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The Mechanism of Killing by the Proline-Rich Peptide Bac7(1–35) against Clinical Strains of *Pseudomonas aeruginosa* Differs from That against Other Gram-Negative Bacteria

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ABSTRACT *Pseudomonas aeruginosa* infections represent a serious threat to worldwide health. Proline-rich antimicrobial peptides (PR-AMPs), a particular group of peptide antibiotics, have demonstrated *in vitro* activity against *P. aeruginosa* strains. Here we show that the mammalian PR-AMP Bac7(1–35) is active against some multidrug-resistant cystic fibrosis isolates of *P. aeruginosa*. By confocal microscopy and cytometric analyses, we investigated the mechanism of killing against *P. aeruginosa* strain PAO1 and three selected isolates, and we observed that the peptide inactivated the target cells by disrupting their cellular membranes. This effect is deeply different from that previously described for PR-AMPs in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, where these peptides act intracellularly after having been internalized by means of the transporter SbmA without membranolytic effects. The heterologous expression of SbmA in PAO1 cells enhanced the internalization of Bac7(1–35) into the cytoplasm, making the bacteria more susceptible to the peptide but at the same time more resistant to the membrane lysis, similarly to what occurs in *E. coli*. The results evidenced a new mechanism of action for PR-AMPs and indicate that Bac7 has multiple and variable modes of action that depend on the characteristics of the different target species and the possibility to be internalized by bacterial transporters. This feature broadens the spectrum of activity of the peptide and makes the development of peptide-resistant bacteria a more difficult process.

KEYWORDS proline rich, antimicrobial peptide, Bac7, cystic fibrosis isolate, *Pseudomonas aeruginosa*, mechanism of action

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Innovative anti-infective agents are urgently needed to overcome the antibiotic resistance problem. Antimicrobial peptides (AMPs) are a large class of innate immunity effectors with a remarkable capacity to kill microorganisms, which are currently in the limelight as potential future anti-infective therapeutics (1). Most AMPs strongly interact with bacterial membranes, leading to a lethal permeabilization of the microbial envelope (2). However, some AMPs kill bacteria mainly by interfering with internal cellular functions and without significant perturbation of cell membranes at microbicidal concentrations (3, 4).

The group of proline-rich peptides (PR-AMPs) is the best known example of these intracellular-acting peptides (5, 6). Some of them have been identified in mammal neutrophils (7), whereas others have been found in hemolymph of insects and crustaceans (8). All of them invariably have a high number of proline and arginine residues,

show similar spectra of activity, including several Gram-negative species, and have similar modes of action. Recently different PR-AMPs, both mammalian Bac7(1–35) and insect oncocin and apidaecins, were demonstrated to efficiently bind to different regions of prokaryote ribosomes (9–11), leading to the inhibition of protein synthesis (12).

PR-AMPs reach the ribosome after having crossed the cell membranes by means of SbmA/BacA, an inner membrane transporter present in several but not all proteobacteria. In *Escherichia coli*, some PR-AMPs appeared to rely exclusively on the SbmA uptake system (13), whereas others, including Bac7(1–35) and oncocin, are quite active also in SbmA-deleted strains (14), likely because of the presence of a second bacterial transport system recently identified as the *yjiL-mdtM* gene cluster (13).

Pseudomonas aeruginosa is an emergent pathogen with high intrinsic antibiotic resistance and is among the most common hospital pathogens (15). Serious *P. aeruginosa* infections are often associated with compromised host defenses such as in neutropenia, severe burns, or cystic fibrosis (CF) (16). These infections demonstrate high morbidity and mortality for the limited therapies, in particular due to the spread of antimicrobial-resistant strains (17). For these reasons, finding alternative prevention and treatment strategies is an urgent priority (18).

A number of *P. aeruginosa* strains, including multidrug-resistant (MDR) strains, show considerable susceptibility to the PR-AMP Bac7(1–35), with MIC values ranging from 0.5 to 32 μM (19). In addition, the artificial long proline-rich antimicrobial peptide dimer A3-APO, is active against strains of *P. aeruginosa* (20). Despite this clear anti-*Pseudomonas* activity and different from other gammaproteobacteria, *P. aeruginosa* lacks the inner membrane protein SbmA as well as the homologue of the *yjiL-mdtM* cluster. Therefore, the mechanism used by this PR-AMP to cross the bacterial membranes and more in general the mechanism of bacterial killing are still puzzling.

In this study, we investigated the activity of Bac7(1–35) against *P. aeruginosa* strains and the mechanism by which the peptide kills these bacteria. We then evaluated the effect of the heterologous expression of the *E. coli* SbmA transporter on the susceptibility to PR-AMPs in this bacterial species, which naturally lacks this transporter. The results highlight a different mechanism of action for PR-AMPs from that previously described against other Gram-negative bacteria, which is also influenced by the expression of SbmA.

RESULTS

Antimicrobial activity of Bac7(1–35) against clinical isolates of *Pseudomonas aeruginosa*. The antimicrobial activity of Bac7(1–35) was tested against a panel of *P. aeruginosa* strains, including the reference PAO1 strain and 11 different multidrug-resistant (MDR) strains obtained from CF patients (21). Bac7(1–35) inhibited the growth of most strains, showing MIC values of between 4 and 32 μM. PAO1 was inhibited at 8 μM, while PA07 and PA08 strains were not susceptible to Bac7(1–35) up to 32 μM (Table 1). Based on these results, PA21, PA05, and PA35 strains, exhibiting low, medium, and high susceptibilities to the peptide, respectively, were selected together with the PAO1 reference strain for a further characterization. The minimal bactericidal concentration (MBC) values of Bac7(1–35) for these four strains were identical to those of the MIC and indicated that the peptide has a bactericidal activity at bacterial growth-inhibiting concentrations. Differently, at subinhibitory concentrations, Bac7(1–35) could not inhibit biofilm formation of the four strains (see Fig. S1 in the supplemental material).

