APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1997, p. 44–49 Vol. 63, No. 1
0099-2240/97/$04.00+0

Antimycoplasma Properties and Application in Cell Culture of Surfactin, a Lipopeptide Antibiotic from *Bacillus subtilis*

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Received 5 July 1996/Accepted 10 September 1996

Surfactin, a cyclic lipopeptide antibiotic and biosurfactant produced by *Bacillus subtilis*, is well-known for its interactions with artificial and biomembrane systems (e.g., bacterial protoplasts or enveloped viruses). To assess the applicability of this antiviral and antibacterial drug, we determined the cytotoxicity of surfactin with a 50% cytotoxic concentration of 30 to 64 μM for a variety of human and animal cell lines in vitro. Concomitantly, we observed an improvement in proliferation rates and changes in the morphology of mycoplasma-contaminated mammalian cells after treatment with this drug. A single treatment over one passage led to complete removal of viable *Mycoplasma hyorhinis* cells from various adherent cell lines, and *Mycoplasma orale* was removed from nonadherent human T-lymphoid cell lines by double treatment. This effect was monitored by a DNA fluorescence test, an enzyme-linked immunosorbent assay, and two different PCR methods. Disintegration of the mycoplasma membranes as observed by electron microscopy indicated the mode of action of surfactin. Disintegration is obviously due to a physicochemical interaction of the membrane-active surfactant with the outer part of the lipid membrane bilayer, which causes permeability changes and at higher concentrations leads finally to disintegration of the mycoplasma membrane system by a detergent effect. The low cytotoxicity of surfactin for mammalian cells permits specific inactivation of mycoplasmas without significant deleterious effects on cell metabolism and the proliferation rate in cell culture. These results were used to develop a fast and simple method for complete and permanent inactivation of mycoplasmas in mammalian monolayer and suspension cell cultures.

Mycoplasmas are causative agents of serious diseases of humans and animals, such as acute respiratory inflammation (including pneumonia) and diseases of the urogenital tract, and seem to be cofactors in the pathogenesis of AIDS (4, 30). These smallest free-living organisms are parasites of eukaryotic cells and are one of the major contaminants that affect tissue culture cells. The most prevalent agents that do this are the mollicute species *Mycoplasma orale* (a human species), *Mycoplasma hyorhinis* (a porcine species), *Mycoplasma arginini* (a bovine species), and *Acholeplasma laidlawii* (a bovine species) (16). Contaminating mycoplasmas affect a variety of cellular processes and cell morphology, deplete the nutrients in the growth medium, and interfere with virus replication (5, 24). For both biological and ecological reasons, it is important to eliminate these agents from cell cultures used for basic research, diagnosis, and biotechnological production. The most effective procedure for eliminating, inactivating, or suppressing mycoplasmas in cell cultures is treatment with antibiotics (6, 22, 27). In general, antibiotic therapies do not result in long-lasting successful decontamination, and undesirable side effects on eukaryotic cells due to cytotoxic effects and the development of resistant mycoplasma strains have been observed (22, 27). Mycoplasmas lack a cell wall but are encircled by a three-layer cytoplasmic membrane, so that antibiotics such as the penicillins, which are common additives in cell culture media and interfere with murein formation in cell walls, are not effective against them.

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Screening for new antimycoplasma agents with novel modes of action is necessary. The secondary metabolites of various bacteria, fungi, and yeasts are some of the most profitable sources of new antibiotics. The soil bacterium *Bacillus subtilis* produces an abundance of substances with antibiotic properties and with diverse structures (31). One of these, surfactin, is a cyclic lipopeptide antibiotic with a molecular weight of 1,036. It contains a mixture of several β-hydroxy fatty acids with chain lengths of 13 to 15 carbon atoms as its lipid portion. The main component is 3-hydroxy-13-methylmyristic acid, which forms a lactone ring system with an anionic heptapeptide (Fig. 1). As a consequence of this amphiphilic structure, surfactin is a powerful biosurfactant with high surface activity (1, 12, 14) and various interesting biological properties. It exhibits antifungal properties, moderate antibacterial properties (3, 26), and hemolysis; inhibits fibrin clot formation (1, 3); induces the formation of ion channels in lipid bilayer membranes (23); inhibits enzymes such as cyclic AMP phosphodiesterase (10); exhibits antiviral and antitumor activities (13, 29); and inhibits starfish oocyte maturation (25).

