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Mechanisms of Action on *Escherichia coli* of Cecropin P1 and PR-39, Two Antibacterial Peptides from Pig Intestine

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Cecropin P1 and PR-39 are two antibacterial peptides isolated from the upper part of the small intestine of the pig. They have been sequenced, and their antibacterial spectra have been investigated (J.-Y. Lee, A. Boman, C. Sun, M. Andersson, H. Jörnvall, V. Mutt, and H. G. Boman, Proc. Natl. Acad. Sci. USA 86:9159–9162, 1989; B. Agerberth, J.-Y. Lee, T. Bergman, M. Carlquist, H. G. Boman, V. Mutt, and H. Jörnvall, Eur. J. Biochem. 202:849–854, 1991). We have now compared these two peptides for their mechanism of action on *Escherichia coli* K-12 by using three strains with different markers. Our results show that cecropin P1, like other cecropins, kills bacteria by lysis and that this reaction requires more peptide to kill more cells. PR-39 requires a lag period of about 8 min to penetrate the outer membrane of wild-type *E. coli*; then killing is quite fast. This lag period was absent in the *envA1* mutant; in this strain the outer membrane was freely permeable to both peptides. PR-39 killed growing bacteria faster than nongrowing cells; for cecropin P1 there was no such difference. It is suggested from isotope incorporation experiments that PR-39 kills bacteria by a mechanism that stops protein and DNA synthesis and results in degradation of these components.

Cecropins in insects and defensins in rabbit neutrophils were the first types of peptide antibiotics identified in animals (for reviews, see references 4 and 12). For some years, these peptides were thought to be species specific, in part because of their chemistry; cecropins lack cysteine and defensins are cysteine rich. However, the subsequent findings of magainins in a frog (33), of a cecropin in pig intestine (19), of defensin-like molecules in insects (18, 22), and of another cysteine-rich peptide in bovine tracheal mucosa (9) made it clear that antibacterial peptides are widespread in nature. Compared with a clonally based immune defense, antibacterial peptides offer several advantages, and it is reasonable to assume that they are an important part of the defense against the natural microflora both in animals and in plants (3).

There are reasons to expect that the stomach and the upper part of the small intestine should have antibacterial factors, and two such peptides, cecropin P1 (19) and PR-39 (1), have been purified from pig small intestine. They were also sequenced and found to be active chiefly against gram-negative bacteria. Moreover, antibacterial peptides have recently been identified in mouse small bowel (cryptdins [10, 25]) and in frog stomach (23), and mRNA corresponding to two new defensins has been found in human Paneth cells (15, 16).

The mechanism of action has been investigated for cecropin A (28, 29), several defensins (20), and magainins (31), as well as for Bac5 and Bac7 (27), two proline-arginine-rich peptides from bovine neutrophils (11). Bac5 and Bac7 are structurally very similar to PR-39 but somewhat larger. The killing mechanism found for most peptides investigated consists of attacks on the outer and inner membranes, ultimately resulting in lysis of the bacteria. Channel formation in artificial membranes is documented for cecropins (6), defensins (17), and magainins (7), but it is not yet quite clear whether this is the primary lethal effect that these peptides exert on bacteria. For Bac5 and Bac7, the data from membrane experiments were interpreted to mean that the peptides affected transport and energy metabolism in the cytoplasmic membrane and that this was the primary lethal effect (27).

Since cecropin P1 and PR-39 were isolated from the same large-scale fractionation of pig intestine and since nothing is known about their mechanism of action, we decided to compare cecropin P1 and PR-39 for their action on *Escherichia coli*. Our results show that these two peptides differ in their mechanisms; cecropin P1 causes an instantaneous lysis of bacteria, while with PR-39 the cells do not lyse. Instead, after a short lag period, bacteria are killed with a mechanism that stops protein and DNA synthesis.

MATERIALS AND METHODS

Antibacterial peptides. The natural form of cecropin P1 (with a free C-terminal carboxyl group) was synthesized by Magainin Pharmaceuticals, Inc. (Plymouth Meeting, Pa.), while PR-39 was prepared from pig small intestine as described previously (1). For cecropin P1, peptide concentration of a stock solution was determined from the extinction of the tryptophan residue (a 1 mM solution has an A_{280} of 5.60; molecular weight = 3,338.9), while PR-39 (molecular weight = 4,719.7) was weighed on an electrobalance (this process does not allow any correction for water content).