Mechanism of action of Bac7(1–35) on *P. aeruginosa* PAO1. We investigated the effects of Bac7(1–35) on membrane integrity by propidium iodide (PI)-uptake assay. Treatment of PAO1 cells with Bac7(1–35) at the MIC (8 μM) resulted in 60% of PI-positive cells within 60 min, indicating a rapid and vast permeabilization of the cells. Twenty percent of PI-positive cells were also observed even at the sublethal (1/2 MIC) Bac7(1–35) concentration (Fig. 1A). Overall the membranolytic effects due to the

TABLE 1 Antimicrobial activity of Bac7(1–35)

Strain	MIC (μM)[a]
P. aeruginosa	
PAO1	8
PA03	16
PA05	8
PA07	>32
PA08	>32
PA09	32
PA10	32
PA14	32
PA21	32
PA22	8
PA31	8
PA33	8
PA35	4
PA36	8
E. coli	
ATCC 25922	1

[a]The MIC was defined as the lowest concentration of peptide that prevented visible growth of bacteria after incubation for 20 h at 37°C. MIC values are representative of three independent experiments with comparable results.

peptide were not so different from those caused by the α-helical BMAP27(1–18), a lytic AMP that permeabilized ~70% of cells after 15 min of incubation (Fig. 1A).

The PAO1 cell viability was then evaluated under the same conditions used in the PI uptake assay. Bac7(1–35) decreased significantly (2 logs) the number of viable cells within 60 min of incubation (Fig. 1B), clearly indicating the simultaneity of events between bacterial killing and membrane damage.

PA21, PA05, and PA35 strains of *P. aeruginosa* were also tested for cell permeabilization (Fig. 1E). A positive correlation between the extent of membrane damage and the susceptibility of the different strains was found with the less susceptible PA21 showing the lowest level of permeabilization (25% of PI-positive cells) and with the more susceptible PA35 exhibiting the highest effect (>70% PI-positive cells) to its membrane.

To exclude the possibility that the observed membrane lysis is only a consequence of cell death, we carried out cell permeabilization and killing kinetics assays also with tobramycin, a nonlytic and protein synthesis inhibitor antibiotic. The aminoglycoside confirmed to do not alter the permeability of the membrane within a time period (30 min) in which it caused a 2- to 3-log decrease of the number of viable PAO1 cells (Fig. 1C and D).

Taken together, these results underline the different mechanism of *P. aeruginosa* killing between Bac7(1–35) and a nonlytic antibiotic, and strongly indicate membrane perturbation as a major mechanism of Bac7 activity against this bacteria.

Internalization of Bac7(1–35)-BY into *P. aeruginosa* cells. Localization of the peptide in PAO1 cells was examined by confocal scanning laser microscopy using the fluorescent derivative Bac7(1–35)-BY. This boron dipyrromethene (BODIPY)-linked peptide has previously been tested on *E. coli* cells (22), and it shows the same MIC values of the unlabeled peptide against PAO1 cells (data not shown). Although fluorescence due to the peptide was present into the cytoplasm, the most intense signal was visible on the membrane, suggesting that the peptide accumulated on the bacterial surface (Fig. 2A). (See the plot profile in Fig. S2 in the supplemental material for a detailed analysis of the fluorescence distribution.) This distribution diverged from that observed in *E. coli* cells, where Bac7(1–35)-BY was homogeneously distributed between the cytoplasm and the membranes (Fig. 2C).

We also investigated the level of Bac7(1–35)-BY internalization by cytometric analyses. After treatment with the peptide at different times, the PAO1 cells were analyzed with or without the presence of trypan blue (TB) to evaluate the aliquot of the

