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Activities of Antimicrobial Peptides and Synergy with Enrofloxacin against *Mycoplasma pulmonis*▽

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We showed in a previous study that associations of antimicrobial peptides (AMPs), which are key components of the innate immune systems of all living species, with the fluoroquinolone enrofloxacin can successfully cure HeLa cell cultures of *Mycoplasma fermentans* and *M. hyorhinis* contamination. In the present work, the in vitro susceptibility of *M. pulmonis*, a murine pathogen, to enrofloxacin and four AMPs (alamethicin, globomycin, gramicidin S, and surfactin) was investigated, with special reference to synergistic associations and the effect of the mycoplasma cell concentration. Enrofloxacin and globomycin displayed the lowest MICs (0.4 μM), followed by gramicidin S (3.12 μM), alamethicin (6.25 μM), and surfactin (25 μM). When the mycoplasma cell concentration was varied from 10^4 to 10^8 CFU/ml, the MICs of enrofloxacin and globomycin increased while those of the three other molecules remained essentially constant. The minimal bactericidal concentration of enrofloxacin (0.8 μM) was also lower than those of the peptides (6.25 to 100 μM), but the latter killed the mycoplasma cells much faster than enrofloxacin (2 h versus 1 day). The use of the AMPs in association with enrofloxacin revealed synergistic effects with alamethicin and surfactin. Interestingly, the mycoplasma-killing activities of the two combinations enrofloxacin (MIC/2) plus alamethicin (MIC/4) and enrofloxacin (MIC/2) plus surfactin (MIC/16) were about 2 orders of magnitude higher than those of the three molecules used separately. These results support the interest devoted to AMPs as a novel class of antimicrobial agents and pinpoint their ability to potentiate the activities of conventional antibiotics, such as fluoroquinolones.

The continuous emergence of clinical bacterial strains resistant to one or several conventional antibiotics has become a major medical and veterinary problem in the last decade and has prompted a search for novel anti-infective agents. Among the compounds currently under investigation for their therapeutic potentials are several antimicrobial peptides (AMPs), and a number of them have reached the phase of clinical evaluation and marketing (for a review, see references 1, 6, 19, and 27). Some of these AMPs are natural components of the defense systems of living organisms, from bacteria to plants and animals (6, 8, 16). These peptides are usually amphipathic and cationic, and their sizes generally range from 6 to 40 amino acid residues. Although they are very diverse in structure, origin, and biosynthesis mode, they share a high affinity for membranes, which are, for most of them, the main cell target (for a review, see references 7 and 27). In spite of the growing wealth of information on AMPs, many issues are still unanswered, particularly those concerning their mechanism of action and the factors responsible for their efficacy and antimicrobial spectrum. In vitro, the action of an antibiotic against a given bacterium depends on many factors, including (i) the chemical nature of the molecule, (ii) the antibiotic concentration, (iii) the bacterial density, (iv) the time of incubation, and (v) the presence or absence of another antibiotic. In this study, the influences of these factors on the antibacterial activities of selected AMPs were first investigated and compared to the activity of a conventional antibiotic, enrofloxacin (EFX). Moreover, as combinations of drugs may enhance antibacterial activity and hamper the development of resistance, the association of AMPs with EFX was also analyzed.

This work focused on mycoplasmas (class *Mollicutes*), which include pathogens causing persistent and chronic respiratory, arthritic, and urogenital diseases in humans and many animal hosts (5, 15, 17). These minimalist bacteria (9) are characterized by the simplicity of their cell envelope, which lacks a cell wall and other components (lipopolysaccharide, outer membrane, and teichoic acids) found in gram-positive or gram-negative bacteria. This simplicity explains why mycoplasmas are attractive organisms for the study of antibiotics such as AMPs, which primarily target the plasma membrane. It also explains why the number of antibiotics that can be used for treating mycoplasmal infections is limited to tetracyclines, the macrolide-lincosamide-streptogramine-ketolide group, fluoroquinolones, and phenicols (2). The present work focuses on *Mycoplasma pulmonis*, which is the etiologic agent of respiratory mycoplasmosis in rodents. This pathogen is considered a very useful model for studying mycoplasmal respiratory infections, including those commonly caused by *Mycoplasma pneumoniae* in humans (17).

