterial metabolism within 30 min. It was observed that Bac7 reaches the best antimicrobial capacity when the pH of the medium ranges between 7 and 8. Moreover, it has been shown that increasing the ionic strength of
the medium decreases the bactericidal activity (Skerlavaj et al., 1990), as also shown for other antimicrobial peptides.

Subsequently, an interesting relationship between the amino acid sequence of Bac7 and its antimicrobial activity emerged. Thanks to the comparison of the activity of different synthetic fragments covering the whole Bac7 length, it has been discovered that the N-terminal domain of Bac7 plays an essential role in the antimicrobial activity. Shortening of the peptide to the first 35 N-terminal residues does not modify its antimicrobial activity, and shortening to the first 23 N-terminal residues just slightly decreases its potency. Conversely, it is sufficient to remove the first four N-terminal amino acids (RIRR) to observe a significant drop in the bactericidal efficiency of Bac7. It has also been shown that the shortest amino acid sequence endowed with significant antimicrobial activity is the 1-16 N-terminal fragment, while surprisingly the 1-15 fragment showed virtually no activity (Benincasa et al., 2004).

Due to these considerations, the fragment Bac7(1-35) represents a useful tool to investigate the mode of action of Bac7, being a molecule that can be produced more easily by chemical synthesis, thus avoiding the cumbersome purification process from the natural source or a long and low-yield synthesis. Even though the antimicrobial potency of the Bac7(1-35) is comparable with the full-length native peptide, the fragment showed a lower permeabilizing activity on bacterial membranes when compared with that of its parent peptide. As Bac7(1-35) efficiently kills bacteria at concentrations around the MIC (i.e. 0.5 µM) without membrane damage, this suggests that its primary target is not the bacterial membrane. However, the fragment can also switch to a lytic mode of action, but only at very high concentrations (i.e. 64 µM). So, the removal of the C-terminal part of the natural Bac7 led out a dual mode of action for Bac7(1-35): a markedly non lytic mechanism when the fragment is used at MIC or lower, and an additional lytic activity at much higher

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Figure 3. Minimum inhibitory concentration (µM) of Bac7 and of its synthetic fragments against different bacterial strains (Benincasa et al., 2004).
concentrations (higher than that required by the original Bac7 to destabilize membranes) (Podda et al., 2006).

The internalization of the peptide by bacterial cells is an essential step for the non-lytic mode of the Bac7(1-35) antimicrobial activity (as for other PR-AMPs). The transport of Bac7(1-35) across the membrane, has been shown to be partially stereo-specific. The D isomer of Bac7(1-35), D-Bac7(1-35), was not efficiently internalized into *E. coli* cells, suggesting the presence of a stereo specific transporter. D-Bac7(1-35) showed a considerably lower antimicrobial activity if compared with the L-isomer. Moreover, the D-Bac7(1-35) residual activity appears only when the peptide reaches high concentrations and in parallel with the permeabilizing effects also observed for the L-Bac7(1-35) (Podda et al., 2006). As observed for Bac7(1-35), a highly different activity of the two enantiomers was also observed for other PR-AMPs. The importance of these data emerges when considering that the antimicrobial activity of the D-enantiomers of lytic AMPs is identical to that of their L-counterparts (Bland et al., 2001). This strengthens the idea that the bactericidal activity of Bac7(1-35) is not primarily due to membrane permeabilizing effects.

![Fig. 4. Localization of Bac7 fragments in *E. coli* cells by immunogold electron microscopy. A) Untreated cells, B) cells treated with 10 μM Bac7(1-35), F) cells treated with 10 μM D-Bac7(1-35). The length of the bars corresponds to 0.1 μm (adapted from Podda et al., 2006).](image_url)

It has also been proposed that the internalization of Bac7(1-35) is an energy-dependent process. Data on antibacterial activity of this peptide towards bacteria treated with the metabolic uncoupler dinitrophenol (DNP), resulted in a reduced bactericidal activity. The non-lytic mechanism of action of Bac7(1-35) occurs only if bacteria are metabolically active, considering that proton motive force is collapsed by the presence of DNP. Keeping in mind that these experiments have been performed using non-permeabilizing concentrations of peptide, overall these data suggest that the non-lytic antimicrobial activity of Bac7(1-35) can only occur if the peptide has been internalized by bacterial cells (Podda et al., 2006).
Screening of a pool of *E. coli* mutants led to identification of the inner membrane protein SbmA as the transporter used by Bac7(1-35) to enter bacterial cells. In fact, it was observed that deletion or specific single mutations of the *sbmA* gene led to a 4-8 fold decrease in the sensitivity of the mutants to Bac7(1-35), to other Bac7-derived fragments and also to other PR-AMPs (i.e., apidecin 1b, and fragments of Bac5 and PR-39).

The *sbmA* protein was already involved into the mechanism of action of antimicrobial molecules. It was in fact previously discovered that its deletion in *E. coli* conferred a resistant phenotype to microcin B17 (Laviña et al., 1986) and microcin MccJ25 (Salomón and Farías, 1995), two polypeptide antibiotics produced by *E. coli* when harbouring the respective plasmids, and completely unrelated to the PR-AMPs. It was also discovered that *sbmA* mutations conferred a resistant phenotype against bleomycin, a glycopeptide antibiotic produced by the bacterium *Streptomyces verticillus* (Yorgey et al., 1994).

Subsequently, by following the uptake of a fluorescent derivative of Bac7(1-35) into the bacterial cells, it was observed that mutants carrying the deletion of the *sbmA* gene, showed a decreased amount of internalized Bac7(1-35). These results indicate that lack of the *sbmA* protein protects bacteria from the bactericidal activity of Bac7(1-35) by reducing its translocation across the inner membrane and, consequently, its concentration inside the bacteria (Mattiuzzo et al., 2007).

The role of *sbmA* in the internalization of Bac7(1-35) in *E. coli* cells has been elucidated, but it is possible that other paths are used by the peptide to enter the bacterial cell. The deletion of *sbmA* decreases the uptake of Bac7(1-35) by *E. coli* cells and their sensitivity to the peptide, but it does not result in a complete resistance neither it does totally prevent the penetration of the peptide into the bacterial cytosol (Mattiuzzo et al., 2007). Further studies are necessary to identify additional transporter(s) used by Bac7(1-35) to enter the bacterial cells.

The intracellular structures that this peptide targets to kill bacteria are still partially unknown. Experiments of affinity binding have been performed immobilizing Bac7(1-35) on a resin and letting flow through a complete *E. coli* lysate (Scocchi et al., 2009). As it has been previously assessed for other insect PR-AMPs (Kragol et al., 2001), also Bac7(1-35) was shown to interact *in vitro* with the chaperone protein DnaK. A high binding affinity between the immobilized Bac7(1-35) and the chaperone has been reported. When the same experiment was repeated with D-Bac7(1-35) isomer, no binding of DnaK was observed, supporting a strong, specific and stereo-dependent interaction of Bac7(1-35) with this chaperone. DnaK works as a complex with DnaJ and GrpE to exert its role of chaperone (Mayer and Bukau, 2005). It has been assayed whether or not Bac7(1-35)
could inhibit the chaperone activity of the whole complex. Results showed that the presence of the peptide at concentrations in the range 10-100 μM, inhibited the DnaK/DnaJ/GrpE-dependent refolding activity of an unfolded luciferase, indicating that the peptide could not only bind DnaK, but also impair its activity. Due to the important role exerted by this protein to prevent protein misfolding and aggregation, the inhibition of the DnaK could represent one of the targets of this peptide. However, it is unlikely that DnaK is the only Bac7(1-35) intracellular target. In fact, when assayed at a permissive temperature, \textit{E. coli} ΔDnaK strains did not show any significant decreased susceptibility to the peptide, suggesting that other intracellular targets are present and other killing mechanisms are used by Bac7(1-35) (Scocchi et al., 2009).