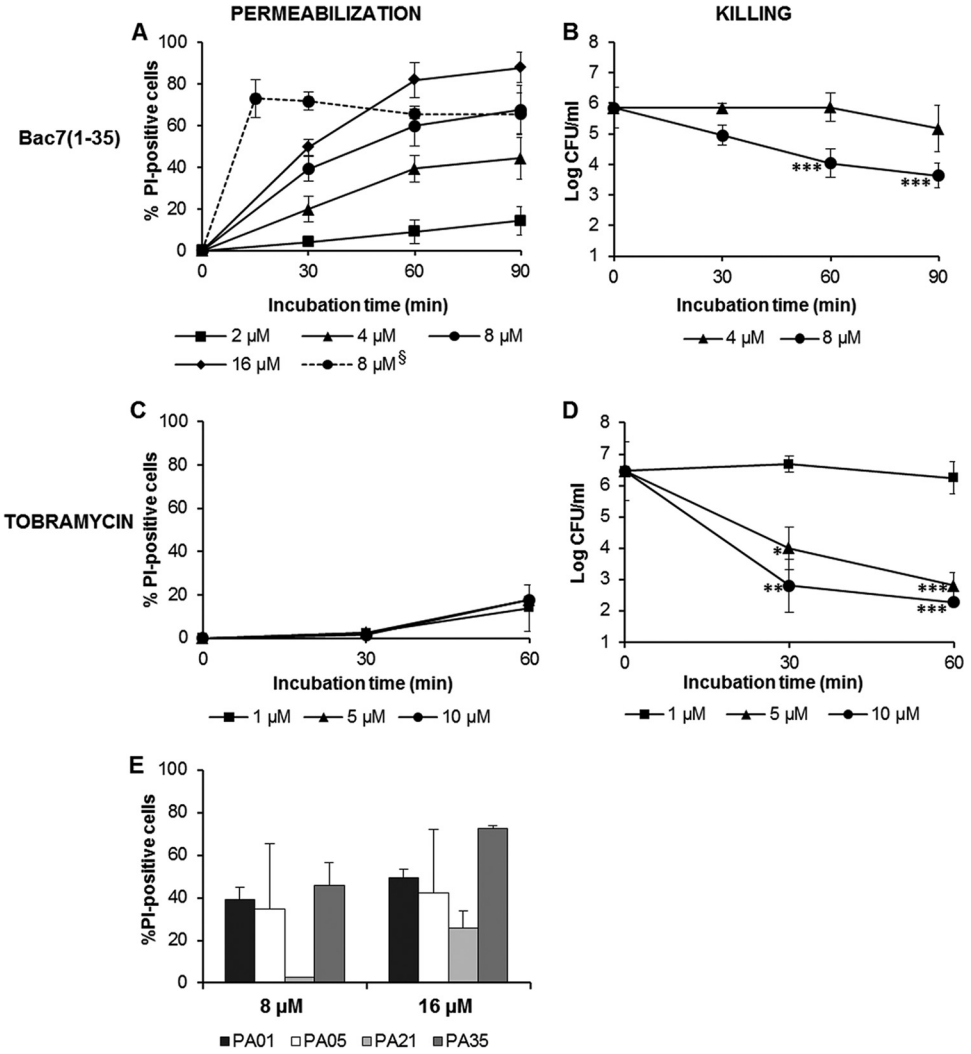

FIG 1 Evaluation of membrane permeabilization and bactericidal activity on *P. aeruginosa* strains. Shown is a comparison between the kinetics of permeabilization and cell killing of PAO1 cells in the presence of Bac7(1–35) (A and B) or tobramycin (C and D). (E) Permeabilization level of PAO1 PA05 PA21 and PA35 strains in the presence of Bac7(1–35) for 30 min. The permeabilization assay and the bactericidal activity (killing) assay were performed on 1×10^6/ml cells in MHB. The level of permeabilization has been reported as percentages of PI-positive cells. Data are means from four independent experiments. The dashed line in panel A (noted by § below the panel) represents the permeabilization kinetics of the BMAP27(1–18) peptide used as a control. *, $P < 0.05$, **, $P \leq 0.01$, and ***, $P \leq 0.001$, versus the starting inoculum (ANOVA with Tukey-Kramer posttest).

extracellularly accessible BODIPY dye by fluorescence quenching. Data confirmed the partial intracellular localization of Bac7(1–35)-BY, even though the high level of quenching (>60%) was consistent with a high exposition of the peptide on the cell surface (Fig. 2D). In contrast, fluorescence of Bac7(1–35)-BY was not quenched at all in *E. coli* cells (<10%) treated under the same conditions (Fig. 2F), according to its known intracellular localization (22). Internalization of Bac7(1–35)-BY was also evaluated in PA35, PA05, and PA21 strains (Table 1). These strains showed very different levels of fluorescence and quenching in the presence of TB (Fig. 2G) at different peptide concentrations, and the absence of any correlation between the level of fluorescence of each strain and the relative susceptibility to Bac7(1–35).

Overall the confocal and cytometric data concur to indicate that Bac7 is mainly localized on the cell surface in *P. aeruginosa* strains instead of reaching the cytoplasm, and this unusual localization for a proline-rich peptide could boost its membranolytic activity.