More specifically, we investigated (i) the influences of the antibiotic concentration, bacterial density, and incubation time on the antimycoplasmal activities of four AMPs (alamethicin, gramicidin S, globomycin, and surfactin) and (ii) the benefits of combining them with EFX, a fluoroquinolone widely used in veterinary medicine and having a potent activity against mycoplasmas (for a review, see reference 2). Among the four AMPs

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TABLE 1. MICs and MBCs of AMPs and enrofloxacin for *M. pulmonis* MpUR1.1[a]

Drug	MIC (μM)/ (μg/ml)	MIC/MIC EFX[b]	MBC (μM)/ (μg/ml)	MBC/MBC EFX[b]
Alamethicin	6.25/12.3	15.6	25/49.1	31.2
Globomycin	0.4/0.26	1	12.5/8.2	15.6
Gramicidin S	3.12/3.6	7.8	6.25/7.1	7.8
Surfactin	25/25.9	62.5	100/103.6	125
EFX	0.4/0.14	1	0.8/0.29	1

[a] MIC determinations were realized in Hayflick medium containing 5% horse serum with the standard concentration (10^6 CFU/ml) of exponential-growth-phase cells of MpUR1.1 For MBC determinations, 0.1-ml aliquots were withdrawn after 2 h for AMPs and 24 h for enrofloxacin and then diluted and plated for viable-cell count. Experiments were performed in triplicate.

[b] The comparison of AMP efficacy with that of EFX is indicated by using the ratio of MICs or MBCs (μM).

mentioned above, alamethicin is a 20-residue amphipathic peptide produced by the fungus *Trichoderma viride* and a member of the peptaibol family (for a review, see reference 25). Alamethicin helices form transmembrane voltage-dependent pores by aggregation in lipid bilayers (for a review, see references 12 and 18). The other AMPs are of bacterial origin and share a cyclic structure. While gramicidin S is a decapeptide (for a review, see reference 24) surfactin is a lipo-octapeptide acting as a surfactant on bacterial membranes (for a review, see reference 22), and globomycin is a lipopentapeptide that specifically inhibits bacterial signal peptidase II (11, 23, 26). These four AMPs were chosen for their structural diversity, for their known antimycoplasmal activities (4, 14), and because additive effects with EFX on mycoplasmas have already been observed in a tissue cell culture model system (20).

MATERIALS AND METHODS

Bacterial strain and culture medium. The *M. pulmonis* MpUR1.1 clone was obtained by randomly picking a colony from a culture of a *M. pulmonis* UAB CTIP isolate as previously described (14). The mycoplasmas were grown in Hayflick medium containing 5% horse serum. For agar plates, Noble agar (DIFCO) at a final concentration of 1% (wt/vol) was added.

Antimicrobial agents. Alamethicin, gramicidin S, surfactin, chloramphenicol, tylosine, mitomycin C, and tetracycline were of commercial origin (Sigma), whereas globomycin was a gift from S. Miyakoshi (Sankyo Co., Japan). Streptomycin and EFX were obtained from Laboratoires Diamant (Paris, France) and Bayer AG (Leverkusen, Germany), respectively.

Determination of the MICs and the MBCs. The antimycoplasmal activities of antibiotics were determined as described previously (4, 14). Briefly, mycoplasmas at an initial concentration of 10^6 CFU/ml were grown in Hayflick medium containing 5% horse serum in 96-well plates in the presence of twofold serial dilutions of the antibiotics. The drug concentrations ranged from 0.4 to 100 μM. A change in the color of the phenol red added to the medium as a pH indicator was used to monitor bacterial growth. The MIC was defined as the lowest antibiotic concentration that completely inhibited the growth of mycoplasmas after 48 h. For minimal bactericidal concentration (MBC) determinations, cells were incubated in the presence of AMPs for 2 h and EFX for 24 h and then plated on solid culture medium. The plates were incubated at 37°C under anaerobic conditions and examined after 4 to 5 days for the appearance of colonies. The MBC was defined as the lowest antibiotic concentration that killed ≥99.9% of the cells. Experiments were performed in triplicate.

Checkerboard titration for antibiotic combination studies. Antibiotic combinations were tested by the checkerboard titration method using 96-well microtiter plates. The drug concentrations ranged from 0.4 to 50 μM, 0.1 to 1.25 μM, 0.05 to 6.25 μM, 0.012 to 1.6 μM, and 0.006 to 0.8 μM for surfactin, alamethicin, gramicidin S, globomycin, and EFX, respectively. The fractional inhibitory concentration (FIC) index for combinations of two antimicrobials was calculated according to the following equation: FIC index = $FIC_A + FIC_B = (A/MIC_A) + (B/MIC_B)$, where A and B are the MICs of drug A and drug B in the combination and MIC_A and MIC_B are the MICs of drug A and drug B alone. The FIC indexes were interpreted as follows: ≤0.5, synergy; >0.5 to 4, indifference; >4, antagonism (13). Experiments were performed in duplicate.