A different approach was also undertaken to try to clarify the effects of Bac7(1-35) on the bacterial cell, i.e. an analysis of the bacterial gene expression in \textit{E. coli} in the presence of sub-lethal doses of the peptide. The peptide influenced the bacterial transcriptional profile: 26 \textit{E. coli} genes were significantly down-regulated and 44 were up-regulated (Tomasinsig et al., 2004). Among the down-regulated genes, many are involved in bacterial metabolism, but the highest levels of repression was observed for genes encoding proteins for maltose transport systems and for the ribose transporter. Regarding the up-regulated genes, the most up-regulated one was the sensor protein BasS. This protein shows high similarity with the PmrB protein of \textit{S. typhimurium}, involved in modifying the LPS molecule by decreasing its negative charge and, as a consequence, reducing the binding of antimicrobial peptides to this macromolecule. \textit{DnaK} was also found to be up-regulated (even if not at high levels). The influence of Bac7(1-35) on the expression of this protein seems in agreement with the biochemical assays that suggest DnaK as an intracellular target for Bac7. Moreover, many genes involved in the transcription/translation processes were found to be up-regulated, suggesting a complex and multi-target mechanism of action for this peptide (Tomasinsig et al., 2004).

Overall, what emerges from both biochemical and genetic approaches is that the mechanism of action of Bac7(1-35) is complex in great part still unclear.

The understanding of the precise molecular mechanism of action of an AMP and of its fragments is crucial for its future therapeutic use alone or in combination with conventional antibiotics, in the fight against antibiotic-resistant pathogens.

Bac7(1-35) should be taken into account for the transfer from academic research to clinic research. In this scenario, this peptide is a good candidate to be studied in order to use it directly as a drug, or to obtain from it some even more effective derivatives. If compared to other antimicrobial peptides,
Bac7(1-35) shows low permeabilising activity on eukaryotic cells at bactericidal concentrations. It was shown that this peptide can be internalized also by eukaryotic cells taking advantage of the micropinocitosys and of a not completely understood membrane-penetration mechanism (Tomasinsig et al., 2006). Once in the cells, Bac7(1-35) accumulates mainly in the nucleoli, forms small aggregates on the cell-membrane and seems to freely diffuse into the cytosol. However, penetration of the peptide into eukaryotic cells does not seem to negatively affect their viability (Tomasinsig et al., 2006). A second aspect of interest for the therapeutic use of Bac7(1-35) is its capability to neutralize endotoxin both in vitro and in vivo. Thanks to this ability, the peptide was shown to protected rats from septic shock by Gram-negative bacteria (Ghiselli et al., 2003). Starting from these positive features, a recent study demonstrated the capacity of Bac7(1-35) to decrease the mortality in a population of mice infected with a lethal dose of Salmonella typhimurium. These promising results have been obtained in spite of partial Bac7(1-35) proteolysis and inactivation by unknown blood components, and in spite of a fast clearance of the peptide from the body because of a quick renal excretion rate (Benincasa et al., 2010). Moreover, regarding the sensitivity to proteases, it is worth nothing that the unmodified Bac7(1-35) shows an half-life which other PR-AMPs have only after ad hoc chemical modifications. (Berthold et al., 2013, Benincasa et al., 2010). All together, these data suggest that Bac7(1-35) or its derivatives could be molecules that in the near future support or replace the classic antibiotic therapy against the threat of drug-resistant microbes.

The aim of this thesis was to better characterise the intracellular mechanism of killing of Bac7(1-35), in order to get a complete scheme of its mode of action, and, as a future prospective, to make possible a therapeutic application of derivatives based on this antimicrobial peptide.
MATERIAL AND METHODS

Bac7(1-35) synthesis
The N-terminal fragment 1-35 of Bac7 was synthesized by the solid phase method on a CEM Liberty microwave-assisted peptide synthesizer using a 2-chlorotrityl chloride resin (substitution 0.20-0.25 mmol/g). Loading of the first amino acid was carried out manually.

The peptide was then cleaved from the resin by incubation for 4 h on a shaker in the following mixture: 87% trifluoroacetic acid (TFA), 8% 3,6-dioxa-1,8-octane-dithiol (DODT), 2% triisopropylsilane (TIPS), 3% water. The cleaved peptide was washed several times with tert-butyl methyl ether (TMBE) and dried with N₂. The crude peptide was then purified using a preparative C₁₈ RP-HPLC column (Waters Delta-Pak™ C₁₈, 5 μm, 300 Å, 25 x 100 mm). The quality of the peptide was checked by mass spectrometry (ESI-MS Bruker Esquire 4000) after purification. The fractions containing the greatest amount of peptide were then collected, freeze-dried, resuspended in 2 ml of 10 mM HCl and re-freeze-dried.

All the peptide solutions used in the assays described below were prepared from a stock solution of the purified peptide dissolved in water. The peptide concentration was determined by at least three methods:

I. by weighing the peptide used to prepare the solution;
II. by using an adaptation of the Waddell method in parallel with a reference;
III. by considering the contribution to the extinction coefficient at 215 nm of Trp, Tyr, His, Phe, Pro, Met and of the peptide bonds (adaptation of Kuipers and Gruppen 2007).

Araasin 1(1-23)
The peptide araasin 1(1-23) was purchased from Biomol International (Exeter, UK).

Bac7(1-35) radioactive labelling
Bac7(1-35)Cys was synthesised, cleaved from the resin and purified as reported above. Two mg of Bac7(1-35)Cys were dissolved in 300 μl of 10 mM HCl (pre-bubbled with N₂), divided in 5 aliquots of 60 μl and immediately frozen at -20 °C until use, to prevent cysteine oxidation and dimers formation. From that point, all the procedures have been done in the dark, to avoid iodoacetamide (IAA) photo-damaging. 25 μl of C¹⁴-iodoacetamide (stock 2 mM C¹⁴-IAA in ethanol, 50-60 mCi/mmol, Biotrend) were diluted in 175 μl of 0,5 M Tris-acetate + 2 mM Na₂EDTA, pH 8, named Tris8 (pre-bubbled with N₂). Then 5 μl of 0,1 mM ascorbic acid were
added, immediately followed by the first 60 μl aliquots of Bac7(1-35)Cys; the mixture was left at room temperature under gentle agitation for 30 min in a N₂ enriched atmosphere. Other two Bac7(1-35)Cys aliquots were added following the same procedure every 30 min. The fourth aliquot of Bac7(1-35)cys was then added after 15 min, immediately followed by 8 μl of non radioactive 10 mM IAA dissolved in Tris8 to reduce the formation of peptide dimers, and by 2 μl of 1 mM ascorbic acid. After 15 min, the last aliquot of Bac7(1-35)Cys was added, immediately followed by 8 μl of non radioactive 100 mM IAA dissolved in Tris8 to reduce the formation of peptide dimers, and by 2 μl of 1 mM ascorbic acid. After 15 min, 100 μl of non radioactive 100 mM IAA dissolved in Tris8 were added to the reaction to completely saturate any free cysteine, immediately followed by 2 μl of 10 mM ascorbic acid; the mixture was vigorously vortexed for 60 sec. Then 8 μl of 0,5 M citric acid were added to block the reaction and the mixture was vigorously vortexed for 20 sec.

The whole reaction mixture was immediately loaded on a desalting column (Hi-trap desalting, GE Healthcare) and separated by HPLC using 100 mM NaCl in MQ-water and a flux of 1 ml/min for the elution. The fractions were collected and analysed by mass-spectrometry. The fraction having the highest content of labelled peptide (Bac7(1-35)Cys-AlchC¹⁴) was frozen and kept for subsequent uses.