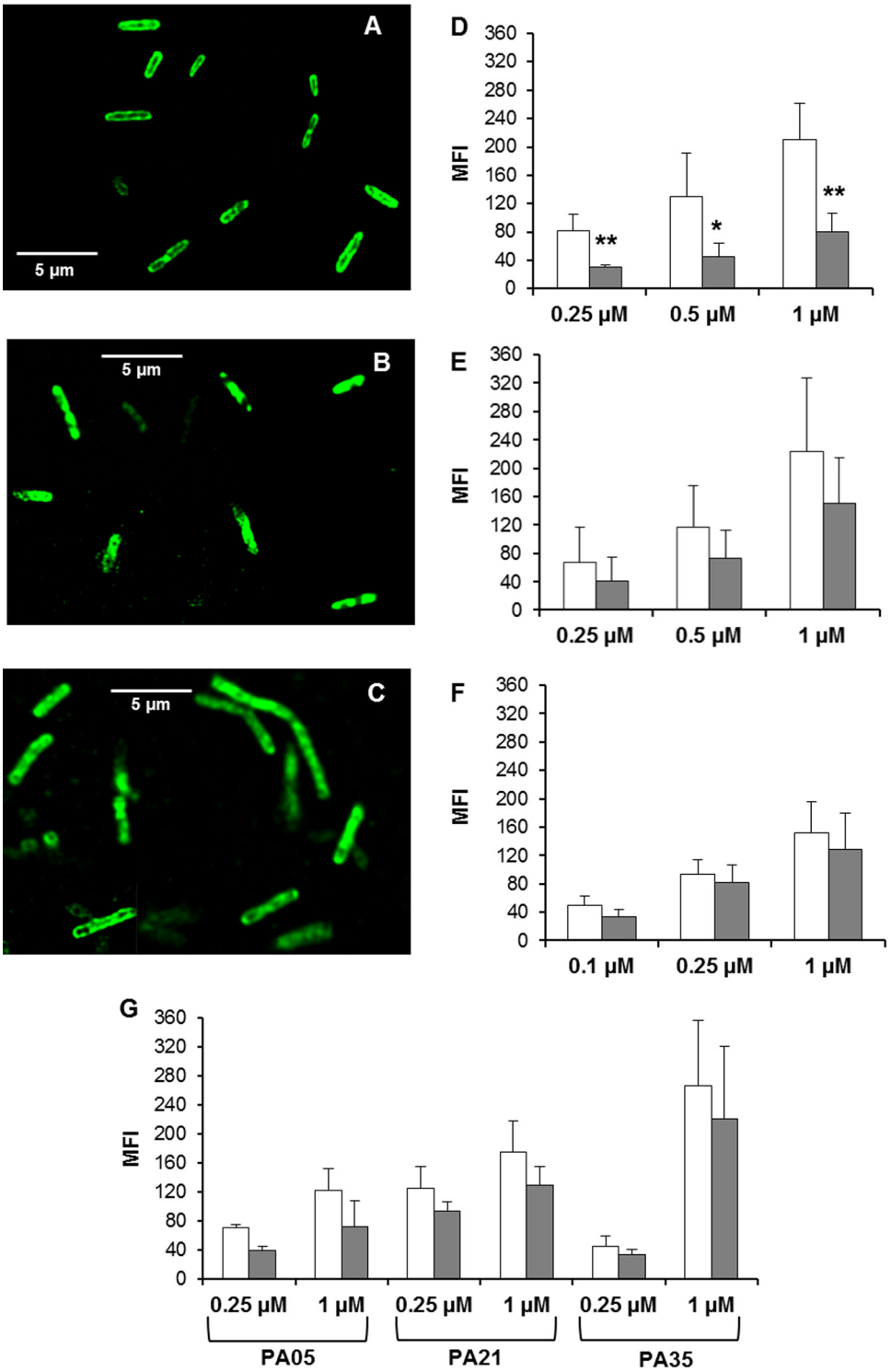

FIG 2 Evaluation of peptide internalization and localization into bacterial cells. (A to C) Confocal microscopy images of PAO1 (A), PAO1(p*sbmA1*) (B), and *E. coli* ATCC 25922 (C) treated with Bac7(1–35)-BY for 30 min. *P. aeruginosa* and *E. coli* cells have been exposed to 1 μM and 0.25 μM peptide, respectively. All images are representative sections from the middle of the bacterial cell. Many fields were examined, and for each experiment, over 95% of the cells displayed the pattern of the respective representative cell shown here. (D to G) Mean fluorescence intensity (MFI) of PAO1 (D), PAO1(p*sbmA1*) (E), *E. coli* ATCC 25922 (F), and PA05, PA21, and PA35 (G) cells exposed to Bac7(1–35)-BY. Bacterial cells (1×10^6 CFU/ml) were incubated with the peptide for 30 min, extensively washed, and analyzed by flow cytometry after (gray histograms) or without (white histograms) incubation with 1 mg/ml TB for 10 min at 37°C. Data are expressed as the average MFI with standard deviation from three independent experiments. *, $P < 0.05$, and **, $P \leq 0.005$, versus the TB-untreated sample (Student-Newman-Keuls multiple comparisons test, ANOVA).

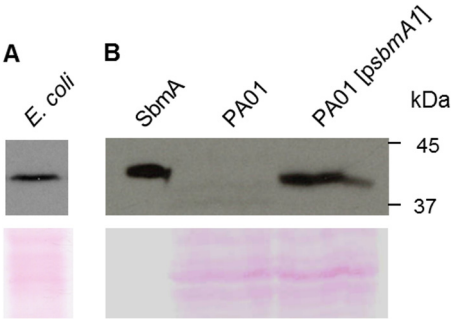

FIG 3 Expression of the *E. coli* SbmA protein in *P. aeruginosa* PAO1. (A and B) Western blot analysis of SbmA in (A) a total lysate of *E. coli* SC122 cells and in (B) total lysates of *P. aeruginosa* PAO1 cells (lane 2) and *P. aeruginosa* PAO1(p*sbmA1*) cells (lane 3). A purified recombinant *E. coli* SbmA protein was also added (lane 1). Western blot analyses were performed by using a polyclonal anti-SbmA antibody.

Heterologous expression of SbmA in *P. aeruginosa* PAO1. A primary role of the membrane protein SbmA in the internalization of proline-rich peptides has clearly been described in *E. coli* and *Sinorhizobium meliloti* (23, 24). However, a gene homologous to *sbmA* is lacking in *P. aeruginosa* genome. For this reason, we investigated the possibility that the *E. coli* SbmA protein could be functional in *P. aeruginosa* and could affect the activity of proline-rich peptides. To this aim, the *E. coli sbmA* gene was cloned into an IncP wide-host-range plasmid and introduced into PAO1 cells by triparental mating to yield the PAO1(p*sbmA1*) strain. PAO1(p*sbmA1*) expressed constitutively the protein at a similar level to that observed in *E. coli* (Fig. 3). Its susceptibility toward different peptides was tested, including both PR-AMPs, such as the mammal-derived Bac7(1–35) and Bac5(1–31) and the insect-derived oncocin 112 and apidaecin 137, as well as to the unrelated lytic α-helical BMAP27(1–18) and cyclic polymyxin B AMPs.