In this paper we show that improvements in the proliferation rates of mycoplasma-contaminated mammalian cells after treatment with surfactin were due to the antimycoplasma activity of this drug. We investigated the mode of surfactin action and developed an efficient method for eliminating mycoplasmas from adherent and nonadherent mammalian cells.

MATERIALS AND METHODS

Source of surfactin. Surfactin was purchased from Biomol (Hamburg, Germany) and Sigma (Deisenhofen, Germany) or was purified from culture supernatants of *B. subtilis* OKB105 by acid precipitation, extraction with methanol, charcoal treatment, and gel filtration with Pharmacia Sephadex LH-20 as described previously (2). For the experiments described below, a 1 mM surfactin

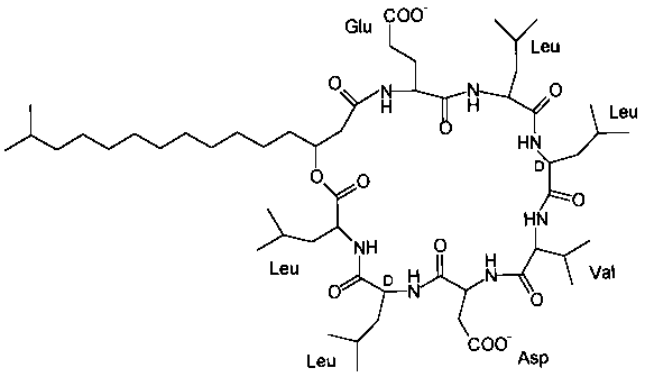

FIG. 1. Structure of surfactin.

solution in phosphate-buffered saline (PBS) was sterilized by heat treatment (121°C, 30 min).

Cells and culture conditions. All cell lines investigated for mycoplasma elimination were continuous cell lines cultured in medium without any antibiotic at 37°C in a humidified atmosphere containing 5% CO_2 in air. Adherent cell lines ML (mink lung), 293 (human embryonal kidney), CV1 (African green monkey kidney), and Hep2 (human larynx carcinoma) were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Uxbridge, Great Britain) supplemented with 5% heat-inactivated fetal calf serum (FCS) (ICN Pharmaceuticals Inc., Irvine, Calif.) in petri dishes (Nunc, Roskilde, Denmark). Suspension cell lines MT-4 (human T-cell leukemia virus type 1 transformed), Molt 4 clone 8 (Molt 4/8), and H9, all of which are human T-lymphoid cell lines, were grown in RPMI 1640 medium (ICN) supplemented with 10% (vol/vol) FCS and 2 mM L-glutamine in tissue culture flasks (Nunc). All suspension cell lines and adherent cell line CV1 were subcultured once a week, and all other adherent cell lines were subcultured twice a week.

Cytotoxicity assay. The 50% cytotoxic concentrations of surfactin for the adherent cell lines were determined by the crystal violet dye uptake assay by using the method described by Flick and Gifford (7). Adherent cells (approximately 10^5 cells/ml) were grown on microtiter plates (200 μl of cell suspension/well) with serial dilutions of surfactin (concentration range, 10 to 70 μM) mixed with DMEM supplemented with 5% (vol/vol) FCS. After the control culture was confluent, the cells were fixed with 1% glutardialdehyde, stained with crystal violet, washed with H_2O, and dried. The dye was dissolved in 100 μl of ethanol-water-acetic acid (50:49.9:0.1). A_{550} values were determined with a microplate reader. The proliferation rates of the nonadherent cell lines after treatment with surfactin were determined by the colorimetric tetrazolium dye reduction assay and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (17). The yellow compound MTT (Sigma) is reduced by mitochondrial dehydrogenases to the water-insoluble blue compound formazan, depending on the viability of the cells. A 20-μl portion of a solution of MTT (5 mg/ml in PBS) was added to every well. The plate was incubated for 4 h at 37°C in a CO_2 incubator. After incubation 150 μl of medium was removed from every well without disturbing the cell clusters. A 100-μl portion of acidified isopropanol (2 ml of concentrated HCl added to 500 ml of isopropanol) was added to each sample, and the preparations were mixed thoroughly on a plate shaker with the cells containing formazan crystals. After all of the crystals were dissolved, the A_{550} values were determined with a microplate reader.