**Determination of the intracellular concentration of Bac7(1-35)**

*E. coli* BW25113 cells were grown overnight in Mueller-Hinton (MH) broth at 37°C under agitation. The day after, 200 μl of this culture were put in 10 ml of new MH broth and incubated at 37°C under agitation until they reached an optical density at 600 nm (OD₆₀₀) of approximately 0.3. Subsequently, the bacterial suspension was centrifuged (2000xg, 20 minutes), the MH medium was discarded and the pellet was resuspended in 5 ml of M9-salt minimal medium. The bacterial suspension was then diluted to the final concentration of 4×10⁸ CFU/ml and incubated for 10 minutes with 10 μM Bac7(1-35)Cys-AlchC¹⁴ at 37°C with agitation. After incubation, part of the culture was centrifuged (6200xg, 8 minutes), the medium was collected for radioactivity measurement and the pellet was resuspended in an equal volume of new M9-salts medium for the measurement. In parallel, another aliquot of the culture, was centrifuged (6200xg, 8 minutes), the medium was discarded and the pellet was resuspended and washed in an equal volume of high-salt PBS (PBS buffer + 500 mM NaCl). The sample was then centrifuged (6200xg, 8 minutes), the high-salt PBS was discarded and the pellet was resuspended in an equal volume of new M9-salts medium for radioactivity determination. The OD₆₀₀ of each sample was measured, in order to normalize the results of the radioactivity count with the number of bacteria present in the samples. An untreated culture of BW25113 and the M9-salt minimal medium were used as blanks. The measurements
were performed, for each experiment, in triplicate mixing 200 µl of each sample with 500 µl of scintillation liquid OptiPhase Supermix (PerkinElmer). A β-counter MicroBeta Trilux (Wallac, Turku, Finland) was used.

The calculation of the intrabacterial amount of Bac7(1-35)Cys-AlchC\textsuperscript{14} was carried out by taking into account the volume of a single K12-derived \textit{E. coli} cell (0.58 fl) (Kubitschek, 1990), the number of bacteria (4×10\textsuperscript{7} CFU) and the volume of sample used for each measurement (200 µl).

**DNA-peptide interaction assays**

The reaction, (as reported in Park et al., 1998) was set up into 200 µl tubes mixing 2 µl of buffer 10× (50% glycerol; 100 mM Tris-HCl pH 7; 10 mM EDTA; 10 mM DTT; 200 mM KCl; 500 µg/ml BSA), 100 ng of plasmidic DNA (pBluescript II KS(+) linearized by using EcoRV, (New England Biolabs), and different aliquots (12.5, 25, 50, 100, 200, 400 and 800 ng) of antimicrobial agent [Bac7(1-35); D-Bac7(1-35), LL-37, BMAP-27, or Polymyxin B], and sterile MQ-H\textsubscript{2}O to a final volume of 20 µl. MQ-sterile H\textsubscript{2}O was used as a negative control in place of the antimicrobial agent. The samples were then incubated for 1 hour at 37° C. The reaction mixture was 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 parallel with a ladder DNA (SharpMass 1 kb DNA ladder, EuroClone).

**In vitro transcription**

The reaction was set up using the commercial kit Riboprobe\textsuperscript{®} System–T7 (Promega) following for all the samples the instructions suggested by the supplier for the positive control, but adding the antimicrobial agents to a final concentration of 50 µM as last component of the reaction before the incubation. As a consequence, the suggested volume of RNAse-free water added to reach the final volume of 20 µl was reduced. For the negative control, no DNA template was added to the reaction. At the end of the reaction, the \textit{in vitro} transcribed RNA was purified using TRIzol\textsuperscript{®} (Life technologies), following the instructions suggested by the supplier for the RNA isolation from “suspension cells” samples. Size and amount of the transcribed RNA were evaluated using a Bioanalyzer 2100 (Agilent) in combination with the commercial kit Agilent RNA 6000 nano kit (Agilent).
**In vitro transcription/translation**

The reaction was set up using the commercial kit S30 T7 High-Yield Protein Expression System (Promega) following for all the samples the instructions suggested by the supplier for the positive control, adding to each reaction (modifying the original protocol) 40 U of RNAses inhibitor (Rna-se Inhibitor Murine, New England Biolabs) and as last component of the reaction the antimicrobial agent to a final concentration of 50 μM. Samples were incubated at 37°C for 60 min under vigorous agitation (1200 rpm).

At the end of the reaction, the presence of the reporter protein (*Renilla reniformis* luciferase) was assessed and quantified using the commercial kit Renilla Luciferase Assay System (Promega) following the instructions of the supplier, in combination with a luminometer (Plate Camaleon, multilabel detection factor, Hidex; software Mikrowin 2000). In addition, the samples were treated as suggested by the T7 High-Yield Protein Expression System instructions, diluted in sample buffer, loaded onto an polyacrilamyde gel at 12,5% and separated by electrophoresis (see below).

**SDS-PAGE Analysis**

The production of the of the proteins was analysed, following the suggestions of the supplier, under denaturing conditions on a gel with a thickness of 0.75 mm prepared with 12.5% acrylamide for the separation (Resolving gel) and of 4% for the loading (Stacking gel) of the samples. The solutions for the Resolving and Stacking gels were prepared as described in Table 1. The gel was then assembled into a vertical electrophoresis system (Biorad) and covered with the Running Buffer. Protein samples were prepared by adding Sample Buffer 4× in order to dilute it to 1×, followed by heating for 10 minutes at 90°C, cooling down on ice for 5 minutes and loading onto the gel (usually 8 μl for each well). The separation was performed at 50 V until the samples reached the interface between stacking and running gel, and then, at 200 V until the bromophenol blue came out from the gel. Gels were stained overnight in the Coomassie Staining Solution with shaking and then destained for 2 h with shaking in the Destaining Solution.

<table>
<thead>
<tr>
<th>Stacking gel (4%)</th>
<th>Final volume 5 ml</th>
<th>Resolving gel (12.5%)</th>
<th>Final volume 10 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% acrylamide solution</td>
<td>0.25 ml</td>
<td>40% acrylamide solution</td>
<td>1.57 ml</td>
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Table 1. List of reagents and volumes used to prepare SDS-PAGE gels.
KEIO library assembly
All the 3985 Keio mutant clones (Baba et al., 2006) were grown separately overnight following the instructions of the supplier. The day after, 5 µl of every mutant strain were picked up and mixed with the others, in order to create a single pool comprising all the 3985 KO strains of the collection, which was divided in aliquots and stored at -80°C.

KEIO library screening
Bac7(1-35) selection.
A frozen aliquot of the Keio mutant pool (approximately 30 µl) was inoculated in 5 ml of MH broth containing 25 µg/ml kanamycin ad incubated at 37°C under agitation for 2 hours (OD_{600}=0.31). Subsequently, 5×10^5 CFU were spread on a MH-agarose plate containing 40 µM Bac7(1-35) and incubated overnight at 37°C. The same amount of CFU was spread on MH-agar plates, as a growth control. The day after, the colonies grown in presence of the peptide were spread on MH-agar containing 50 µg/ml kanamycin to assess their identity as KEIO KO mutants. The clones were then cultured overnight in MH containing 50 µg/ml kanamycin and stored at –80°C.
**Arasin1(1-23) selection**

A frozen aliquot of the Keio mutant pool (approximately 30 μl) was inoculated in 10 ml of MH broth containing 25 μg/ml kanamycin and incubated at 37°C under agitation for 4 hours (OD_{600}=0.53). Subsequently, 5×10^5 CFU were spread on a MH 50% agarose plate containing 10 μM arasin1(1-23) and incubated overnight at 37°C. The same amount of CFU was spread on MH 50%-agarose plates, as a growth control. The day after, colonies grown in the presence of peptide were spread on MH-agar containing 50 μg/ml kanamycin, to assess their identity as KEIO KO mutants. The clones were then cultured overnight in MH containing 50 μg/ml kanamycin and stored at –80°C.