Time-kill assay. Aliquots of exponentially growing mycoplasmas (2.5×10^8 CFU/ml in Hayflick medium) were resuspended in fresh Hayflick broth at approximately 10^8, 10^6, and 10^4 CFU/ml and exposed to antimicrobial agents alone at 1× MIC for 1, 2, or 24 h at 37°C. Then, 0.1-ml samples were serially diluted in Hayflick medium and plated onto agar plates to obtain viable colonies. The control experiment consisted of plating cultures of MpUR1.1 without antibiotics. For combination studies, antimicrobial agents were used at their MIC or at concentrations equal to MIC/2 for EFX, MIC/4 for alamethicin, and MIC/16 for surfactin according to checkerboard titration results. Experiments were performed in duplicate.

RESULTS

Effects of the mycoplasma concentration on the antibacterial activities of AMPs. The in vitro efficacies of EFX and the AMPs against *M. pulmonis* were first evaluated by measuring their growth inhibition and killing activities. MICs of AMPs and of EFX, determined at standard mycoplasma concentrations (10^6 CFU/ml), ranged from 0.4 to 25 μM (Table 1). Specifically, the following order of decreasing activities was recorded: EFX and globomycin (0.4 μM) > gramicidin S (3.12 μM) > alamethicin (6.25 μM) > surfactin (25 μM). The MBCs were determined under the same conditions (Table 1). For the selected antibiotics, the MBC/MIC ratio was in the range from 2 to 4, except for globomycin, for which it was higher (MBC/MIC = 31).

Having defined the susceptibility of *M. pulmonis* for these antibiotics under standard conditions, different factors likely to affect this efficacy were changed. The first to be evaluated was the initial bacterial concentration in the assay. When this concentration varied from 10^4 CFU/ml to 10^8 CFU/ml, the MICs of EFX and globomycin were increased eightfold (Fig. 1).

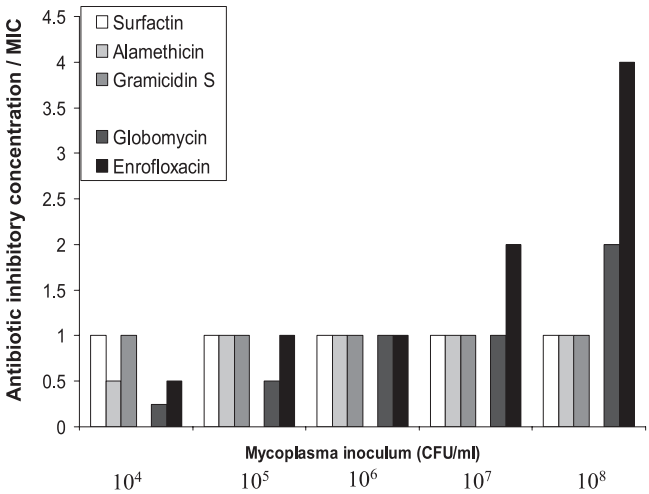

FIG. 1. Effects of variations in the *M. pulmonis* concentration on the MICs of AMPs and enrofloxacin. Various concentrations (10^4 to 10^8 CFU/ml) of exponential-phase growing cells of MpUR1.1 were incubated with twofold dilutions of antibiotics. Inhibitory concentrations of antibiotics were determined by monitoring changes of color of phenol red after 24 and 48 h. For each cellular concentration, the inhibitory-concentration/MIC ratio was calculated. At the standard concentration (10^6 CFU/ml), the ratio was equal to 1. The experiment was performed three times, and the results did not vary from one experiment to the next.