Data clearly indicated that the expression of SbmA in PAO1 increased specifically the susceptibility to proline-rich peptides, with MIC values decreased 2- to 4-fold for both Bac7(1–35) and oncocin 112, but not for the unrelated BMAP27(1–18) and polymyxin B peptides (Table 2). The sensitivities of the two strains to AMPs were also tested by growth inhibition assay. Growth of the SbmA-expressing strain PAO1(p*sbmA1*) was more inhibited than that of the wild-type strain by all of the proline-rich peptides, including apidaecin 137 and Bac5(1–31) (Fig. 4). On the contrary, the extent of inhibition was unchanged by BMAP27(1–18) and polymyxin B (Fig. 4), thus underlining that the expression of SbmA did not affect the activity of all of the AMPs but specifically of the proline-rich family.

Localization of Bac7(1–35)-BY was examined in PAO1(p*sbmA1*) cells. The peptide appeared distributed into the cytoplasm and was less visible on the surface of the cells than in the wild-type strain (Fig. 2B [see the relative plot profile in Fig. S2]). In addition, it is worth noting that the fraction of the internalized peptide in PAO1(p*sbmA1*) increased, as suggested by the lower level of quenching (~35%), in comparison to the wild-type strain (~60% of reduction), and the unchanged level of fluorescence intensity of the cells (Fig. 2D). These results indicate that in SbmA-expressing *P. aeruginosa* cells the uptake of Bac7(1–35) is enhanced lowering the amount of peptide located on the cell surface.

TABLE 2 Antimicrobial activities of different proline-rich and lytic peptides against *P. aeruginosa* expressing *E. coli* SbmA

	MIC (μM)[a]					
Strain	**Bac7(1–35)**	**Bac5(1–31)**	**Apidaecin 137**	**Oncocin 112**	**BMAP27(1–18)**	**Polymyxin B**
PAO1	8	>64	>64	>64	8	1
PAO1(p*sbmA1*)	2–4	64	>64	8–16	8	1

[a]MIC was defined as the lowest concentration of peptide that prevented visible growth of bacteria after incubation for 20 h at 37°C. MIC values are representative of three independent experiments with comparable results.

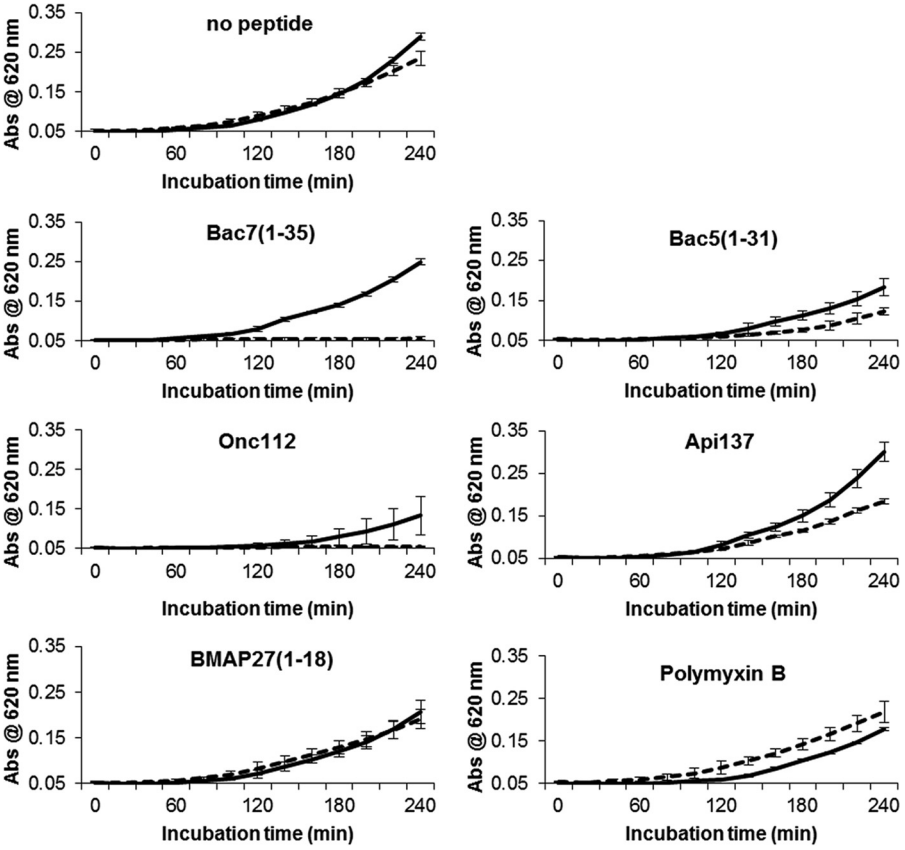

FIG 4 Growth kinetics of *P. aeruginosa* PAO1 and PAO1(p*sbmA1*) in the presence of different proline-rich and lytic peptides. Bacterial suspensions (1×10^6 cells/ml) of PAO1 (solid lines) and PAO1(p*sbmA1*) (dashed lines) were grown for 4 h in the presence of 1/16 MIC of peptides Bac7(1–35) (1 μM), Bac5(1–31) (4 μM), oncocin 112 (Onc112 [4 μM]), or apidaecin 137 (Api137 [4 μM]) or with 1/16 the MIC of BMAP27(1–18) (0.5 μM) or 1/4 the MIC of polymyxin B (0.25 μM), and the absorbance (Abs) at 620 nm was measured every 10 min. The results are means ± standard deviations from three independent experiments. As a control, the growth kinetics of the PAO1 strain carrying empty pMP220 plasmid in the presence of Bac7(1–35) were also assessed and gave similar results to those obtained with the wild-type PAO1 strain (data not shown).