Bacterial strains and media. *E. coli* D21 and D22 are K-12 derivatives. Strain D21 is resistant to ampicillin (*ampA1*) and to streptomycin (*strA*), and it carries as additional markers *pro*, *his*, and *trp*. Strain D22 is an *envA1* mutant of D21 isolated after ethyl methanesulfonate treatment (24). Strain D22 has a barrier defect in the outer membrane (5), but the details are not understood despite the cloning of the *envA1* gene (2) and a recent investigation showing the leakage of periplasmic enzymes (32). Strain CR34 carries the following markers: *thy*, *leu*, *thr*, *thi*, *lac*, and the streptomycin resistance gene. Its origin is not clear, but it was obtained from Howard Goldfine in 1974, and it has since then been kept in

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our strain collection. *E. coli* ATCC 25922 was obtained in 1992 from Richard Darveau and is a clinical isolate used to assay an antibacterial peptide related to human platelet factor IV (8).

We normally used the rich Luria-Bertani (LB) medium in liquid cultures, in agar, and in agarose plates. Overnight plates were used to infect the inoculum cultures, which were grown in sidearm flasks on a shaker at 37°C. A check was always made with a Klett photometer to ascertain that the exponential growth rate was as expected before such a culture was used to infect a second culture used in an experiment. The Casamino Acids medium contained 0.2% glucose, 0.2% Casamino Acids, and basal medium E (30). For growth of strain D21, it was supplemented with tryptophan (20 μg/ml). All medium components were from Difco, and growth temperature was always 37°C. The minimal medium for strain CR34 contained medium E, 0.2% glucose, and the appropriate growth factors.

Measurements of bacterial growth and inhibition zone assay. Whenever possible, growth was monitored by a reading of the optical density in a Klett photometer with a red filter (W66). With this instrument and strain D21, 100 Klett units is equal to 4×10^8 cells per ml. With dilute cultures, 5 to 10 μl was withdrawn, diluted, and plated. Results are given as CFU per unit volume.

For the inhibition zone assay, we used thin agarose plates seeded with the appropriate test bacterium (about 4×10^5 cells in 6 ml of 1% agarose with LB medium). Small wells (diameter, 3 mm; volume, 3 μl) were punched in the plates and loaded with a dilution series of each sample. After overnight incubation at 30°C, inhibition zones were recorded and lethal concentrations were calculated as described in reference 13.

Incorporation of labelled precursors into macromolecules. [*methyl*-^{3}H]thymidine (25 Ci/mmol) and L-[4,5-^{3}H]leucine (70 Ci/mmol) were from Amersham. The volume of the cultures for isotope incorporation was 3 ml, and from these, 50-μl aliquots were withdrawn at the indicated times and transferred to 5-ml ice-cold trichloroacetic acid (TCA) (10%) with carrier bacteria (6×10^7 cells) and nonlabelled thymidine or leucine in at least a 1,000-fold excess. After standing in ice for 45 min or more, the samples were warmed to 37°C for 15 min and then cooled again in ice for at least 10 min before the precipitates were collected on Whatman glass microfiber GF/C filters (diameter, 2.5 cm). The precipitates were washed on the filters six times with 5 ml of cold 10% TCA with nonlabelled thymidine or leucine and once with 5 ml of 95% ethanol. The filters were then dried, and 4 ml of scintillation cocktail (Packard Scintillator 299) was added. Counting was performed in a Beckman LS3801 instrument by using the tritium program.

RESULTS

Comparison of susceptibility to different strains of *E. coli*. We first used the inhibition zone assay to compare the activities of cecropin P1 and PR-39 on three auxotrophic derivatives of *E. coli* K-12 and on a clinical isolate of *E. coli*, ATCC 25922. The results in Table 1 show that both peptides were about equally active on strains D21 and CR34. The *envA* strain D22 was slightly more sensitive than the parent strain D21, especially for PR-39. For both of the peptides, the clinical strain ATCC 25922 was slightly less sensitive than the K-12 derivatives, but the overall impression is that all strains tested were sensitive. Tetracycline was included in this assay in order to compare two animal antibiotics with a conventional broad-spectrum agent. On a molar basis, the former two are somewhat more potent; on a weight basis, they are somewhat less potent.