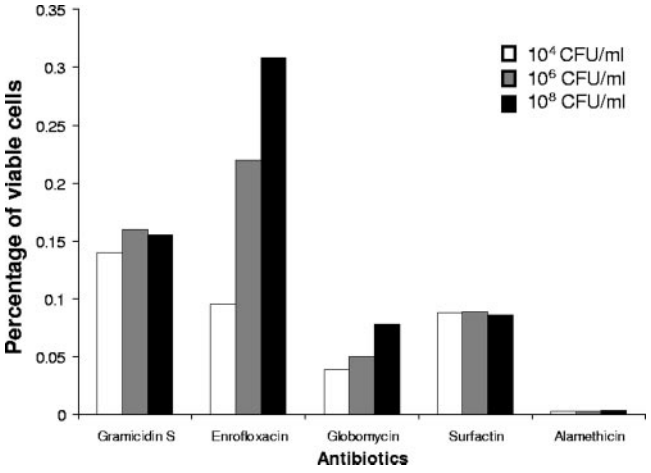

FIG. 2. Effects of variations in the *M. pulmonis* concentration on the bactericidal activities of AMPs and EFX. Cultures of *M. pulmonis* at three different concentrations (10^4, 10^6, and 10^8 CFU/ml) were incubated with antibiotics (concentration equal to the MIC). Aliquots were withdrawn after 24 h and diluted to obtain a viable count expressed as a percentage of the viable count of the control culture (without antibiotic). Average values from two independent experiments are shown. The values did not vary significantly from one experiment to the next (at most, there was a 10% variation in the number of CFU/ml).

Similar results were obtained with other conventional antibiotics, such as chloramphenicol, streptomycin, tylosin, mitomycin C, and tetracycline (data not shown). In contrast, within the same range of mycoplasma concentrations, the MICs remained the same for gramicidin S and surfactin and increased, but only twofold, for alamethicin (Fig. 1). The same question was addressed by a time-kill assay using the selected antimicrobial agents at their MICs at three different cellular concentrations: 10^4, 10^6, and 10^8 CFU/ml (Fig. 2). When used alone, EFX and globomycin killed three times and two times fewer bacterial cells, respectively, in the presence of 10^8 CFU/ml than in the presence of 10^4 CFU/ml, whereas the percentages of killing by gramicidin S, surfactin, and alamethicin did not change (Fig. 2). These results indicate that the AMPs retained the same efficacy even when the concentration of the bacterial inoculum increased, with the exception of globomycin, which was similar in this regard to conventional antibiotics.

The time courses of antimycoplasma activities of AMPs and EFX. Due to their nonspecific actions on cell membranes, AMPs kill bacteria more rapidly than conventional antibiotics. With the aim of verifying this on *M. pulmonis*, we followed the time courses of cell killing by the selected AMPs and by EFX. Antibiotics were added at a final concentration equal to their MIC to a log-phase *M. pulmonis* culture (10^6 CFU/ml), and the counting of viable cells was realized after 1, 2, and 24 h of incubation. The results were expressed as the percentage of surviving cells (Fig. 3). After 1 hour of incubation, the decreasing order of viable cells was 98% for gramicidin S, 89% for EFX, 68% for globomycin, 43% for surfactin, and 31% for alamethicin. After 2 hours, the bactericidal activities of all of the antimicrobial agents were enhanced, but without modifying the order of efficacy (Fig. 3). In contrast, after 24 h of incubation, the order of efficacy was altered, since the percentages of viable cells were 0.22 for EFX, 0.16 for gramicidin S, 0.08 for surfactin, 0.05 for globomycin, and virtually 0 for alamethicin (Fig. 3). These data show that the highest antibacterial activity against *M. pulmonis* was obtained after 24 h of incubation for both EFX and AMPs and that the most active AMP used alone was alamethicin.

Effects of the combination of EFX and AMPs on *M. pulmonis*. Combinations of the four AMPs with EFX were tested in order to enhance mycoplasma killing. The efficacies of these combinations were assessed using the checkerboard method and a time course killing assay.

In the checkerboard method, the FIC indexes were equal to 0.56 for the EFX-surfactin association, 0.75 for EFX-alamethicin, and 1 for both EFX-globomycin and EFX-gramicidin S associations (Fig. 4). Actually, the FIC of the EFX-surfactin combination suggested a synergy of action of the two compounds, since it proved to be quite close to the threshold (FIC ≤