**In vivo incorporation of radioactive isotopes**

BW25113 E. coli cells were grown overnight in Mueller-Hinton (MH) broth at 37°C under vigorous agitation. The day after, 200 μl of this culture were put in 10 ml of new MH broth and incubated at 37°C under vigorous agitation until they reached an optical density at 600 nm (OD_{600}) of approximately 0.3. The bacterial suspension was then centrifuged (2000xg, 20 minutes), the MH medium was discarded and the pellet was resuspended in an equal volume of M9-salt minimal medium containing 5mM glucose and 1% v/v MH broth, in order to guarantee the metabolic activity of bacteria. The bacterial suspension was then diluted to a concentration of 1.5×10^7 CFU/ml and 650 μl of this culture were incubated into 15 ml tubes at 37° in a water bath for the whole experiment. After a pre-incubation of 5 minutes to acclimatize bacteria, samples were treated as follows.

Leucine incorporation: at time 0 min, 7.5 μl of ^3^H-Leucine (1 mCi/ml, Perkin Elmer) were added to 650 μl of bacteria. After quick mixing by pipetting, 100 μl of sample were immediately picked up, diluted in 5 ml of ice-cold tricloroacetic acid (TCA, 10% in water), added with 100 μl of BSA (10 mg/ml in water), vortexed a few seconds and put on ice for 150 min. 4.5 μl of 100 μM Bac7(1-35) were added 11 min after the addition of the ^3^H-Leucine (time 11), to reach the final concentration of 1 μM Bac7(1-35). Samples were taken and treated as described above at time 10, 20, 30, 40 and 50 min, shifting the picking up from the different series of samples of 1 min. A positive control was set up in a different sample and using the same timing, by adding 9 μl of 10 mM kanamycin to reach the final concentration of 200 μM kanamycin. Finally, a negative control was set up in a different sample by adding 9 μl of sterile MQ-water instead of antimicrobial agents.

Uridine incorporation: at time 0 min, 6.5 μl of 100 μM Bac7(1-35) were added, to reach the final concentration of 1 μM Bac7(1-35). A positive control was set up in a different sample and using the
same timing, by adding 5.2 μl of 10 mg/ml rifampicin, to reach the final concentration of 100 μM rifampicin. After 20 min, 4 μl of 3H-Uridine (1 mCi/ml, Perkin Elmer) were added to 650 μl of bacteria and quickly mixed by pipetting; 100 μl of the sample were immediately picked up, diluted in 5 ml of ice-cold TCA (10% in water), added with 100 μl of salmon-sperm DNA (10 mg/ml in water), vortexed a few seconds and put on ice for 150 min. Samples were taken as above at time 24, 28, 32, 36 and 40 min, shifting of 1 min the picking up from the different series of samples. As a negative control, 6 μl of sterile MQ-water were added in a different sample instead of antimicrobial agents.

Thymidine incorporation: at time 0 min, 6.5 μl of 100 μM Bac7(1-35) were added, to reach the final concentration of 1 μM Bac7(1-35). A positive control was set up in a different sample and using the same timing, by adding 6.5 μl of 10 mM nalidixic acid, to reach the final concentration of 100 μM nalidixic acid. After 20 min, 4 μl of 3H-Uridine (1 mCi/ml, Perkin Elmer) were added to the 650 μl of bacteria and quickly mixed by pipetting; 100 μl of sample were immediately picked up, diluted in 5 ml of ice-cold TCA (10% in water), added with 100 μl of salmon-sperm DNA (10 mg/ml in water), vortexed a few seconds and put on ice for 150 min. Samples were taken as above at time 24, 28, 32, 36 and 40 min, shifting of 1 min the picking up from the different series of samples. In a different sample, as a negative control, 6.5 μl of sterile MQ-water were added instead of antimicrobial agents.

Filtration: after the precipitation, samples were incubated for 15 min at 37°C and filtered on 0.22 μm filters (0.22 μm GSTF, cellulose ester, Millipore) pre-washed with 5 ml 10% TCA using an under vacuum multiple filtering device (Millipore). The tubes were then washed using 5 ml of new ice-cold 10% TCA, and the TCA used for the washing was filtered on the same filters. The filters were then washed by filtering 5 ml of new ice-cold TCA, directly poured on the filter. The filters were then dried for 40 min on paper, put separately into 4 ml of scintillation fluid (Ecolite(+) liquid scintillation cocktail, MP biomedicals), and stored for 60 min at room temperature. Radioactivity was then quantified by using a β-counter (2200 CA TRI-CARB liquid scintillation analyzer, Packard), measuring each sample for 10 min.

**Colony PCR**

Minimal amounts of bacterial colonies were suspended in 50 μl sterile MQ-water and heated at 90°C for 5 min to lyse the cells. Subsequently, samples were cooled on ice for 5 min and 2 μl lysate were used as template for PCR. The following components were mixed for the PCR reaction (50
μl): 5XGoTaq buffer (("Green" containing electrophoresis loading buffer, Promega); 25 mM MgCl2 (Promega); 2,5 U GoTaq DNA polymerase (Promega); 10 mM dNTPs; primers sbmA-FW (ATGTTTAAGTCTTTTTTCC) (0,5 μM) and sbmA-RV (AGCTCAAGGTATGGGTTAC) (0,5 μM); 1 μl of bacterial lysate and water. The amplification was carried out on a EuroClone One Advanced thermocycler using the following program: pre-denaturation (95°C, 5 min), 30 amplification cycles (95°C, 1 min; 62°C, 1 min; 72°C, 65 sec), final elongation (72°C, 5 min). The products were separated by electrophoresis on 1% agarose gel (50 V, 1 h) and visualised on a UV trans-illuminator.

**Bacterial growth kinetics**

To determine the growth inhibition of the bacteria exposed to various peptide concentrations, we chose an OD-based approach to measure the cell density changes over the incubation time on a microtiter plate. Mid-log phase bacteria cultures were diluted in 50% MH broth to 2 × 10^6 CFU/ml and 100 μl of each dilution were added to 100 μl of peptide solutions, previously prepared on a microtiter plate, or to 100 μl of medium with no peptide, as a control. Thus a suspension of 10^6 CFU/ml, with the peptide at half of the original concentration, was placed in a plate reader (Tecan Sunrise, Switzerland) and incubated at 37°C throughout the assay with intermittent shaking. The OD change was measured at the wavelength of 620 nm with 30-minute intervals for 8 hours. During the incubation, the plate was covered with a sterile plastic film, to prevent evaporation. Each single experiment was performed in triplicate and data collected by Magellan 4 software (Tecan) and averaged.