TABLE 1. LCs of two intestinal peptides for strains of *E. coli*

Agent	LC (μM)[a] for:			
	D21	D22	CR34	ATCC 25922
Cecropin P1	0.36	0.19	0.40	0.93
PR-39	0.28	0.06	0.38	0.94
Tetracycline	0.9	1.2	2.4	1.2

[a] Defined as the lowest concentration of an agent that can kill a target bacterium. Details about the strains and the inhibition zone method of determining LCs are given in Materials and Methods. Tetracycline (T-3258; Sigma) is included for comparative purposes only.

We next investigated the kinetics of killing by incubating about 10^8 cells of D21 with each of the peptides. In order to obtain the same degree of killing after 15 min, we had to use 12.5 μM cecropin P1 and PR-39 at twice the concentration. This is shown in Fig. 1, which also illustrates that with PR-39 there was a lag of about 8 min before viable counts began to drop. We suspected that this lag period was due to a barrier in the outer membrane, and we therefore compared the rate of killing of the *envA* mutant D22 with the rate of killing of its parental strain, D21. Figure 2A shows that with PR-39 the lag is present in D21 but absent in D22. The barrier in the outer membrane does not show up with cecropin P1 (Fig. 2B), but for both of the peptides it is clear that in this type of assay strain D22 is much more sensitive than the parental strain, D21. In fact, the difference between the strains is so large that it was difficult to find a peptide concentration allowing us to record the killing of both strains with the same amount of peptide. For Fig. 2, we have used the same amounts of cells (measured as Klett units) in both experiments, but since D22 forms short chains its viable counts are reduced by a factor of 3 to 5.

Lysis of liposomes with cecropin A has been shown to be a stoichiometric reaction (28). Figure 3 shows that with cecropin P1 the log of the survival of strain D21 was proportional to the concentration of cecropin P1 added,

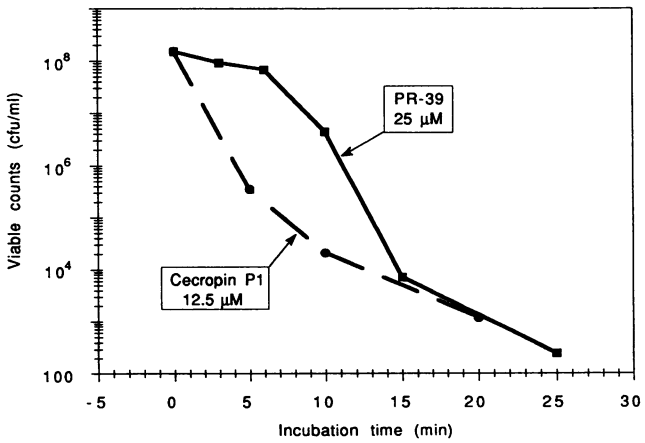

FIG. 1. Rate of killing of *E. coli* D21 at 37°C with cecropin P1 and PR-39, two antibacterial peptides from pig intestine. The bacteria were pregrown in the rich LB medium, and peptides were added at zero time. Aliquots were withdrawn at the times indicated, diluted, and spread on plates which were incubated overnight, and then the colonies were counted.