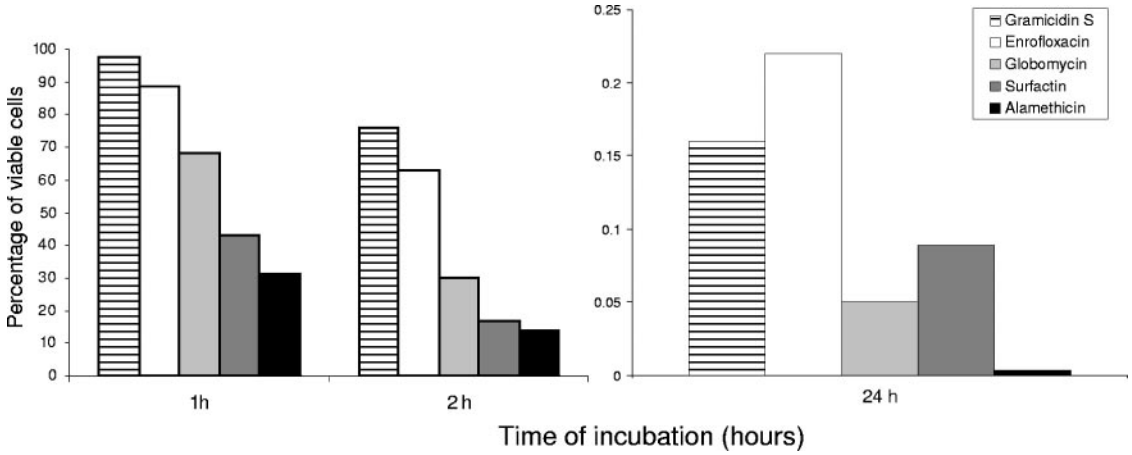

FIG. 3. Rates of killing of *M. pulmonis* by AMPs and enrofloxacin. Log-phase bacteria (about 10^6 CFU/ml) were obtained in Hayflick medium, and concentrations equal to the MICs of antibiotics (Table 1) were added at time zero. Aliquots were withdrawn at the indicated times (1, 2, and 24 h) and diluted to count viable cells. The percentage of viable cells was equal to 100% for the control (i.e., MpUR1.1 without antibiotic). Average values from two independent experiments are shown. The values did not vary significantly from one experiment to the next (at most, there was a 6.4% variation in the number of CFU/ml).