We also evaluated the kinetics of permeabilization due to different concentrations of Bac7(1–35) when SbmA is expressed in *P. aeruginosa* cells (Fig. 5). Interestingly, a decrease of nearly 50% of permeabilized cells was observed in the PAO1(p*sbmA1*) strain with respect to the wild-type strain (Fig. 5B). Conversely, BMAP27(1–18) permeabilized both strains to the same extent (see Fig. 1A and 5A), indicating that the protective effect of SbmA on the membrane permeabilization is specific for proline-rich peptides.

DISCUSSION

P. aeruginosa infections represent an urgent and worldwide health problem that needs to be addressed through the development of new therapeutic agents. PR-AMPs such as Bac7(1–35) and optimized apidaecins have been proved to be active against several strains of this opportunistic pathogen (19, 25). Here we showed that it is active also against *P. aeruginosa* strains isolated from CF patients, and it acts through a complex mechanism that involves membranolytic activity.

Hosts and pathogens can coexist in CF for years, leading to genotypes of the *P. aeruginosa* strains present in CF infections that differ from those of wild-type *P. aeruginosa* (26). These strains are characterized by high mutation rates *in vivo* that are linked to the evolution of antibiotic resistance (27). It is worth noting that Bac7(1–35) possesses good antimicrobial activity against almost half of these CF strains that are resistant to at least three groups of antibiotics. Despite their genotypes not having been characterized yet, this result indicates that mutations that are responsible for the

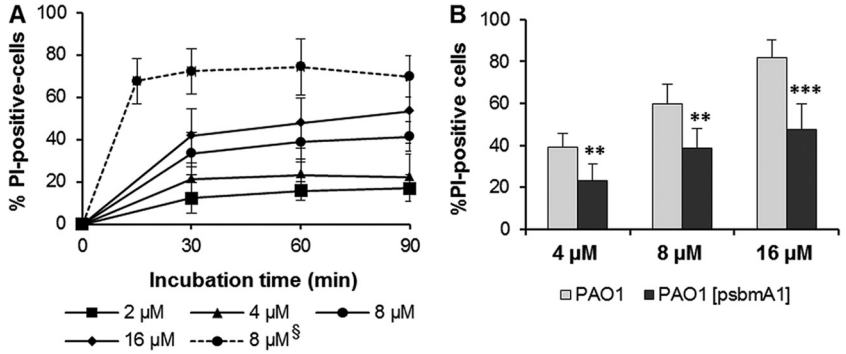

FIG 5 Evaluation of membrane permeabilization of the SbmA-expressing *P. aeruginosa* strain by Bac7(1–35). (A) Permeabilization kinetics of PAO1(p*sbmA1*) cells in the presence of Bac7(1–35) at different concentrations. (B) Comparison between membrane permeabilization of PAO1 (light gray histograms) and PAO1(p*sbmA1*) (dark gray histograms) cells after 60 min of incubation with Bac7(1–35). The assay was performed on 1×10^6/ml cells in MH broth, and the membrane permeabilization has been reported as percentages of PI-positive cells. Data are means from four independent experiments. The dashed line in panel A (noted by § below the panel) represents the permeabilization kinetics using the peptide BMAP27(1–18) as a control.**, $P < 0.005$, and ***, $P < 0.0005$, versus the PAO1 strain (Student-Newman-Keuls multiple comparisons test, ANOVA).

resistance to the currently used antibiotics could not lead to resistance to this peptide, suggesting it has a different mode of action.

The mechanism of action of PR-AMPs, such as Bac7 and other proline-rich peptides, has previously been well depicted in *E. coli* and *Salmonella enterica* serovar Typhimurium (22, 28). PR-AMPs cross the plasma membrane, exploiting the inner membrane protein SbmA (23, 29), and enter the cytoplasm, where they inhibit vital functions such as protein synthesis (12). Bac7(1–35) and other PR-AMPs have also shown membranolytic activity in *E. coli* and *S.* Typhimurium, but only at much higher concentrations than their MIC values (16- to 64-fold); however, lytic effects due to PR-AMPs occur over longer times than those observed by most AMPs with lytic activity (30). For this reason, membrane damage has been described as a secondary effect at killing concentrations (30). No other species have been studied in details in relation to the killing mechanism.

Here we show that this peptide affects *P. aeruginosa* strains by a different mechanism. *P. aeruginosa* strains are susceptible to Bac7(1–35) at micromolar concentrations despite the fact they do not have a gene homologue to SbmA or other PR-AMPs' known transporters. This apparent discrepancy can be explained by the fact that killing mechanism of Bac7(1–35) against *P. aeruginosa* relies mainly on membrane permeabilization occurring at the MIC and that this effect on membrane integrity is responsible for the bacterial cell death. Fifty percent of PAO1 cells are permeabilized within 30 min of incubation with the peptide, a value that is not so different from that observed for canonical lytic AMPs. On the contrary, less than 5% of *E. coli* and *S. enterica* serovar Typhimurium cells are permeabilized within the same time period with Bac7(1–35) used at near MICs (22, 31). Moreover, the temporal correlation between membrane damage and cellular death here evidenced for Bac7(1–35) against PAO1 cells is a typical feature of the activity of membranolytic peptides.

Significant fluorescence quenching has been observed in the peptide-treated *P. aeruginosa* strains, a feature that has never been observed previously in *E. coli* or *S. enterica* serovar Typhimurium cells (22, 31), where the internalization of the peptide is very efficient. These data are consistent with an extracellular localization of the peptide, and this observation is strengthened also by the images obtained by confocal microscopy showing the localization of most of the peptide on the bacterial surface, possibly on the membranes. Again these results suggest that the killing of *P. aeruginosa* cells is due to a membrane-related event rather than to inhibition of an internal target.