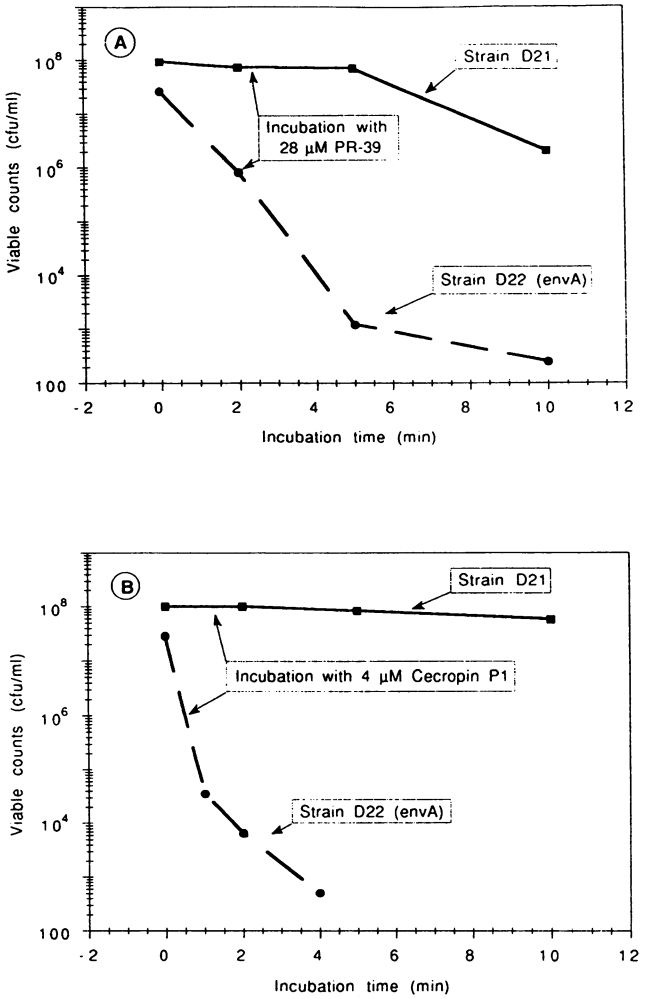

FIG. 2. Comparison of the rate of killing at 37°C of *E. coli* D21, which has the wild-type envelope, and D22, which has the *envA1* mutation that makes the outer membrane permeable to peptides. (A) PR-39; (B) cecropin P1.

which is consistent with a stoichiometric killing reaction. For PR-39, the lag period makes it difficult to determine a similar plot. However, it is still obvious that to kill more cells more PR-39 peptide is also needed.

Killing of growing and nongrowing bacteria. Attacins (a family of larger antibacterial proteins from insects) kill only growing bacteria, and they have no detectable action on buffer-suspended cells (13); this applies also to the defensins (20). We therefore compared the killing rates of PR-39 on growing and nongrowing cells. The inoculum was grown exponentially to a density of 4×10^8 cells per ml (100 Klett units) in Casamino Acids medium with tryptophan (because strain D21 requires this amino acid). The cells were harvested by filtration and washed with Casamino Acids medium (without tryptophan) on the filter and resuspended in the same medium. They were then diluted 2,000 times and divided into four separate cultures. To two of these, PR-39 was added, and to one of them, tryptophan was also added. The two other cultures were controls, one with and one without tryptophan. Figure 4 shows that PR-39 killed growing cells considerably faster than it killed nongrowing cells. In both cases the lag period was obvious, but it was longer in

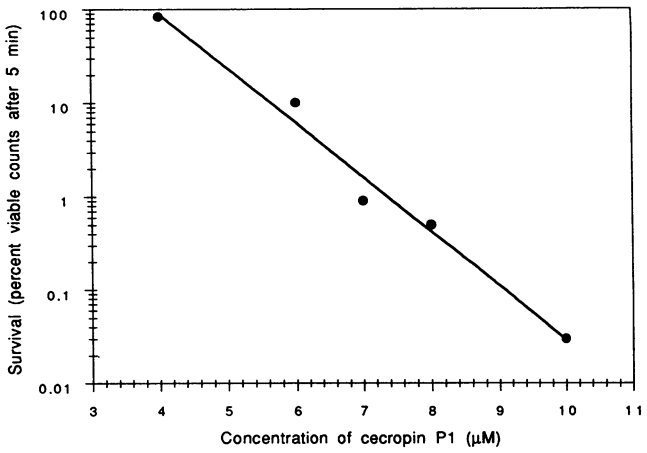

FIG. 3. Variation in the number of surviving bacteria (*E. coli* D21) with the concentration of cecropin P1. Log-phase bacteria (about 10^8 cells per ml) were incubated in LB medium at 37°C with different amounts of cecropin P1, and aliquots were removed at different times and diluted for viable counts. The interpolated values for survival after 5 min of incubation were read from the respective killing curves.

the nongrowing cells. The control without tryptophan stayed constant (data not shown), while the one with complete medium increased in cell density. For killing with cecropin P1, there was no difference between growing and nongrowing cells (data not shown).