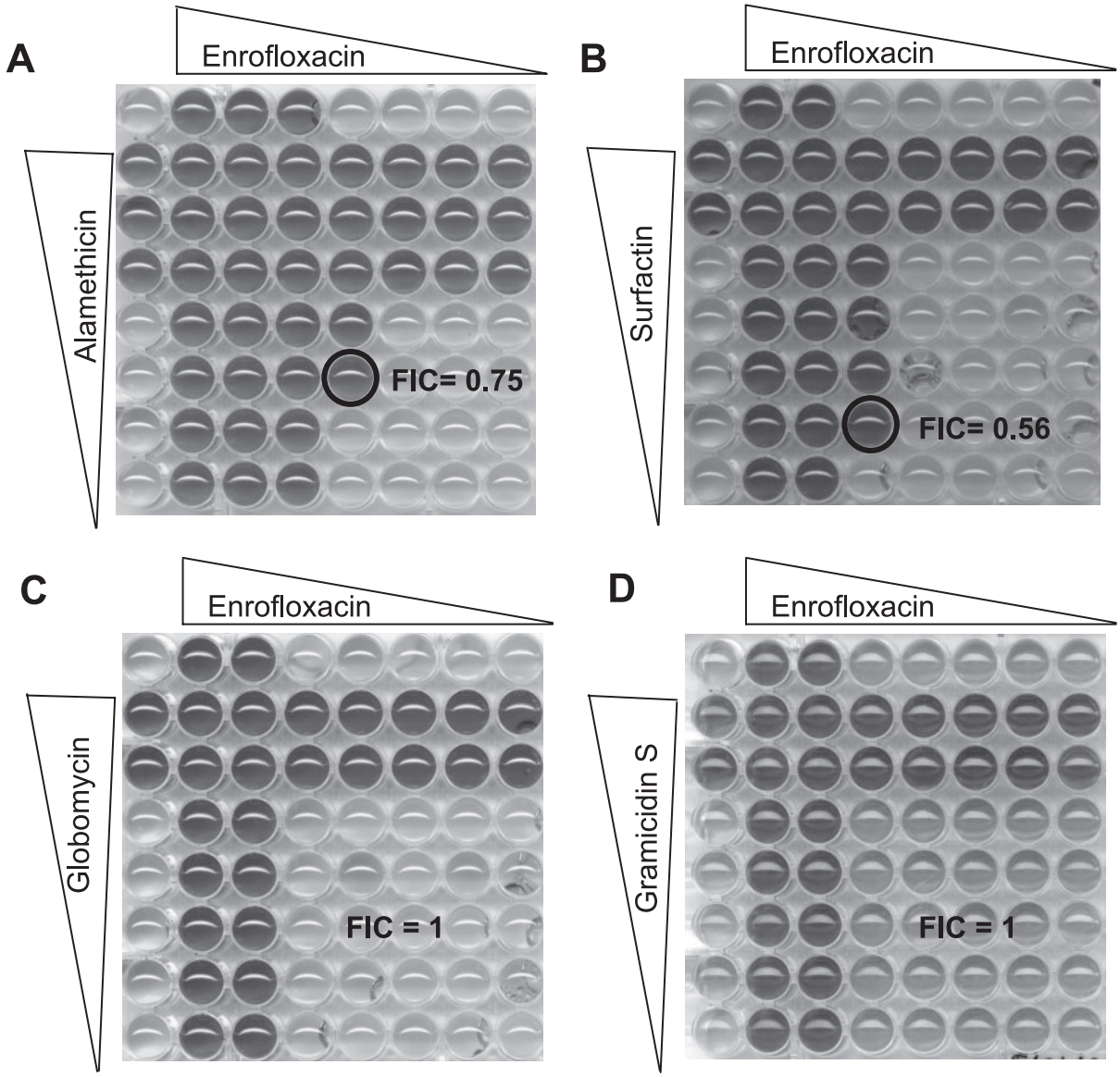

FIG. 4. Effects of combinations of enrofloxacin and AMPs on *M. pulmonis* growth by the checkerboard system. A culture of *M. pulmonis* (10^6 CFU/ml) was incubated with twofold dilutions of antibiotic associations from 2× MIC to MIC/32. The inhibition of growth was monitored by the color change of Hayflick medium. FIC indexes were subsequently calculated (see Materials and Methods) for each of the combinations: EFX with alamethicin (A), with surfactin (B), with globomycin (C), and with gramicidin S (D). Experiments were performed in duplicate. No significant changes were observed from one experiment to another.

0.5) defined by current international standards for synergistic action (21).

Two different experiments were performed using the time course killing method: (i) with concentrations of antimicrobial agents equal to the MIC and (ii) with concentrations equal to MIC/2, MIC/4, and MIC/16 for EFX, alamethicin, and surfactin, respectively. These concentrations were chosen according to the results obtained with the checkerboard method: *M. pulmonis* growth was inhibited using combinations of EFX at a MIC/2 concentration and with alamethicin at a MIC/4 concentration (Fig. 4A). The EFX-surfactin combination inhibited mycoplasma growth at concentrations equal to MIC/2 for EFX and MIC/16 for surfactin (Fig. 4B).

A time course killing assay using EFX and AMPs at their MICs indicated that whatever the time of incubation (1, 2, or 24 h), the number of viable cells detected after the action of EFX-gramicidin S or EFX-globomycin combinations on *M. pulmonis* was similar to that obtained after the action of each of the AMPs alone (Fig. 5A). In contrast, for the EFX-surfactin combination, the number of viable cells dropped by almost 2 orders of magnitude (≈90-fold) after 24 h compared to the action of surfactin alone (Fig. 5A). The EFX-alamethicin combination showed the highest efficacy, even after 1 hour (2.4% of viable cells versus 31% with the AMP alone). After 2 h of incubation, cell killing was close to 99.9%, meaning that the number of viable cells was 100 times lower than with the AMP alone (Fig. 5A).