By cytometric analysis, we observed an intense fluorescence in peptide-treated *P. aeruginosa* strains, with marked difference among the strains, but always higher than

that detected on the susceptible *E. coli* strains analyzed under the same conditions. *P. aeruginosa* produces at least three exopolysaccharides, namely, alginate, Psl, and Pel (32, 33), which could influence the binding of Bac7(1–35) to the surface of the cells. Alginate is produced by a subset of strains that are often isolated from lungs of chronically colonized CF patients, and its overproduction is responsible for the mucoid phenotype of *P. aeruginosa* strains (32). Psl and Pel are often associated with nonmucoid strains and are necessary for biofilm formation (34). The anionic alginate could interact electrostatically with Bac7(1–35) enhancing the binding of the peptide, while the cationic Pel could repulse it (35). In this respect, it has been shown that alginate in solution inhibits the antimicrobial activity of structurally different peptides, including Bac7(1–35) (36). We do not know whether the strains analyzed in this study express one of these biopolymers; however, we found that the amount of peptide bound to each strain is nonpredictive of the antibacterial activity. It is worth noting that PA21 is the less susceptible strain and at the same time binds to Bac7(1–35) very efficiently (Fig. 2G). On the contrary, PA35 showing the highest level of peptide-derived fluorescence is also the more susceptible to Bac7(1–35). Further studies will be necessary to understand this complex relationship between different binding ability and bacterial killing capacity.

When a copy of the *E. coli sbmA* gene was introduced into the PAO1 cells, we found that the level of expression of the SbmA protein in *P. aeruginosa* was comparable to that in *E. coli*, allowing a direct comparison of the effects of SbmA expression on the susceptibility to the peptide. The presence of a functional SbmA transporter made *P. aeruginosa* cells more susceptible to different proline-rich peptides of both mammalian and insect origins. In addition, the decreased level of fluorescence quenching in the strain expressing SbmA and the confocal microscopy images are consistent with a more efficient SbmA-mediated internalization of Bac7(1–35).

Our results show that the *sbmA* gene can be efficiently expressed in *P. aeruginosa*, resulting in functional transport, and confirm that the SbmA protein is a stand-alone transporter that does not need other subunits to translocate proline-rich peptides across the membrane (29).

Previously an heterologous expression of SbmA was achieved in *Sinorhizobium meliloti*, an alphaproteobacterium symbiotic with leguminous plants, showing that SbmA is functionally interchangeable with its homolog BacA of *S. meliloti* (24). BacA is essential for bacteroid development (37), and it has been proposed to protect bacteria from host-derived NCR peptides during the instauration of symbiosis (38). BacA is thought to reduce the extent of cytoplasmic membrane damage due to nodule-specific cysteine-rich (NCR) peptides, possibly by mediating their uptake into the cytoplasm, thus reducing their local concentration at the membrane level. The BacA homolog SbmA could have a similar effect when expressed in *P. aeruginosa* cells. That the percentage of PI-positive cells in bacteria expressing SbmA was significantly lower than that in the wild-type PAO1 cells suggests that expressed SbmA could decrease the amount of surface-bound Bac7(1–35) by mediating its internalization into the cytoplasm and reducing the damaging effect onto the bacterial cell membrane.

Overall, these results indicate that a peptide antibiotic such as Bac7(1–35) has a complex and variable mode of action, where membranolytic effects and intracellular activity may participate both in bacterial killing in different proportions according to the different bacterial species and also different strains. Otvos et al. also demonstrated that slight changes in amino acid composition of the nonlytic PR-AMP pyrrhocoricin resulted in derivatives with an altered mode of action. It was shown that the dimeric pyrrhocoricin derivative Pip-pyrr-MeArg (39) and also the mixed pyrrhocoricin-drosocin dimer (40) kill bacteria better than the native proline-rich peptide due to improved activity on bacterial membranes (39).

In conclusion, we have shown that the proline-rich peptide Bac7(1–35) has the ability to kill *P. aeruginosa* CF clinical isolates switching from a mode of action mainly based on intracellular activity, as observed in *E. coli* and *S. enterica*, to a mechanism mainly based on membrane damage, as shown here against *P. aeruginosa* strains.

Therefore, we showed that Bac7(1–35) and possibly other PR-AMPs are capable of inhibiting bacteria via a multimodal mechanism, which could be varied depending on the nature of the target bacteria, the composition of their cellular membrane, and the presence of suitable transmembrane transporters. In this respect, the presence of SbmA/BacA plays the role of a "switch" allowing or not the internalization of the peptide and hence changing the peptide effects. These features point out the potential of Bac7(1–35) as an antibiotic molecule, since its multimodal mode of action makes the development of resistance in bacteria a more difficult and slow process.

MATERIALS AND METHODS

Antimicrobial peptides. Bac7(1–35) and its BODIPY fluorescently labeled derivative [Bac7(1–35)-BY] were prepared as previously described (14). Bac5(1–31) and BMAP27(1–18) were chemically synthesized as previously described (41). Apidaecin 137 and oncocin 112 were generously provided by Ralf Hoffmann (13, 42).