Simultaneous recording of killing and lysis. Insect cecropins lyse bacteria, and lysis of buffer-suspended *E. coli* was in fact the assay used in the isolation of cecropins A and B (14). A culture of strain D21 growing exponentially in LB medium was divided at a density of about 50 Klett units. One of the cultures received cecropin P1 to a final concentration

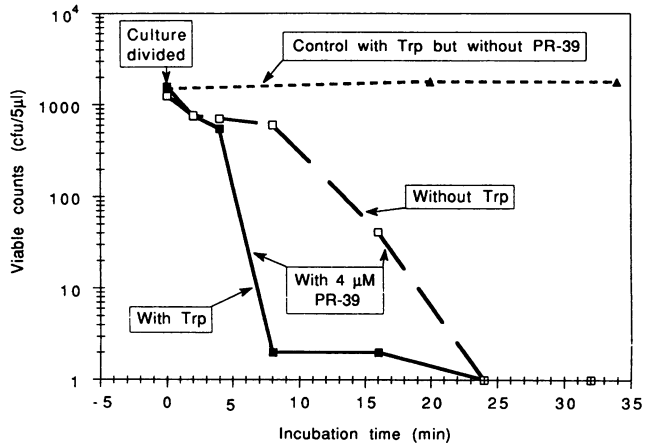

FIG. 4. Comparison of the killing by PR-39 of growing and nongrowing cells of *E. coli* D21. A log-phase inoculum culture growing in Casamino Acids medium with tryptophan (Trp) was filtered, washed with medium without Trp, resuspended, and at zero time diluted 2,000 times into four cultures, three of them supplemented as indicated. The fourth was a control (without Trp or PR-39) that stayed constant (not shown). The 24- and 32-min plates were in fact sterile. However, to allow the computer program used for the figure to work, the zero values in the protocol were changed to 1.

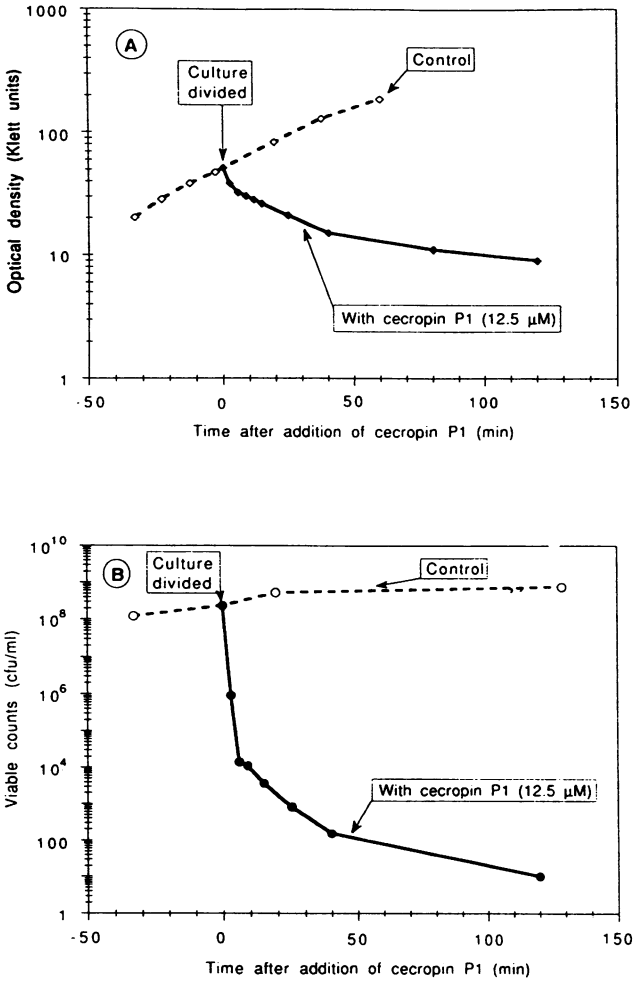

FIG. 5. Simultaneous recording of optical density (A) and viable counts (B) for *E. coli* D21 to which cecropin P1 was added at zero time. A log-phase inoculum at 100 Klett units was diluted five times and allowed to grow to 50 Klett units. This culture was then divided; cecropin P1 was added to one part, and an equal volume of water was added to the other. Optical density was monitored for both of the cultures, and aliquots were removed for viable counts.