**Minimum inhibitory concentration (MIC)**

In order to determine the lowest peptide concentration that inhibits the growth of the tested strains (Minimum Inhibitory Concentration, MIC), an o/n culture in MH [50% MH for arasin1(1-23) or 100% for Bac7(1-35)] and, the day after, the dilution 1:50 of the culture in the same fresh medium were set up to obtain mid-log phase cells. The culture was diluted to 5×10^5 CFU/ml. The peptide was diluted serially in the wells of a round-bottom microtiter plate in 50 μl of MH broth [50% for arasin1(1-23) or 100% for Bac7(1-35)], and then 50 μl of bacterial suspensions previously prepared into the same medium were added to the wells, thus diluting the peptide and the bacteria to half of their initial concentrations in each well. The whole plate was covered with a sterile adhesive plastic film to minimise evaporation and incubated for 24 hours at 37°C. The MIC was considered the lowest peptide concentration preventing visible growth of the bacteria.
RESULTS

_E. coli_ deletion mutant pool preparation and search for new Bac7(1-35) resistant mutants._

Previously, it was demonstrated that deletion of _sbmA_, a gene encoding an inner membrane protein, confers resistance to Bac7(1-35) to _E. coli_ (Mattiuzzo et al., 2007). In order to further investigate the mechanism of action of this peptide, we tried to obtain additional mutants showing decreased susceptibility to Bac7(1-35). We prepared a pool of 3985 different KO mutants of _E. coli_, collecting together all the single KO mutants from the Keio Collection (Baba et al., 2006). This pool was then screened on solid medium using the lethal concentration of 40 μM Bac7(1-35). By this selection, starting from approximately 5·10⁴ CFU, we collected 48 putatively resistant clones. Then we checked for the presence of the _sbmA_ gene in all the selected clones by colony-PCR. Unfortunately, 48 clones out of 48 presented the deletion of the _sbmA_ gene (data not shown). The lack of resistant clones presenting the deletion of genes different from _sbmA_ suggests that there are no other non-viable genes involved into the Bac7(1-35) mechanism of action. As a consequence, it is reasonable to believe that one of the main strategies of resistance against this peptide known to date, is the reduction of its internalization by downregulating, inactivating or deleting the SbmA protein.

Search for arasin1(1-23) resistant mutants.

With the aim to obtain a more general view of the PR-AMPs mechanism of action, we decided to carry out in parallel a similar screening of the KEIO library but using arasin1(1-23), an uncharacterized fragment derived from a different PR-AMP previously isolated from the spider-crab (_Hyas araneus_) (Stensvåg et al., 2008) (Paulsen et al., 2013).

**SRWPSPGRPRPFPGRPKPIFRPRPCNCYAPPCCDRWRH**

Figure 5. Amino acid sequence of the whole mature peptide Arasin1. The fragment 1-23 is shown in bold.

We thought that if PR-AMPs such as Bac7(1-35) and arasin1(1-23) share some internal target and/or mechanism of action, this second screening with arasin1(1-23) could allow us to isolate some mutants not yet identified using Bac7(1-35). Any possible overlapping in the mechanism of resistance to these two peptides could be potentially useful for also understanding the mechanism of
action of Bac7(1-35). Our intention was thus to correlate any obtained result with the data accumulated during the search of the Bac7(1-35) intrabacterial target.

During the preliminary experiments for the selection, we noticed that the activity of arasin1(1-23) is salt-sensitive (data not shown). Because of its salt-sensitivity, all the following assays with this peptide were performed in MH 50% both in solid and in liquid medium.

The Keio collection was employed to identify single genes that could mediate the antibacterial action arasin1(1-23). Plating bacterial mutants (approximately $5 \times 10^4$ CFU) on 50% MH agar supplemented with the lethal concentration of 10 μM of arasin1(1-23), resulted in the survival of nine putative resistant clones named KeAR1 to 9. A preliminary Minimum Inhibitory Concentration assay by micro-dilution (MIC) showed that the sensitivity to arasin1(1-23) of all the isolated mutants was lower than that of the wt strain (data not shown).

Knowing the importance of the SbmA protein for susceptibility not only to Bac7(1-35) but also to several other PR-AMPs [Mattiuzzo 2007], we screened KeAR1-9 clones for the presence of an intact copy of $sbmA$ gene by using colony-PCR. We detected a band of 1221 bp corresponding to the $sbmA$ gene only in three of the clones (KeAR5, KeAR6 and KeAR9), while it was absent in all the other cases (Fig. 6).

![Figure 6. PCR analysis of $sbmA$ on KeAR1-9 isolated clones. Line 1: DNA ladder. Line 2: negative control, no bacteria. Line 3: positive control, wt BW25113. Line 4 positive control for Keio mutant BW25113ΔwaaY. Line 5 BW25113Δ$sbmA$. Line 6 to 14: KeAR1 to KeAR9 clones.](image)

By sequencing a randomly selected $sbmA$-negative clone (KeAR1), we verified if the $sbmA$ gene was interrupted . Western-blot analysis of total protein lysates of all the KeAR mutants, using a specific anti-SbmA antibody, confirmed the expression of SbmA only in the KeAR5, KeAR6 and KeAR9 clones (data not shown). Thanks to a collaboration with Dr. Stensvag’s group of the University of Tromsø (Norway), these three clones were subjected to sub-cloning and sequencing of genomic DNA. The gene deletions were localized in correspondence of the genes $ygdD$ (KeAR5 and KeAR9) and $nrdE$ (KeAR6). $ygdD$ is an inner membrane protein of unknown function, $nrdE$ is a component of the subunit α of a ribonucleoside-diphosphate reductase.
In order to better characterise the resistance to arasin1(1-23) of the isolated clones, more precise MIC and bacterial growth kinetics assays were performed. The mutants for all genes showed a reduced sensitivity to arasin1(1-23) if compared to the wild-type in terms of MIC (Table 2) and in terms of Growth kinetic (Fig. 7), growing better and faster in the presence of peptide than the wild-type strain.

Table 2. MIC assays of arasin(1-23) in 50% MH against wild-type *E. coli* BW25113 and deletion mutants. Results are the average of at least three independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>BW25113</th>
<th>BW25113ΔsbmA</th>
<th>BW25113ΔrdE</th>
<th>BW25113ΔygdD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC Arasin 1(1-23)</td>
<td>8 μM</td>
<td>32 μM</td>
<td>16 μM</td>
<td>16 μM</td>
</tr>
</tbody>
</table>

Figure 7. Bacterial growth kinetics of wt and mutant strains of *E. coli*. Growth kinetics were performed in 50% MH in absence and presence of 8 μM arasin1(1-23). Wild-type BW25113 (black diamonds), BW25113ΔygdD (green triangles), BW25113ΔnrdE (violet crosses), BW25113ΔsbmA (red squares). Results are the average of at least three independent experiments.

These data demonstrate that the single deletion of the genes *nrdE* and *ygdD* can reduce the sensitivity of *E. coli* to arasin1(1-23). As a consequence, our results suggest an involvement of the proteins encoded by these genes into arasin 1(1-23)’s mechanism of action.

**Looking for a common mechanism of action of PR-AMPs**

Having assessed the involvement of NrdE and YgdD in the mechanism of action of the arasin1(1-23), we returned to Bac7(1-35) in order to investigate if these proteins could somehow influence the bacterial sensitivity also to this antimicrobial peptide. Indeed, as both arasin1(1-23) and Bac7(1-35) belong to PR-AMPs, it would be very interesting to find some overlap between the mechanism of action of these two molecules.
We thus performed some MIC assays on the isolated clones lacking the \textit{nrdE}, \textit{ygdD} and \textit{sbmA} genes using Bac7(1-35) in complete medium (data not shown), but only the BW25113Δ\textit{sbmA} clone showed a decreased sensitivity (in agreement with the results previously reported by Mattiuzzo et al., 2007) if compared to the wild-type BW25113. These data suggest that even if arasin1(1-23) and Bac7(1-35) belong to the PR-AMPs, the only shared mechanism regards the internalisation via the SbmA protein. Arasin1(1-23) and Bac7(1-35) probably act by targeting different intra-bacterial structures or processes.