Mycoplasma detection. (i) Cytochemical staining of DNA with DAPI. The cell cultures used for cytochemical staining of DNA with 4′,6-diamidino-2-phenylindole (DAPI) (Boehringer, Mannheim, Germany) (21) were grown on coverslips in petri dishes to 70% confluence. The culture medium was removed, and the cells were fixed with methanol at room temperature. The cells were incubated for 5 min at 37°C with a DAPI staining solution (1 μg of DAPI per ml of methanol) and rinsed with methanol. Using PBS as the mounting medium, we examined the cells with a fluorescence microscope fitted with a Zeiss filter combination consisting of type BP 365 and 520-560 filters. Mycoplasmas appeared as bright yellow-green spots against a dark cytoplasmic background next to the fluorescence signal of the stained nuclear DNA of the mammalian cells.

(ii) Immunological detection: enzyme-linked immunosorbent assay (ELISA). Immunological detection and identification of *M. orale*, *M. hyorhinis*, *A. laidlawii*, and *M. arginini* were performed with a commercially available mycoplasma detection kit (Boehringer). Biotinylated polyclonal antibodies directed against specific mycoplasma antigens were reacted with the cells. Binding of the antibodies was visualized by the streptavidin-alkaline phosphatase assay. After enzymatic hydrolysis of 4-nitrophenyl phosphate, the yellow nitrophenol product was quantified with a microplate reader (model EAR 400 AT; SLT-Labinstruments, Grödig, Austria) at a wavelength of 405 nm.

(iii) PCR amplification of mycoplasma rRNA. The templates used for mycoplasma PCR were extracts of cell-free culture media prepared by boiling. A 100-μl portion of a cell culture supernatant was transferred into a sterile Eppendorf tube and boiled for 5 min. The tube was centrifuged briefly, and the supernatant was diluted 1:10 with sterile double-distilled water. A mycoplasma PCR primer set obtained from Stratagene (La Jolla, Calif.) was used to differentiate mycoplasma types, and the primer set of van Kuppeveld et al. (28) was used to identify groups, as described elsewhere (18). Each final PCR mixture (total volume, 50 μl) contained 10 μl of template and 40 μl of amplification mixture containing 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 at 25°C), 0.01% Tween 20, 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 50 μM, each primer at a concentration of 0.2 μM, and 1.25 U of *Thermus aquaticus* DNA polymerase (InViTek, Berlin, Germany). Each reaction mixture was overlaid with 2 drops of mineral oil. PCR amplification was performed with a Perkin-Elmer thermal cycler. The initial 5-min denaturation step at 94°C was followed by a 1.75-min annealing step at 55°C. Next there was a 3-min primer extension step at 72°C, followed by a 45-s denaturation step at 94°C and a 1.75-min annealing step at 55°C. The remaining 40 cycles each consisted of extension for 3 min at 72°C, denaturation for 45 s at 94°C, and annealing for 45 s at 55°C. The posttreatment steps consisted of 10 min at 72°C and then 10 min at 27°C. The amplified PCR products were separated by standard agarose gel electrophoresis in 2 and 4% (wt/vol) agarose gels for the Stratagene system and the system of van Kuppeveld et al., respectively, and were visualized by ethidium bromide staining.

Electron microscopy. ML cells were propagated in DMEM supplemented with 5% FCS. After the culture was confluent, the surfactin concentration in the medium was adjusted to 12.5, 25, or 50 μM. After 1 h of incubation at 37°C, the cells were fixed with 2.5% glutardialdehyde in PBS and concentrated by gentle centrifugation after successive washes with PBS. The fixed pellets were enclosed in an agar block, dehydrated in ethanol, and embedded in Epon by standard techniques (8). Ultrathin sections were examined at 80 kV with a Zeiss model EM 902 electron microscope.

Mycoplasma elimination procedures. Reproducible decontamination of adherent and nonadherent cell lines was performed as follows. The surfactin used in the procedures described below was diluted in PBS to a concentration of 1 mM, autoclaved at 122°C for 30 min, and directly added to the medium.

(i) Adherent cell lines. Approximately 10^6 freshly trypsinized cells of an adherent cell line were transferred into a petri dish (diameter, 10 cm) containing 10 ml of DMEM supplemented with 5% (vol/vol) FCS and 40 μM surfactin. The cells were maintained in this medium for one passage (approximately 3 to 8 days) under normal growth conditions. After this, the cells were subcultured in surfactin-free standard medium.