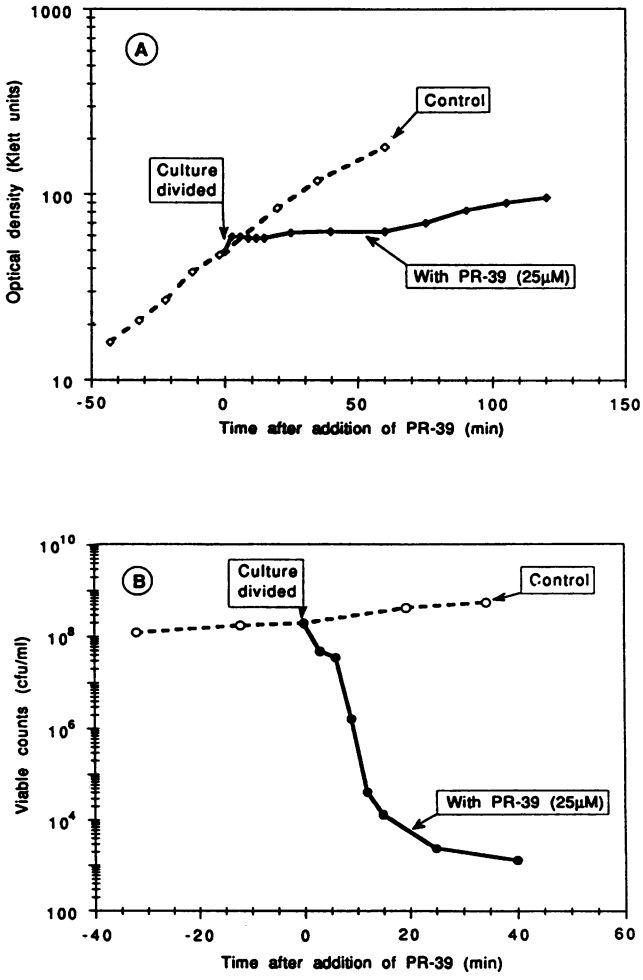

FIG. 6. Simultaneous recording of optical density (A) and viable counts (B) for *E. coli* D21 to which PR-39 was added at zero time. All details were as described for Fig. 5.

of 12.5 μM, and the same volume of water was added to the other culture. For both of the cultures, cell density was monitored with a Klett photometer, and aliquots were removed and diluted for viable counts. Figure 5A shows that Klett values dropped immediately after the addition and then continued to fall at a low rate. The simultaneous recording of viable counts (Fig. 5B) showed a drop of 4 orders of magnitude within the first three min, after which the rate decreased. We conclude that cecropin P1, like insect cecropins, lyses bacteria, and as before, killing is very fast. The fact that optical density drops more slowly may indicate that fairly large fragments of the lysed bacteria remain in the suspension of killed cells.

A similar experiment was performed with PR-39 but at twice the concentration used for cecropin P1 (see above). Figure 6A shows that in this case there was no lysis; instead, bacteria with PR-39 swelled during the rest of the experiment (130 min). The simultaneous recording of viable counts (Fig. 6B) showed again first a short lag, then a rapid drop (more than 3 orders of magnitude in 9 min), and finally a slower phase of killing. We noticed that on the plates which were used for the last viable counts, a few more colonies came up after 42 h. A number of these were picked, subcultured, and tested for the markers of D21; for one of the cultures, the lethal concentration (LC) for PR-39 was determined. In every respect, these isolates behaved as did the original strain, D21. We conclude that (i) PR-39 does not cause any lysis linked to the reaction by which the bacteria are killed and (ii) the killing reaction is probably reversed to a low degree by a rapid dilution.

Effects of PR-39 on the synthesis of DNA and proteins. An exponentially growing culture of CR34 (which requires thymine and leucine) in minimal medium (generation time, 67 min) at 50 Klett units was diluted 400 times for a 3-ml culture with [^{3}H]thymidine. After 4 h of growth, this culture was divided; 25 μM PR-39 was added to one part, and an equal volume of water was added to the other (the control). At different times, samples of 50 μl were withdrawn and added to ice-cold TCA (see Materials and Methods). All samples were filtered and washed, and the precipitates were finally counted in a scintillation counter. Figure 7 shows that in the cultures with PR-39, thymidine incorporation stopped within 3 min and that degradation of TCA-insoluble material was obvious after 40 min. In the control culture, there were