Once having assessed that working on arasin1(1-23) would not provide insights on the Bac7(1-35) mechanism of action, but would open a new and wide research-field, we decided to focus our attention to Bac7(1-35) only.

\textbf{Intracellular concentration of Bac7(1-35)}

Before starting any \textit{in vitro} assay to investigate the mechanism of action of Bac7(1-35), it was mandatory to estimate the concentration that the peptide could reach into the bacterial cytoplasm. A precise evaluation of its intracellular amount was very important to simulate \textit{in vitro} conditions and concentrations that are as close as possible to those present in Bac7-treated bacteria.

To this aim, a C\textsuperscript{14}-labeled radioactive derivative of Bac7(1-35) was prepared: Bac7(1-35)Cys-AlchC\textsuperscript{14}. We estimated the amount of Bac7(1-35)Cys-AlchC\textsuperscript{14} that had penetrated into bacteria by measuring the radioactivity intensity of the pellet with respect to that remaining in the medium. \textit{E. coli} BW25113 cells were incubated in liquid medium in the presence of 10 \textmu\text{M} Bac7(1-35)Cys-AlchC\textsuperscript{14}. The bacterial suspension was then centrifuged, the medium was collected and the bacterial pellet was washed and then resuspended. Using the same volume of collected supernatant and resuspended pellet, we measured the radioactivity. We found that a significant amount of the peptide was intracellular (Fig. 8). Since a prokaryotic cell volume is a minimal fraction the total volume, we calculated that inside bacteria the peptide reached a concentration of approximately 3,7 mM. An increase of the peptide concentration into bacterial cells of more than 300-fold suggests an active import mechanism for the internalization of Bac7(1-35) into \textit{E. coli} and/or binding of the peptide to intracellular structures.
Bac7(1-35) inhibits the transcription/translation process

It has been previously shown that the administration of sub-lethal amounts of Bac7(1-35) to an *E. coli* culture, led to the up-regulation of many genes involved into the transcription/translation process (Tomasinsig et al, 2004). It may be, that the over-expression of these genes represents the physiological response of stressed bacteria trying to restore some mechanisms whose functionality has been reduced by the peptide. Moreover, the positive charge of Bac7(1-35) suggested its electrostatic attraction to nucleic acids and to the nucleoid region, where the transcription/translation process takes place.

To verify this hypothesis, *in vitro* transcription/translation experiments were performed. Increasing amounts of Bac7(1-35) were added to *in vitro* transcription/translation reactions in order to assess any peptide’s inhibiting activity on these processes. The inhibitory effect of the peptide was evaluated by measuring the level of transcription/translation of a reporter gene: luciferase from *Renilla reniformis*. The production of luciferase was thus estimated by SDS-PAGE and by luminometry. Results showed that Bac7(1-35) inhibits the transcription/translation process, and that it acts in a concentration-dependent manner (Fig. 9). 50 μM Bac7(1-35) was the minimum peptide concentration needed to completely inhibit luciferase expression (values comparable to those of the negative control, in which the DNA template was absent). Moreover, it was observed that the peptide started to exert a significant inhibiting activity already at 1 μM, a concentration that is significantly lower than the concentration that can be reached into the bacterial cytoplasm.
Figure 9. Transcription/translation assay. Synthesis of luciferase in absence and in presence of 50 μM, 10 μM, 1 μM and 0,1 μM of Bac7(1-35) estimated A) by SDS-PAGE (black arrow indicates the luciferase band), and B) by luminescence production after reaction with the substrate. Values are indicated as percentage of the positive control. No luciferase-encoding DNA was added to a reaction as negative control. (B ) results are the average of at least three independent experiments.

The inhibition of transcription/translation is specific for Bac7(1-35)

The following step was to assess if the observed inhibiting activity was specific for Bac7(1-35). We performed in vitro transcription/translation experiments, but adding other molecules instead of Bac7(1-35) to the reactions, at the fixed concentration of 50 μM. Kanamycin was chosen as a known inhibitor of bacterial translation. LL-37 and BMAP-27 were chosen as two cationic cathelicidins showing similar size and similar net charge respectively, but unrelated structure and different mechanism of action from that of Bac7(1-35). D-Bac7(1-35), a less active (Podda 2006) D-stereoisomer of the original peptide, was chosen to investigate if the peptide’s chirality could influence its inhibiting activity of the transcription/translation process. We found that only D-Bac7(1-35) and kanamycin gave results similar to Bac7(1-35), completely blocking the transcription/translation process (Fig. 10). On the contrary, LL-37 and BMAP-27 decreased less impressively the expression of the reporter gene, suggesting an unspecific interaction with the transcription/translation process. Interestingly, the Bac7(1-35) D-isomer, known as less bactericidal
(Podda et al., 2006), showed an in vitro activity comparable to the L-isomer.

![Figure 10](image)

Figure 10. Transcription/translation assay. Luciferase production in absence or in presence of 50 μM D-Bac7(1-35), 50 μM LL-37, 50 μM BMAP-27 and 50 μM kanamycin estimated by A) SDS-PAGE (black arrow indicates the luciferase band), and B) luminescence production after the reaction with the substrate. Values are indicated as percentages of the positive control. No antimicrobial agents were added to the reaction in the positive control. No luciferase-encoding DNA was added to the reaction as negative control. (B) Results are the average of at least three independent experiments.

Knowing that Bac7(1-35) inhibited the transcription/translation process, we decided to dissect this complex mechanism in order to understand at which level the peptide exerts its action.

**Dissecting the mechanism of action of Bac7(1-35): DNA interaction assay**

As first we evaluated the in vitro binding of Bac7(1-35) to DNA. An EMSA assay in agarose gel was performed on a digested plasmid in the presence of peptide. To assess if the interaction could be reduced to a simple electrostatic interaction between the positively charged Bac7(1-35) and the negatively charged DNA, also in this case D-Bac7(1-35), LL-37 and BMAP-27 were used as a control. Each peptide was co-incubated with the same amount of digested plasmid. We observed a slight band-retardation effect similar for all the peptides at low peptide/DNA ratio, without any evident DNA-specificity for Bac7(1-35) (Fig. 11). Differently, at high peptide/DNA ratio we observed a DNA-retention in the agarose wells in the presence of each of the peptides, suggesting a peptide-induced precipitation of the nucleic acid. Moreover, this precipitation seems to have a threshold effect (Fig. 11). The similar behaviour of the different AMPs suggests that the binding of
Bac7(1-35) to DNA is likely due to electrostatic interactions, and that this interaction is not specific of one of the peptides used. Thus, it seems unlikely that Bac7(1-35) can exert an inhibitory activity at the transcription/translation level only by unspecific electrostatic binding to DNA. However, the capacity of Bac7(1-35) to aggregate the nucleic acids may contribute to a general unspecific disturbing activity on the viable intracellular processes of bacteria. In conclusion, it is reasonable to believe that other mechanisms take part to the transcription/translation inhibition.

Dissecting the mechanism of action of Bac7(1-35): in vitro effect on transcription

We then tried to establish whether Bac7(1-35) was inhibiting transcription, translation or both. To this aim, we added 50 μM of Bac7(1-35) to a commercial in vitro transcription kit (see material and method section). This is the lowest peptide concentration capable of inhibiting transcription/translation. Moreover, the previously mentioned D-Bac7(1-35), LL-37, BMAP-27 and kanamycin were used as a control at the same concentration. After the transcription reaction, the newly synthesised RNA was purified and quantified. No significant inhibition of the transcription process due to the presence of Bac7(1-35) was found (Fig. 12), suggesting that the inhibiting action of the peptide may target only the translation process. In addition, this result demonstrates that Bac7(1-35) does not affect the activity of the T7 polymerase, thus suggesting that binding of the peptide to DNA affects the transcription process only poorly.