(ii) Suspension cell lines. Depending on the proliferation rate, 1×10^5 to 5×10^5 cells of a suspension cell line were transferred into a centrifuge tube containing the elimination mixture (5 ml of RPMI 1640 medium, 60 μM surfactin, 5% FCS, and 50% 0.125% [wt/vol] trypsin–5 mM EDTA in PBS). The mixture was vortexed and shaken gently for 30 min at room temperature. After centrifugation at $600 \times g$ for 10 min, the supernatant was discarded. The cells were resuspended in 5 ml of RPMI 1640 medium supplemented with 30 μM surfactin and 5% (vol/vol) FCS. The cells were incubated in this medium for 3 days in a 25-ml culture flask under normal growth conditions and pelleted by centrifugation for 10 min at $600 \times g$, the supernatant was discarded, and the cells were resuspended in 5 ml of the elimination mixture. Then the steps from vortexing to incubation for 3 days in a 25-ml culture flask under normal growth conditions were repeated. After 3 days of cultivation in surfactin-containing medium, the cells were transferred into surfactin-free growth medium.

After surfactin treatment, the cultures were grown for at least four passages before samples were taken for mycoplasma detection.

RESULTS

Effect of surfactin on cell proliferation. Recently, we investigated the antiviral properties of surfactin (29) by using surfactin-containing media and ML cells for virus titration. In the virus-free control cultures we observed a significant increase in cell proliferation, and the confluent culture was vital and healthy and had less contrast than an untreated culture. When mycoplasma-free cell cultures were treated with the drug, no changes in morphology or proliferation rate could be detected. In order to investigate the effect of surfactin in more detail, several cell lines were collected from different sources and tested for mycoplasma infection. Positive cell lines were exposed to surfactin at various concentrations. The cytotoxic effect of this drug was measured by the crystal violet technique for adherent cell lines or by the MTT assay for suspension cell lines (Fig. 2). The 50% cytotoxic concentrations of surfactin for the adherent and nonadherent cell lines were as follows: ML, 40 μM; 293, 30 μM; Hep2, 42 μM; CV1, 50 μM; Molt 4/8, 35 μM; MT-4, 30 μM; and H9, 43 μM. At concentrations greater

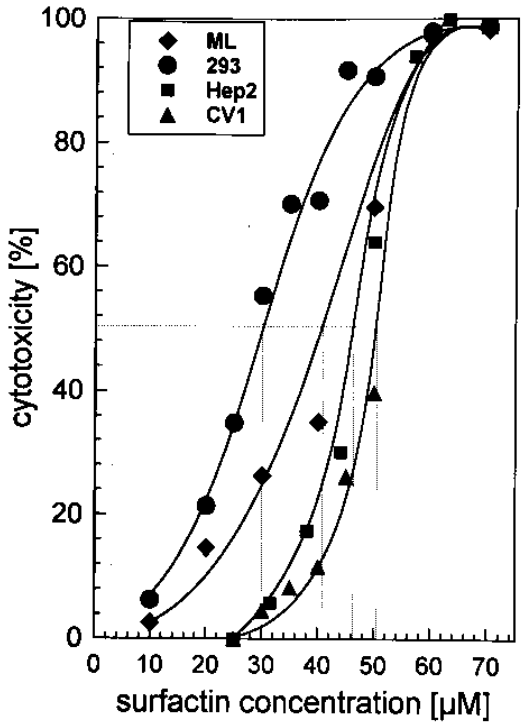

FIG. 2. Cytotoxic effects of surfactin on different cell lines. The inhibitory effects of different doses of surfactin on cell growth were determined by the crystal violet assay. Cell lines ML, 293, Hep2, and CV1 were treated with surfactin at concentrations ranging from 10 to 70 μM. No surfactin was added to the control culture. The percent growth reduction was calculated from the extinction difference between a surfactin-treated cell culture and the control. The 50% cytotoxic surfactin concentrations are indicated by the dotted lines.