Bacterial strains and growth conditions. The bacteria used in this study were multidrug-resistant *P. aeruginosa* strains (PA03, PA05, PA07, PA08, PA09, PA10, PA14, PA21, PA22, PA31, PA33, PA35, and PA36) isolated from the respiratory tract of CF patients at the Bambino Gesù Pediatric Hospital of Rome (21). Each CF isolate, except PA21 and PA31, was resistant to at least three of the following groups of antibiotics (defined as multidrug resistant [MDR]): β-lactams with or without β-lactamase inhibitor, aminoglycosides, fluoroquinolones, folate pathway inhibitors (trimethoprim-sulfamethoxazole), tetracyclines, and macrolides. As reference strains, *Pseudomonas aeruginosa* PAO1 and *Escherichia coli* ATCC 25922 were used. The PAO1(p*sbmA1*) strain was prepared in this study as described below. The inoculum was incubated overnight at 37°C with shaking in Mueller-Hinton broth (MHB) supplemented with 300 μg/ml of tetracycline only for the PAO1(p*sbmA1*) strain. For the assays, the overnight bacterial cultures were diluted 1:30 in fresh MHB and incubated at 37°C with shaking for approximately 2 h.

The *E. coli sbmA* gene, including 187 bp of the promoter region, was obtained from the pMAU1 plasmid (23) and inserted into the broad-host-range pMP220 plasmid (43). The resulting plasmid, p*sbmA1*, was mobilized from *E. coli* into *P. aeruginosa* PAO1 via triparental mating with the helper strain of *E. coli* (pRK2013) (44). Transconjugants were then selected on LB agar in the presence of 300 μg/ml tetracycline, 100 μg/ml ampicillin, and 25 μg/ml nalidixic acid. The presence of the plasmid and expression of SbmA were verified by PCR and Western blotting, respectively.

Evaluation of the antimicrobial activity of peptides. MIC determinations were carried out in MHB on mid-log-phase bacteria (1×10^5 to 5×10^5 CFU/ml) as previously described (19). The MIC was taken as the lowest concentration of antimicrobial peptide resulting in the complete inhibition of visible growth after 20 h of incubation at 37°C. The minimum bactericidal concentration (MBC) was determined by spreading 25 μl of the bacterial suspension in the wells corresponding to the MIC or 2- and 4-fold the MIC onto MHB agar plates and by counting the viable colonies after 24 h. The MBC was taken as the lowest concentration resulting in killing of at least 99.99% of the original inoculum.

The bacterial growth curves were obtained using mid-log-phase bacteria at 1×10^6 CFU/ml in MHB, in the presence of increasing peptide concentrations, monitoring the optical density at 620 nm at 37°C every 10 min for 4 h in a microplate reader with intermittent shaking (Tecan Trading AG, Switzerland).

The bactericidal activity was determined using a mid-logarithmic-phase bacteria suspension, diluted in fresh MHB to a final concentration of 1×10^6 CFU/ml, and incubated at 37°C with different peptide concentrations. After a 2-h incubation, samples were removed, diluted in phosphate-buffered saline (PBS), plated on Mueller-Hinton agar, and incubated overnight to allow the colony counts.

Data are expressed as means $\pm$ standard deviation (SD). Significance was evaluated by an analysis of variance (ANOVA) between groups with a Tukey-Kramer posttest. *P* values of <0.05 were considered statistically significant.

Flow cytometric analysis. The flow cytometric assays were performed with a Cytomics FC 500 instrument (Beckman-Coulter, Inc., Fullerton, CA), as described previously (14, 22). Integrity of bacterial cell membrane was assessed by measuring the propidium iodide (PI) uptake by flow cytometry, as previously described (30). Briefly, mid-log-phase bacterial cultures, diluted at 1×10^6 CFU/ml in MHB, were incubated at 37°C for different times with increasing concentrations of Bac7(1–35). PI was added to all samples at a final concentration of 10 μg/ml. At the end of the incubation, the bacterial cells were analyzed by flow cytometry.

For the uptake evaluation, cultures of mid-log-phase bacteria, diluted to 1×10^6 CFU/ml in MHB, were incubated at 37°C for different times with different concentrations of peptide and analyzed as extensively described (14, 22).

Data analysis was performed with the FCS Express3 software (De Novo Software, Los Angeles, CA). Data are expressed as means $\pm$ SD. Significance of differences among groups was assessed by using the program Instat (GraphPad Software, Inc.) and performed by an ANOVA followed by the Student Newman-Keuls posttest. *P* values of <0.05 were considered statistically significant.

CSLM. Confocal scanning laser microscopy (CSLM) analyses were performed by using a Nikon C1-SI confocal microscope, as described previously (14, 22). *P. aeruginosa* PAO1, PAO1(p*sbmA1*), and *E. coli* ATCC 25922 cells treated for 30 min with 1 μM (*P. aeruginosa* strains) or 0.25 μM (*E. coli* strain) Bac7(1–35)-BY were prepared following the same protocol used for the flow cytometric assay (described above) without any fixation. Briefly, after the incubation with the peptides, the cells were washed three times with buffered high-salt solution, and 10 μl of each bacterial suspension was placed between two

cover glasses to obtain an unmovable monolayer of cells. The image stacks collected by CSLM were analyzed with the EZ-C1 Free Viewer (Nikon Corporation) and the Image J 1.40g (Wayne Resband, National Institutes of Health, USA) software. Images were then deconvolved using Huygens software with the classical CMLE algorithm.

Protein analysis. Total protein lysates were prepared starting from 10 ml of mid-log-phase bacteria that were pelleted in a 2-ml tube by centrifugation at 8,000 × *g* for 5 min and resuspended in the appropriate volume of sample buffer (3% SDS [wt/vol], 0.1 M dithiothreitol [DTT], 7.5% glycerol [wt/vol], and 0.0125% bromophenol blue in 0.125 M Tris-HCl [pH 6.8]) in order to normalize the concentration of cells per milliliter. Bacteria were lysed by freezing them at −20°C, sonication for 10 s at 35 kHz, and heating at 80°C for 10 min. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and detected as previously reported. Purified recombinant SbmA (29) was loaded as a control.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01660-16.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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