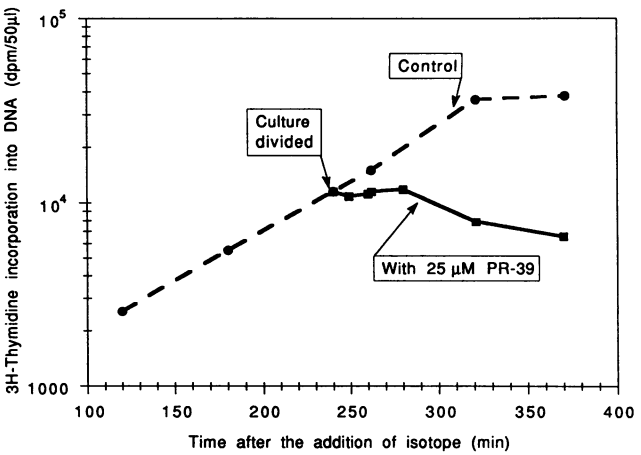

FIG. 7. Effect of PR-39 on DNA synthesis in growing *E. coli*. A log-phase culture of strain CR34 in minimal medium was diluted 400 times into a 3-ml culture in the same medium but with less thymidine (1 µg/ml) to which was added 60 µl of the stock solution of [^{3}H]thymidine. At the times indicated, 50-µl aliquots were removed, TCA precipitated, and washed, and [^{3}H]thymidine incorporation was determined as described in Materials and Methods. Thymidine becomes growth limiting towards the end of the experiment.

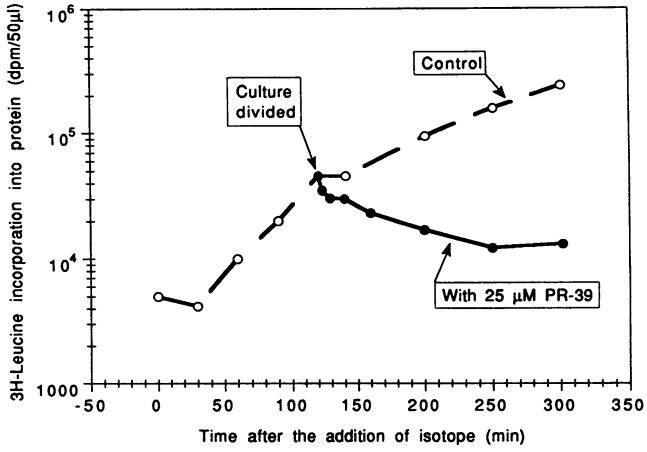

FIG. 8. Effect of PR-39 on protein synthesis in growing *E. coli*. A log-phase culture of strain CR34 in minimal medium was diluted 200 times into a 3-ml culture in the same medium but with less leucine (2.5 µg/ml) to which was added 75 µl of the stock solution of [^{3}H]leucine. At the times indicated, 50-µl aliquots were removed, TCA precipitated, and washed, and [^{3}H]leucine incorporation was determined as described in Materials and Methods.

parallel increases in thymidine incorporation and in viable counts. For the culture with PR-39, a 9-min treatment gave a drop in viable counts of more than 3 orders of magnitude. Since the isotope was added nearly four generations before the addition of PR-39, and since we record TCA-insoluble counts, it is likely that the incorporation shown in Fig. 7 represents DNA synthesized.

Starting again from a log-phase culture of CR34, a similar experiment was performed with the incorporation of [^{3}H]leucine. Isotope was present from the start of the dilution, and samples were withdrawn at different times. After 120 min, the culture was divided, and PR-39 and water were added to each part. Samples were withdrawn for an additional 180 min, filtered, and washed as before. The results in Fig. 8 show that during the first 2 h there was an increase in labelled protein that was somewhat faster than the generation time and that then there was a normal increase during the next 3 to 4 hours. In the culture with PR-39, [^{3}H]leucine incorporation began to drop immediately after the addition of the peptide. After 9 min with PR-39, incorporation in the TCA precipitate was reduced to 66% of the value at the time of the addition of the peptide, and after 130 min it had dropped to 27%. Parallel viable counts showed that the control culture passed through almost four generations and that it also increased its leucine incorporation in a corresponding way. In the culture that received PR-39, viable counts dropped in 9 min from 2.5×10^6 to 800 CFU/ml. Thus, PR-39 stops protein synthesis instantaneously and also gives rise to protein degradation. Moreover, there does not seem to be a lag period with strain CR34.

DISCUSSION

Like other cecropins, the porcine cecropin P1 is composed of an amphipathic N-terminal part with a tryptophan, a single lysine plus a lysine doublet, and an arginine at conserved positions, while the C terminus is rather hydrophobic (19). The solution structure was investigated with nuclear magnetic resonance, and in a lipophilic environment the peptide is an extended helix with a flexible part around an SEG segment (26). Thus, the structure of cecropin P1 predicts a membrane-active function, which is in agreement with the results from earlier studies of cecropin A (28, 29).