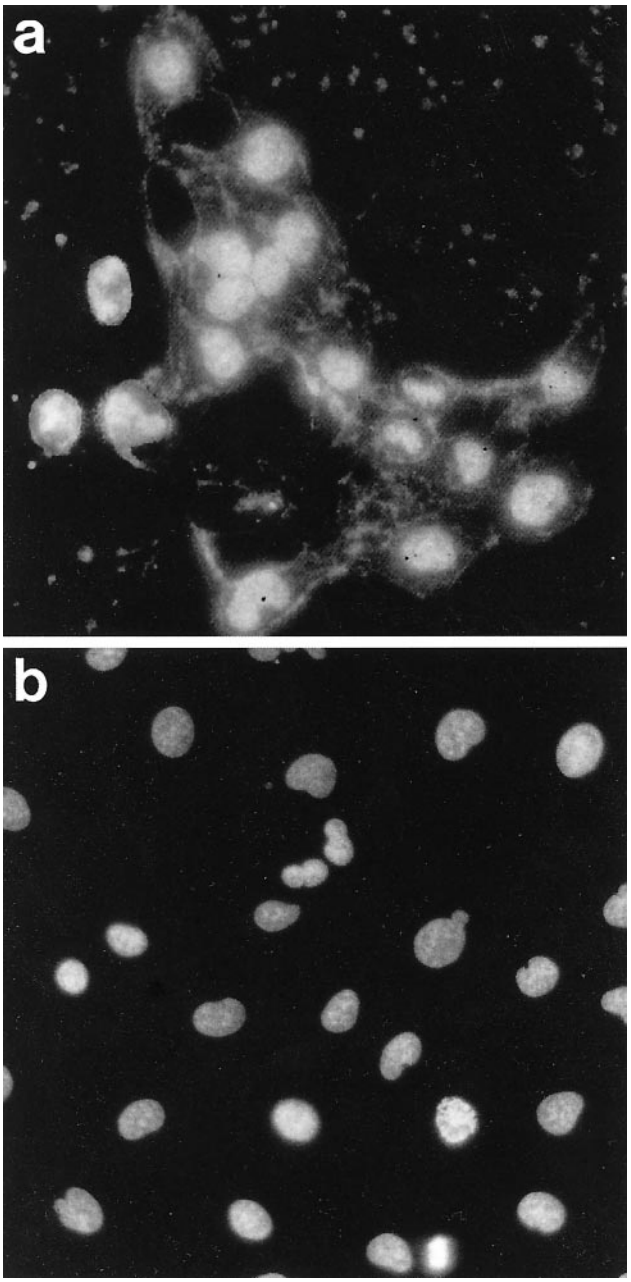

FIG. 3. DNA fluorescence staining of surfactin-treated cells with DAPI: ML cells heavily contaminated with *M. hyorhinis* (a) and mycoplasma-free cultures (b) after treatment with surfactin. Mycoplasmas were detected after the DNA in the culture was stained with the fluorochrome dye DAPI; they appear as small fluorescent spots against a dark background in the cytoplasm and intercellular spaces.

than 70 μM (100% cytotoxic concentration), no cells survived after one passage. Surfactin concentrations less than 10 to 25 μM had no toxic effects on the cells.

Mycoplasma detection and differentiation. Stock cultures and surfactin-treated cultures were tested for mycoplasma infection by the DAPI staining method, which showed that all of the stock cultures were mycoplasma positive (Fig. 3a), while the cultures treated with the antibiotic were free of mycoplasmas (Fig. 3b). The fluorescence DAPI test is only useful for screening, as only massive mycoplasma contaminations can be detected. In order to enhance the detection limit for mycoplasmas, the cultures were tested by the highly sensitive PCR and ELISA techniques two passages after treatment at the earliest. The species of contaminating mycoplasmas were identified by the ELISA for all of the cell lines investigated. The mycoplasma species residing in adherent cell lines ML, 293, Hep2, and CV1 was identified as *M. hyorhinis*. The mycoplasmas in suspension cell lines Molt 4/8, H9, and MT-4 belonged to *M. orale*. The Stratagene PCR method permits differentiation between PCR products derived from different mycoplasma species. Figure 4 shows typical fingerprint results obtained with two strains of mycoplasmas. For *M. orale* in MT-4, Molt 4/8, and H9 cells, one band at 650 bp was observed, and for *M. hyorhinis* in ML, 293, Hep2, and CV1 cells, four bands at 700, 600, 250, and 150 bp were detected. No double infections were observed with either method.

Biological activity of surfactin against mycoplasmas. To understand the mode of action of the drug, we treated a confluent ML cell culture that was highly contaminated with *M. hyorhinis* with surfactin at several concentrations below the 80% cytotoxic concentration (12.5, 25, and 50 μM) and investigated the effects of the drug by transmission electron microscopy (Fig. 5). Mycoplasmas incubated in a cell culture without surfactin were visible as intact particles at the surfaces of the ML cells. After incubation of mycoplasmas with 12.5 μM surfactin at 37°C for 1 h, we observed formation of small holes in the mycoplasma membrane and swelling of the particles. Especially mycoplasmas attached to the cell surface were directly affected by the drug. At a surfactin concentration of 25 μM bursting of the particles was induced. Also, mycoplasmas which