Figure 11. DNA-peptide interaction assay. Increasing amounts of Bac7(1-35), D-Bac7(1-35), LL-37 and BMAP-27 (shown on the top of the gels and reported in nanograms) were co-incubated with 100 ng of linearized pBluescript plasmid and separated on 1% agarose gel.
Figure 12. *In vitro* transcription assay. RNA production (showed as percentage of the positive control) in the presence of 50 μM Bac7(1-35), D-Bac7(1-35), LL-37, BMAP-27 and kanamycin. In the negative control (CTRL-) no template DNA was added to the reaction. Results are the mean of at least three independent experiments.

**Effects of Bac7(1-35) on the incorporation of radioactive macromolecular precursors in *E. coli* cells.**

Having obtained *in vitro* evidence that Bac7(1-35) inhibits the translation process, we verified whether this effect also occurs in living bacteria. In order to verify any Bac7(1-35) interfering activity with the synthesis of proteins, RNA, and DNA, we measured the incorporation of radioactive leucine, uridine and thymidine in bacterial macromolecules after exposing *E. coli* cells to 1 μM peptide. As a positive control, for every tested biosynthetic pathway, we also used a well-known antibiotic capable to inhibit it, i.e. kanamycin for translation, rifampicin for transcription and nalidixic acid for DNA duplication. The concentration of 1 μM was chosen because it falls in the MIC range of Bac7(1-35) against the *E. coli* BW25113 strain.

After exposure to Bac7(1-35), bacteria showed a significant decrease in the incorporation of radioactive leucine (Fig. 13). Conversely, in peptide-treated bacteria, the incorporation of radioactive uridine and thymidine did not show any significant decrease when compared with the untreated controls. These results indicate that only protein synthesis is strongly affected by Bac7(1-35), confirming the inhibition of translation observed in the *in vitro* tests. Moreover, to get additional data supporting the link between the peptide and the reduced level of protein synthesis, we repeated the experiment of radioactive leucine incorporation, in the presence of Bac7(1-35), also using the *E. coli* BW25113ΔsbmA mutant that lacks the SbmA protein. Deletion or specific mutations of this inner membrane protein reduce the uptake of Bac7(1-35) in *E. coli* cells and, as a
consequence, also the sensitivity to this peptide (Mattiuzzo 2007). A lower inhibitory effect on the protein synthesis was thus expected with this mutant. We noticed that, in presence of Bac7(1-35), the deletion mutant showed a higher level of leucine incorporation compared to the wild-type, underlining a direct link between internalisation (and activity) of Bac7(1-35) and inhibition of protein synthesis in *E. coli* (Fig 13D).

![Figure 13](image_url)

Figure 13. Internalization of radioactive precursors in *E. coli* BW25113. A) Incorporation of leucine-³H in an untreated culture (diamonds), in the presence of 1 µM Bac7(1-35) (squares) or of 200 µM kanamycin (crosses). B) Incorporation of thymidine-³H in an untreated culture (diamonds), in the presence of 1 µM Bac7(1-35) (squares) or of 100 µM nalidixic acid (crosses). C) Incorporation of uridine-³H in an untreated culture (diamonds), in the presence of 1 µM Bac7(1-35) (squares) or of 100 µM rifampicin (crosses). D) Incorporation of leucine-³H in untreated cells (diamonds), in the presence of 1 µM Bac7(1-35) (squares) or of 200 µM kanamycin (crosses). Black arrows indicate the time of addition of the radioactive precursor; white arrows indicate addition of the antimicrobial peptide (in the graphs in which the white arrow is not shown, the antimicrobial compound was added 20 min before addition of the labelled precursor). Results are the mean of at least three independent experiments.
DISCUSSION

Data concerning the intrabacterial concentration and activity of both lytic and non-lytic antimicrobial peptides are often unclear, especially for the latter category. Actually, for lytic peptides, the targeting to intrabacterial structures has been reported at peptide concentrations that do not completely lyse the membrane. On the other hand, even though the membrane was not destroyed, its integrity under these conditions was already compromised. As a consequence, it is not easy to understand whether the internalization of the peptide is just a consequence of the membrane destabilization, or if the translocation into the cytoplasm is an independent bactericidal mechanism taking place before lysis. Moreover, some peptides are assumed to cross biological membranes, but often these evidences were obtained in vitro, with experiments based on liposomes or other forms of artificial membranes. These conditions can mimic the bacterial membrane, but do not ensure that what is observed really happens when peptides are added to whole living bacteria.

Concerning the non-lytic peptides, such as the PR-AMPs, the identity of their intracellular targets and their molecular mechanism of action are not yet clearly understood, even though their internalization in bacterial cells is less ambiguous. In this study we investigated the mechanism of action of Bac7(1-35) with the aim of identifying its intracellular targets.

In order to find genes involved in the Bac7(1-35) mechanism of action, we screened a library containing almost all the viable knock-out mutants of E. coli against the peptide fragment. All the isolated, resistant clones obtained after screening showed the deletion of the sbmA gene. This gene codes for a protein already known to be involved in the internalization of Bac7(1-35) in E. coli cells (Mattiuzzo et al., 2007). Here we assessed that to date sbmA is the only non-essential gene whose deletion was found to significantly decrease the sensitivity of E. coli to Bac7(1-35). Moreover, deletion of sbmA was the most abundant mutation also found among the clones isolated by a screening performed using arasin1(1-23), a fragment of a different PR-AMP isolated from the spider crab. Even though the physiological function of SbmA is still unknown, our results highlight that the exploitation of this protein for peptide internalization is a common feature shared by many components of the proline-rich family of AMPs and confirms the crucial role of this protein into their mode of action.
In addition, it is worth noting that the involvement of SbmA seems to be the only shared step in the mechanism of action of PR-AMPs from different sources as they seem to diverge in the downstream steps, as indicated by the results obtained with Bac7(1-35) and arasin1(1-23). Deletion of some genes decreasing the sensitivity of *E. coli* cells to arasin1(1-23) did not protect bacteria from Bac7(1-35) activity, suggesting a different mode of action of these two PR-AMPs without a clear overlapping of the killing mechanism. Further comparative studies are necessary to establish whether or not the internalization via SbmA is really a point of convergence for the activity of many (or maybe all) the PR-AMPs against *E. coli* and other Gram-negative bacteria.

Another information that is important for the understanding of the mode of action of Bac7(1-35) is the concentration that it can reach within the target bacteria. Knowledge of the intrabacterial concentration of the peptide is a crucial point because this is the effective concentration acting on the bacterial internal target(s) under non-lytic conditions. Using a radioactively-labelled Bac7(1-35) derivative, we found an impressive accumulation of the peptide within the cytosol of peptide-treated bacteria. We calculated the intracellular concentration of Bac7(1-35) in the millimolar range when the peptide was added at 10 μM in the external medium. The two-order of magnitude increase in the intracellular concentration suggests that Bac7(1-35) may be actively imported by bacteria, and/or implying that it does not easily reach an equilibrium, but that it could be quickly sequestered once in the cytoplasm, likely by binding to cytosolic components.

Concerning the difference between the internal and external concentrations, it has already been proposed that AMPs can reach on the bacterial membrane a concentration considerably higher than that of free peptide into the medium (Melo et al, 2009). Our results, although concerning the intracellular and not the surface concentration, are in agreement with this observation, indicating that the active peptide concentration within the cell is much higher than that added to the medium.