We here demonstrate that there was an instantaneous lysis and killing when cecropin P1 was added to growing cells of *E. coli* D21 (Fig. 5A and B). The peptide must act on both the outer and the inner membranes, and the time it takes to disrupt the outer membrane is too short to be reflected in our kinetics (Fig. 1 and 5B). However, the time required to penetrate the outer membrane is well illustrated (Fig. 2B) by the difference in kinetics between strain D21, which has a wild-type envelope, and strain D22, in which the *envA1* gene makes the outer membrane permeable to lysozyme (from outside to inside [5]) and to periplasmic enzymes (from inside to outside [32]). Since these enzymes are much larger molecules than cecropin P1 and PR-39, it is reasonable to assume that in the *envA1* strain D22, both of the peptides can freely pass through the outer membrane.

PR-39 was given its name because it is a 39-residue peptide with 49% proline and 24% arginine. There is no sequence homology between PR-39 (1) and two other proline-arginine-rich peptides, Bac5 and Bac7 (11), but all three share certain amino acid motifs like PRP and PP repeated many times but in different ways (1). Despite these similarities, the mechanisms of action seem to differ. Bac5 and Bac7 show membrane activities which were interpreted to mean that these peptides interfere with transport and energy metabolism in the cytoplasmic membrane (27).

From our experiments with strain D21, which has a wild-type envelope (Table 1 and Fig. 1, 2A, and 4), it is clear that there is a penetration barrier in the outer membrane (absent in the *envA1* strain D22) (Fig. 2A) which PR-39 takes about 8 min to overcome. Unexpectedly, this barrier was also absent in strain CR34, which made it possible to record a significant drop in leucine incorporation 3 min after the addition of PR-39 (Fig. 8) and also, within the same time, a stop in thymidine incorporation (Fig. 7). Since in both these experiments the labelled precursors were added about 2 to 4 generations before the addition of PR-39, and since we have

recorded TCA-insoluble counts, it seems reasonable to conclude that in the target bacteria, protein synthesis was halted and protein degradation started within 3 min and that DNA synthesis was also stopped within the same time. Within this first 3 min, there was a 75% decrease in viable counts, and after 9 min the viable counts had dropped more than 3 orders of magnitude (results not shown).

If protein synthesis is stopped in a bacterium, DNA synthesis should cease when all ongoing rounds of replications of the chromosome are completed (21). In a nonsynchronized cell population growing in a minimal medium, this means that if protein synthesis is stopped, then DNA synthesis should increase only by about 50% before coming to a stop. Our results (Fig. 7 and 8) show that both protein synthesis and DNA synthesis were effectively stopped within 3 min, which for DNA synthesis was faster than expected from the completion of ongoing rounds of replication. These findings indicate that the lethal action of PR-39 may be to stop protein synthesis and induce degradation of some proteins required for DNA replication. In fact, it is possible that a PR-39-induced proteolytic activity could be the primary action that stops both protein and DNA synthesis. Consistent with this suggestion is the finding that actively growing cells are killed more rapidly than nongrowing cells (Fig. 4), which was observed in an experiment performed with a minimum difference in media. To date, there is no other antibacterial peptide of animal origin for which a similar mechanism has been suggested.

Our departure point was that cecropin P1 and PR-39 are produced in the same upper part of the small intestine of pigs, which is almost sterile. The LCs of the peptides (Table 1) are the same for all strains with the possible exception of strain D22, which is somewhat more susceptible to PR-39 than to cecropin P1. However, other experiments (such as the one for Fig. 1) indicate that cecropin P1 is at least twice as potent as PR-39. However, part of this difference could come from the fact that the water content in the freeze-dried peptide may give an error as to the true concentration. For both of our peptides, increasing amounts of peptide are needed in order to kill increasing numbers of bacteria (Fig. 3). Finally, it should be stressed that our mode of recording antibacterial activities may not reflect the in vivo situation in the intestine. We have looked for a synergism between our two peptides without finding any evidence for cooperativity. However, if the in vivo function of the peptides is the control of the normal intestinal flora (in conjunction with other antibacterial peptides), it could still be an advantage for the host to have agents working with different mechanisms of action.

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