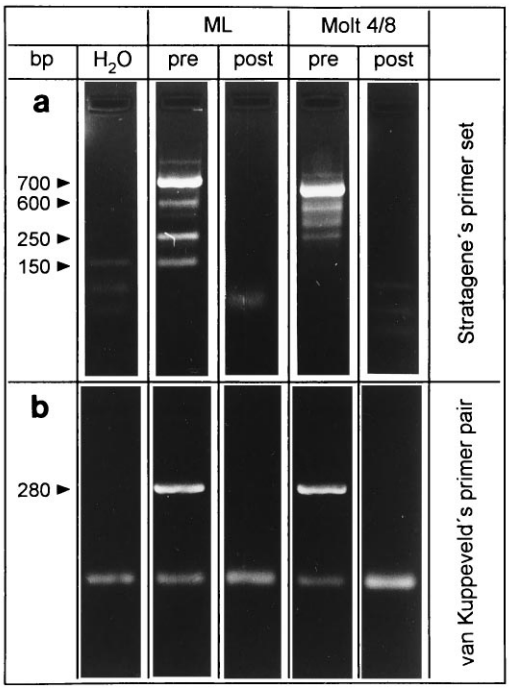

FIG. 4. Gel electrophoretic analysis of PCR amplification products of cell culture supernatants before and after surfactin treatment. Only mycoplasma rRNA was amplified by PCR when the type-specific Stratagene mycoplasma primer set was used (a) or the species-specific primer pair of van Kuppeveld et al. was used (b). Templates obtained from the culture medium containing surfactin-treated cells (post) produced no PCR signals. The Stratagene primer set revealed the *M. hyorhinis*-specific pattern (700, 600, 250, and 150 bp) for the untreated ML culture (pre) and an *M. orale*-specific pattern (650 bp) for the untreated Molt 4/8 culture (pre). The PCR of van Kuppeveld et al. (28) was considered mycoplasma positive if a single 280-bp product was amplified.

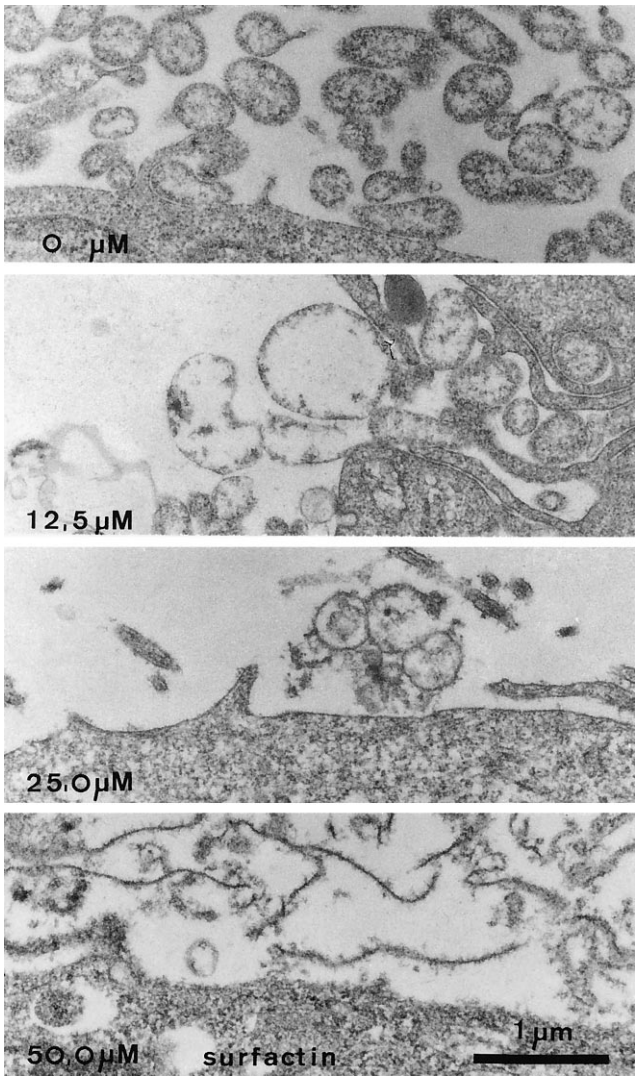

FIG. 5. Thin-section electron micrographs of mycoplasma-contaminated ML cells before and after addition of surfactin. When an ML cell culture that was highly contaminated with *M. hyorhinis* was confluent, surfactin was added to the culture at final concentrations of 12.5, 25, and 50 μM, and the preparations were incubated for 60 min at 37°C. No surfactin was added to the control culture. Interaction of the membrane-active surfactant with the outer part of the lipid membrane bilayer induced permeability changes. At the higher concentrations the drug finally caused the mycoplasma membrane system to burst by a detergent effect.

were hidden in pockets and clefts of the cell membrane began to disintegrate. Finally, at a surfactin concentration of 50 μM disruption of the mycoplasma lipid bilayer included total disintegration of the membrane systems, which led to bursting of all microorganisms.