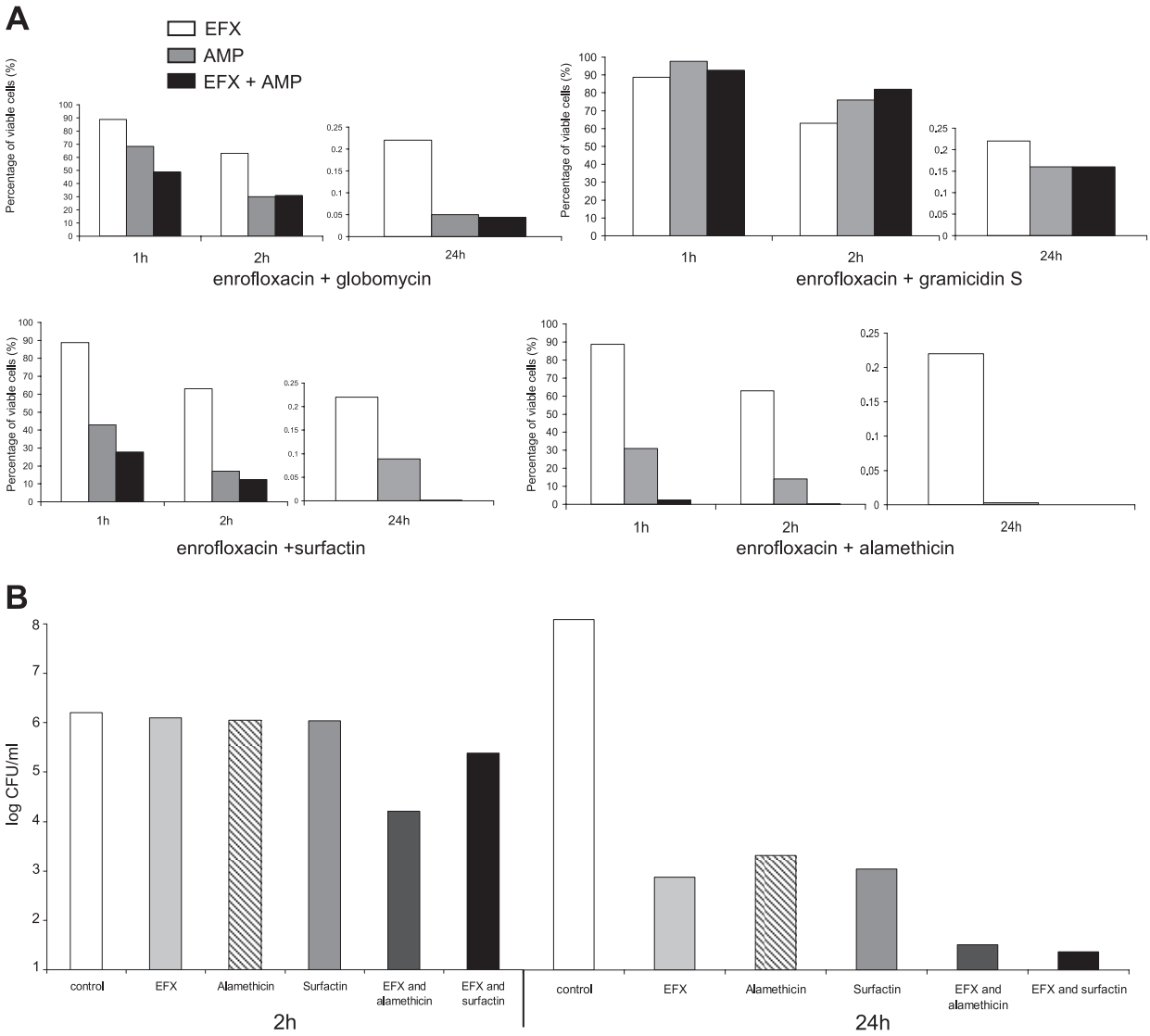

FIG. 5. Effects of the combination of enrofloxacin and AMPs on *M. pulmonis* growth by the viable-cell count technique. (A) Log-phase bacteria (about 10^6 CFU/ml) were obtained in Hayflick medium. The viable-cell count was assessed by incubating mycoplasma with each antibiotic alone or with a combination of concentrations of enrofloxacin with globomycin, gramicidin S, surfactin, and alamethicin equal to the MIC. Aliquots were withdrawn after 1, 2, and 24 h and diluted for a viable count. Average values from two independent experiments are shown. The values did not vary significantly from one experiment to the next (at most, there was a 6.2% variation). (B) The same combination experiments were performed using EFX at MIC/2, alamethicin at MIC/4, and surfactin at MIC/16. Viable-cell counts were performed after 2 and 24 h. Average values from two independent experiments are shown. The values did not vary significantly from one experiment to the next (at most, there was a 5% variation in the number of CFU/ml).

We then assessed the killing activities of these two combinations after 2 and 24 h, this time using concentrations less than the MIC (Fig. 5B). The mycoplasma-killing activities of the two combinations enrofloxacin (MIC/2) plus alamethicin (MIC/4) and enrofloxacin (MIC/2) plus surfactin (MIC/16) were almost 2 orders of magnitude higher than those of the three molecules used separately, indicating synergistic effects between EFX and the two AMPs.