The processes of transcription and/or translation were the first candidates as possible Bac7(1-35) targets. In fact, overexpression of several genes linked to these processes were observed after the exposure of *E. coli* cells to sub-lethal amounts of Bac7(1-35) (Tomainsig et al., 2004). We speculated that the up-regulation of these genes could represent the bacterial response to the stress caused by binding of the peptide to its target(s). In addition, it seems reasonable that a highly cationic peptide could bind to and have the negatively charged nucleic acids as a target or that it could be electrostatically attracted by the nucleosome where it can find its final target(s).

The *in vitro* experiments of coupled transcription/translation assay in the presence of increasing amounts of Bac7(1-35) indicated that the peptide strongly inhibits these processes already at concentrations significantly lower than those reached by Bac7(1-35) in the bacterial cytoplasm. We
estimated that the minimum peptide concentration necessary to completely inhibit the transcription/translation of a reporter gene is approximately 50 μM (Fig. 9).

We then demonstrated that the inhibition of the transcription/translation is a specific effect of Bac7(1-35). To this aim, we tested two different α-helical cationic cathelicidins, LL-37 and BMAP-27, resembling Bac7(1-35) respectively in the number of amino acid residues or in the electrostatic charge. Both LL-37 and BMAP-27 did not significantly affect the transcription/translation processes, indicating that the inhibition due to Bac7(1-35) was a specific effect of the peptide and not simply an unspecific interference of whatever cationic peptides (Fig 10).

We also investigated if the inhibitory activity of Bac7(1-35) with respect to transcription/translation was stereo-specific. Unexpectedly, we found that D-Bac7(1-35) also showed an inhibitory activity comparable to that of its L-enantiomer (Fig 10). Since D-Bac7(1-35) has an antimicrobial activity considerably lower than that of its all-L countepart and it is not easily internalized by E. coli cells (Podda et al., 2006), our results indicate that the reduced activity of the all-D peptide is largely due to its poor internalization. Since D- and L-enantiomers of Bac7(1-35) present a comparable inhibitory activity in vitro on the transcription/translation, we propose that the stereospecificity observed for the activity of Bac7(1-35) has to be only restricted to its transport across the bacterial membrane, and does not likely influence its intracellular molecular mechanism of action.

We further dissected the inhibition of the transcription/translation process in order to precisely find the target of Bac7(1-35) among the many molecular actors involved in this complex biosynthetic pathway.

We first evaluated the interaction between Bac7(1-35) and DNA. To this aim, we used a medium roughly approximating the salt composition and protein concentration of the bacterial cytosol. We observed that Bac7(1-35) interacts with DNA but in a non specific manner. A similar extent of interaction was observed for its D-enantiomer and also for the α-helical peptides LL-37 and BMAP-27 (Fig. 11). These results on binding to DNA are congruent with previous observations concerning other AMPs not belonging to the PR-rich group (Park et al., 1998) (Park et al., 2000) (Hsu et al., 2005). However, some additional considerations have to be taken into account in the case of Bac7(1-35): firstly, Bac7(1-35) shows an interaction with DNA similar to that of other peptides but, among the AMPs tested, it is the only one that reaches a high intracellular concentrations under non lytic conditions; secondly, the most evident result in the DNA/peptide interaction experiments was the aggregation effect occurring at a 2:1 peptide/DNA ratio by weight (Fig. 11). This result suggests that Bac7(1-35) can bind and aggregate DNA when its amount is twofold by weight of that of the
nucleic acid. We calculated that even at the highest concentration reached in the bacteria by Bac7(1-35) (scale of mM), this peptide/DNA ratio is not achieved. Moreover, a certain degree of transcription/translation inhibition is detectable also in the presence of amounts of peptide substantially lower than the amount needed to aggregate DNA (Fig. 9). On the whole, these considerations suggest that the inhibiting activity of Bac7(1-35) on the transcription/translations processes cannot be simply explained as a “disturbing activity” caused by binding of the peptide to DNA eventually followed by DNA aggregation.

Concerning the peptide binding to DNA, it seems likely that this interaction is just due to electrostatic attraction between the positively charged peptide and the negatively charged phosphate groups of the nucleic acid. If this speculation is correct, an interaction of Bac7(1-35) with RNA is also likely, concurring even more to a general disturbing activity.

We then assessed that Bac7(1-35) used at a concentration inhibiting the transcription/translation process did not affect synthesis of RNA (Fig. 12), suggesting that the inhibitory activity of Bac7(1-35) is only exerted on translation. For comparison, we also used in parallel the following peptides: D-Bac7(1-35), BMAP-27 and LL-37. LL-37 was the only peptide showing a significant inhibitory activity on in vitro transcription. However, this peptide is known to form aggregates and to be “sticky” (Zelezetsky et al., 2006), so it is possible that it binds to RNA polymerase reducing its function. However, the synthesis of RNA in the presence of LL-37 was only reduced and not completely blocked, thereby allowing the synthesis of an amount of RNA still enough to maintain the translation process. This could explain why no significant inhibition was observed. The slight inhibition of the transcription/translation process observed for both enantiomers of Bac7(1-35), in our opinion, could be due to an unspecific previously described interaction between the peptides and the DNA.

To confirm the in vitro inhibiting activity of Bac7(1-35) on the translation process, we tested the incorporation of radioactive precursors the in E. coli cells in the presence of 1 μM Bac7(1-35) (Fig. 13). This peptide concentration was chosen because it is close to the MIC value for the strain of E. coli used, and because it is virtually not permeabilizing for bacterial membrane (Podda et al., 2006). We observed that Bac7(1-35) at 1 μM affects significantly only leucine incorporation, suggesting that the peptide inhibits specifically the protein synthesis, while RNA and DNA syntheses remain unaffected. The effect of Bac7(1-35) on protein synthesis was confirmed by following leucine incorporation in an E. coli strain deleted for the sbmA gene (Fig. 13D). In agreement with the fact that in the sbmA-deletion mutants the internalization of the peptide is decreased with respect to the wild-type (Mattiuzzo et al., 2007), we observed a higher incorporation of leucine in the presence of 33
Bac7(1-35) in the mutant compared to the wild-type strain. The higher level of leucine incorporation in the mutant can be explained by the lower intracellular concentration of peptide in the mutated strain.

Our results on precursor incorporation in bacterial macromolecules differ for some aspects from those previously reported by Skerlavaj et al. (1990). In this paper, the Authors observed that the native peptide Bac7 (60 residues in length) inhibited the synthesis of both RNA and protein at concentrations around 1.5 μM, and caused membrane permeabilization. Based on these data, they proposed a lytic mechanism of action for Bac7, which determined an inhibition of protein and RNA syntheses. Even though the experimental conditions are not perfectly superimposable, the results here described for the fragment 1-35 confirm that it has a different mode of action compared to the whole peptide, i.e. Bac7(1-35) at low concentration acts via a non-lytic mechanism, as proposed by Podda et al. (2006), while the whole peptide also exerts membrane permeabilizing effects already in the micromolar range of concentration. In addition, we identified the translation as an important intracellular target of Bac7(1-35), finding evidence of a specific, non stereo-dependent inhibition of protein synthesis.

This work contributes to the understanding of the molecular mechanism of action of Bac7(1-35). We demonstrated that it targets specifically protein synthesis, a biosynthetic pathway that is involved in the mechanism of action of many conventional antibiotics, their improvement and de novo design. Following the identification of the translation process as an intracellular target of Bac7(1-35), now it remains to be identified the precise molecular identity of the peptide’s targets (i.e. mRNA, tRNAs or some ribosomal component). Once these molecules will be identified, many applications for Bac7(1-35) will be possible into the field of biomedicine. Even though further studies are necessary to reach a complete understanding of the Bac7(1-35) mode of action, this peptide could become a promising starting point for the design of novel antibiotics for therapeutic applications.
REFERENCES