Mycoplasma elimination procedure. On the basis of the observations described above, we developed a procedure for removing mycoplasmas from mammalian suspension and monolayer cells by using the lytic effect of surfactin on these organisms. In order to determine the optimal dose and duration of exposure, adherent cell lines Hep2, ML, 293, and CV1 were treated with 10, 20, 30, 40, 50, and 60 μM surfactin over a period of one or two cell passages. After lipopeptide treatment, cells were cultivated in the absence of antibiotics so that a low level of infection would be detectable after two passages by each of the methods. Contamination with mycoplasmas was assessed by using at least three different procedures. For all cell lines treatment with 10 or 20 μM surfactin for two passages did not completely eliminate mycoplasma contamination, but after incubation for one passage with 30 μM surfactin for ML cells and with 40 μM surfactin for CV1 and Hep2 cells, the cultures were free of mycoplasmas. For cell line 293 two consecutive treatments with 40 μM surfactin were necessary for effective elimination of all viable *M. hyorhinis*. The viability of these cells was significantly lower than the viability of CV1 and Hep2 cells after one passage with 40 μM surfactin and the viability of ML cells after one passage with 30 μM surfactin. A surfactin concentration of 50 or 60 μM led to complete removal of mycoplasmas, but the cells were either dead or seriously damaged, which led to unacceptably low proliferation rates. Therefore, we recommend using a surfactin concentration of 40 μM for two passages as a standard elimination procedure for adherent cell lines in order to be sure that all cell lines are devoid of mycoplasma infections. The efficiency of the treatment is shown in Table 1 and Fig. 4.

Using the results obtained with the adherent cell lines, we determined the duration of surfactin exposure and the concentrations required for treatment of suspension cell lines Molt 4/8, H9, and MT-4, as described above. By double treatment with surfactin at concentrations of 30 to 50 μM we obtained complete elimination of mycoplasmas in all cell lines. Unfortunately, these results were not reproducible when several cell cultures were tested in different mycoplasma elimination experiments. A surfactin concentration of 50 or 60 μM led either

TABLE 1. Mycoplasma elimination by surfactin[a]

Cell line	Detection assay	Passage(s) tested for mycoplasma contamination
Adherent cell lines		
ML	DAPI	2, 5, 15, 20
	ELISA	6
	PCR (Stratagene method)[b]	2, 5, 15, 20
	PCR (van Kuppeveld method)[c]	2, 5, 15, 20
CV1	DAPI	2, 3, 4
	ELISA	3
	PCR (Stratagene method)	3, 5
	PCR (van Kuppeveld method)	2, 5
293	DAPI	8, 17, 28
	PCR (Stratagene method)	4, 12, 28
	PCR (van Kuppeveld method)	4, 12, 14, 18, 25, 28
Hep2	DAPI	8, 20
	PCR (Stratagene method)	2, 5
	PCR (van Kuppeveld method)	4, 9, 16, 20
Suspension cell lines		
Molt 4/8	ELISA	10
	PCR (Stratagene method)	4, 9, 15, 20
	PCR (van Kuppeveld method)	3, 4, 6, 9, 15, 20
MT-4	ELISA	10
	PCR (Stratagene method)	4, 6, 20
	PCR (van Kuppeveld method)	3, 4, 6, 10, 16, 20
H9	ELISA	10
	PCR (Stratagene method)	3, 5, 9, 20
	PCR (van Kuppeveld method)	3, 5, 7, 9, 13, 16, 20

[a] The effectiveness of the elimination process was controlled during several cell passages by using different mycoplasma detection methods. All of the cultures tested after surfactin treatment were free of contaminants. In the cell passages tested no mycoplasma recontamination occurred during cultivation.
[b] PCR performed with primers obtained from Stratagene.
[c] PCR performed by the method described by van Kuppeveld et al. (28).

to cell death or to the reduction of proliferation rates below acceptable levels after one to two passages.