DISCUSSION

After determining the MICs and MBCs of alamethicin, globomycin, gramicidin S, and surfactin against *M. pulmonis* (Table 1), we assessed the importance of the bacterial-cell concentration on the activities of these AMPs. Under our standard conditions (10^6 CFU/ml) and on a molar basis, the MIC of globomycin was equal to that of EFX while the MICs of the three other AMPs were from 7.8 to 62.5 times higher than that of EFX; under the same conditions, the MBCs of the peptides were 7.8 to 125 times higher than that of EFX, suggesting better efficacy of the fluoroquinoline than of the AMPs. However, the increase in the initial mycoplasmal concentration from 10^4 to 10^8 CFU/ml increased the MICs of EFX and globomycin but increased those of the other three AMPs very little or not at all. Although the mechanism underlying the lack

of sensitivity of AMPs to the variation of the bacterial-cell density remains unknown, one can hypothesize that at the MIC and above, a large fraction of AMP molecules remain in solution while only a small fraction probably act on the membrane, affecting its integrity. Hence, increasing bacterial density raises the recruitment of new peptide monomers from the solution, which in turn destabilize the mycoplasmal membrane without significantly modifying the overall active concentration of AMP that is able to inhibit bacterial growth. Whatever the rationale underlying this phenomenon, the independence of AMP activities with respect to the target cell concentration is a significant advantage over the mode of action of conventional antibiotics, which are dependent on the cell concentration. In contrast to membrane-active AMPs, the main and specific target of globomycin is signal peptidase II, an enzyme involved in lipoprotein maturation. Indeed, as demonstrated in two previous studies performed on the mollicute *Spiroplasma melliferum*, the action of globomycin on the cell membrane is quite marginal, even when used at high concentrations (3, 4). Hence, as for conventional antibiotics acting with high affinity on a specific protein target, the concentration of globomycin required for inhibiting or killing bacterial cells is proportional to their concentration.

This study was also aimed at identifying possible synergistic interactions between EFX and the four AMPs. The checkerboard method and the time course killing assay showed indifferent interactions between the fluoroquinolone EFX and globomycin or gramicidin S. In contrast, this method revealed that combining EFX with alamethicin or surfactin led to FIC indexes of <1, indicating possible additive effects between these molecules. These results were confirmed by the time course killing assay of the EFX-alamethicin and EFX-surfactin combinations. When, in these associations, the three molecules were used at their respective MICs, it was possible to kill virtually all the mycoplasma cells after 2 h with the EFX plus alamethicin combination and after 24 h with the EFX plus surfactin combination. Furthermore, the mycoplasma-killing activities of the two combinations enrofloxacin (MIC/2) plus alamethicin (MIC/4) and enrofloxacin (MIC/2) plus surfactin (MIC/16) were almost 2 orders of magnitude higher than those of the three molecules used separately. Consistent with the fact that the association of EFX with alamethicin was capable of curing a HeLa cell culture of *Mycoplasma fermentans* in 30 min and of *Mycoplasma hyorhinis* in 24 h (20), this result indicated synergistic effects between EFX and alamethicin or surfactin against *M. pulmonis*.

Hence, the two salient results of our study are that, in vitro, (i) AMPs retain the same level of activity over a large range of *M. pulmonis* concentrations and (ii) there is a synergistic action between the fluoroquinolone enrofloxacin and two AMPs (alamethicin and surfactin). Associating EFX with either of these two AMPs allowed lower concentrations of EFX and AMPs to be used than when each molecule was taken alone. This is important because, although AMPs are broad-spectrum antimicrobials displaying rapid bactericidal activity, from the perspective of therapeutic applications these advantages are counterbalanced by limited in vivo stability (notably for AMPs composed of L-α-amino acid residues) and possible toxicity toward mammalian cells (see reference 19 for a recent review of this subject). Toxicity remains a major concern, because the MICs of AMPs are usually 1 or 2 orders of magnitude higher than those of many conventional antibiotics. This was confirmed in this study when the MIC and MBC of EFX were compared with those of the AMPs (Table 1). Furthermore, the activities of AMPs can be hampered in vivo by different biological factors, such as enzymatic cleavage or absorption by serum lipoproteins. Fortunately, a better knowledge of the structure-activity relationships of AMPs makes it feasible to design molecules with a better therapeutic index. For example, Dartois et al. (10) have recently designed cationic cyclic peptides displaying a broad antimicrobial spectrum and a prolonged systemic activity in the mouse. Additionally, although the pharmaceutical usefulness of AMPs has still to be fully established, several molecules are presently under clinical development (1, 19, 27), and one may expect still better achievements in the future from the association of conventional antibiotics with engineered AMPs. We believe that this track should be explored in the case of mycoplasmas.

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