To eliminate the possibility that mycoplasmas hidden in intercellular spaces, as well as in pockets and clefts of the cell membrane, would escape contact with the drug, we used trypsin to detach the cells from each other and to smooth the cell surfaces. By also using a pretreatment with 60 μM surfactin for 30 min, we were able to effectively eliminate all mycoplasmas from suspension cells (Fig. 4 and Table 1). A pronounced decrease in the viability of the culture cells tested of approximately 50 to 85% was observed after the second treatment, but enough viable suspension cells could be recovered for further subcultivation. Under these conditions our results were nicely reproducible.

We tested the reemergence of residual mycoplasmas by cultivating the cells for up to 28 passages. As Table 1 shows, in the periods investigated all of the cell lines could be cultivated so that they were free of contamination. In no case was any permanent growth inhibition of mammalian cells detected. The surviving cells regained the normal rate of growth and compensated for the loss in cell numbers after one to two passages. The initial cell density and the FCS concentration were important factors for the success of this procedure. Higher cell densities and FCS concentrations in the reaction mixture decreased the efficiency of elimination of mycoplasmas, but lower concentrations decreased the level of viability.

DISCUSSION

Our investigation of the mycoplasmacidal effect of surfactin was initiated by the observation that mycoplasma-contaminated adherent and nonadherent cells exhibited improved proliferation rates and changes in morphology after treatment with the drug. These effects of surfactin were described previously by Hosono and Suzuki (11) with Chinese hamster ovary (CHO-K1) cells and were similar to the effects of dibutyryl cyclic AMP. This activity was interpreted as being due to inhibition of cyclic AMP phosphodiesterase by surfactin (10). Our electron microscopic studies provided evidence that surfactin affects the envelopes of contaminating mycoplasmas. Obviously, surfactin disrupts the plasma membrane, which is its primary site of activity. It causes leakage, at higher concentrations it leads to complete disintegration of the membrane systems, and finally it causes the mycoplasmas to burst. Eradication of the contaminants resulted in native morphology and a native proliferation rate of the mammalian cells.

Previous studies with artificial membranes (15, 23), protoplasts (26), and eukaryotic cells (11) demonstrated that surfactin binds readily to cell membranes with a high degree of selectivity, depending on the membrane lipid composition. The fatty acid portion of surfactin is anchored in the lipid bilayer, showing high affinities to cholesterol and phospholipids (11, 16). In *Mycoplasma* species cholesterol was found at levels comparable to those in the plasma membranes of eukaryotic cells (25 to 30% [wt/wt] of the total membrane lipids) (19, 20). The higher levels of membrane phospholipids (especially phosphatidylglycerol, phosphatidylcholine, and phosphatidylethanolamine) found in *Mycoplasma* cells compared with eukaryotic cells may result in the greater susceptibility of mycoplasma membranes to surfactin (19, 20). Surfactin is active against *M. hyorhinis* and *M. orale* at concentrations greater than 30 μM. A critical micellar concentration of 10 μM was determined for surfactin (12) in 0.1 M $NaHCO_3$ (pH 8.7). Obviously, surfactin interacts with the mycoplasma membrane in micellar form, inducing an osmotic influx of medium and ultimately complete disruption of the cells. Our observations of the lytic effect of surfactin on mycoplasmas were utilized to develop a procedure for elimination of mycoplasmas from mammalian cell cultures. All adherent cell lines and suspension cells tested could be successfully cleansed of two of the most common mycoplasmas associated with such cell cultures, *M. hyorhinis* and *M. orale*, respectively. The efficiency of the mycoplasma elimination procedure which we developed was demonstrated by several highly sensitive techniques. No reemergence of the contaminants was detected, which meant that the mycoplasmas were completely eradicated and not merely arrested in growth by a bacteriostatic effect of the drug. All cultures from which *Mycoplasma* species were eliminated were grown under conditions under which recontamination by other contaminated cell cultures was not possible (i.e., in a separate incubator or laminar airflow biohazard cabinet).

Compared with other mycoplasma elimination protocols performed with antibiotics, such as ciprofloxacin and enrofloxacin (quinolone derivatives; trade names, Ciprobay and Baytril, respectively), tiamulin and minocycline (pleuromutilin and tetracycline derivative, respectively; combined to form the commercially available product BM-Cyclin), or Mycoplasma Removal Agent (a 4-oxo-quinoline-3-carboxyl acid derivative) (6, 9, 22, 27), the method described here has the advantage of being more effective. Therefore, cells do not have to be protected against reemergence of contaminants, and time-consuming and labor-intensive replenishment of the antibiotic during cultivation is not necessary. Indeed, the only antibiotics

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