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# Characterization of a New Lipopeptide Surfactant Produced by Thermotolerant and Halotolerant Subsurface *Bacillus licheniformis* BAS50

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**Strain BAS50, isolated from a petroleum reservoir at a depth of 1,500 m and identified as *Bacillus licheniformis*, grew and produced a lipopeptide surfactant when cultured on a variety of substrates at salinities of up to 13% NaCl. Surfactant production occurred both aerobically and anaerobically and was optimal at 5% NaCl and temperatures between 35 and 45°C. The biosurfactant, termed lichenysin A, was purified and chemically characterized. A tentative structure and composition for the surfactant are described. Lichenysin A is a mixture of lipopeptides, with the major components ranging in size from 1,006 to 1,034 Da. The lipid moiety contains a mixture of 14 linear and branched β-hydroxy fatty acids ranging in size from $C_{12}$ to $C_{17}$. There are seven amino acids per molecule. The peptide moiety is composed of the following amino acids: glutamic acid as the N-terminal amino acid, asparagine, valine, leucine, and isoleucine as the C-terminal amino acid, at a ratio of 1.1:1.1:1.0:2.8:1.0, respectively. Purified lichenysin A decreases the surface tension of water from 72 mN/m to 28 mN/m and achieves the critical micelle concentration with as little as 12 mg/liter, characterizing the product as a powerful surface-active agent that compares favorably to others surfactants. The antibacterial activity of lichenysin A has been demonstrated.**

Many microorganisms produce effective biosurfactants which reduce the interfacial tension between oil and brine to less than 0.01 mN/m (29). However, these biosurfactants are produced by aerobic organisms, and little information exists at present about anaerobic biosurfactant producers. Some *Clostridium* and *Desulfovibrio* strains isolated from various petroleum reservoirs produce extracellular compounds that lower the surface tension of medium to about 50 to 55 mN/m (9, 15). The archaebacterial ether-linked phytanyl membrane lipid of the extremely halophilic bacteria has been shown to have surfactant properties (32). Jenneman et al. isolated *Bacillus licheniformis* JF-2 from oil field injection water, which has properties that are potentially useful for in situ microbially enhanced oil recovery (16). JF-2 grows anaerobically and produces a biosurfactant, lichenysin, in a medium supplemented with glucose and 0.1% $NaNO_3$ (15). It also grows in medium with NaCl concentrations of up to 10%, at temperatures of up to 50°C, and at pHs from 4.6 to 9.0. Furthermore, growth was not inhibited by the presence of crude oil. The biosurfactant produced was not affected by the temperatures, pHs, or NaCl and calcium concentrations typical of many reservoirs (25). The lichenysin produced by *B. licheniformis* JF-2 (termed here lichenysin B) has been patented for applications in enhanced oil recovery (24) and has been used in coreflood experiments (35).

Lichenysin B is a cyclic lipopeptide and belongs to the most effective biosurfactants discovered so far (21, 24). The structure and activities of at least six such agents have been characterized in detail. The best-characterized lipopeptide surfactants are surfactin, which has been isolated from several strains of *Bacillus subtilis* and *Bacillus pumilis* (1, 10, 19, 28, 36); lichenysin B (21, 25), lichenysin C (17), and biosurfactant BL86 (12), which have been isolated from different strains of *Bacillus licheniformis*; viscosin, produced by *Pseudomonas fluorescens* (20); and arthrofactin, produced by *Arthrobacter* sp. strain MIS38 (27). All of these surface-active lipopeptides consist of several amino acids covalently bound with the carboxy and hydroxy groups of β-hydroxy fatty acids. They vary in amino acid composition, position of the lactone ring, and lipid portion.

We show here that *B. licheniformis* BAS50, isolated from a deep oil well, can grow and produce a surfactant which substantially changes the surface tension of the culture medium under conditions that exist in many petroleum reservoirs. In the present work, we also reported the isolation and structural analysis of this new surface-active compound.

## MATERIALS AND METHODS

**Microorganisms and culture conditions.** Strain *B. licheniformis* BAS50 was used throughout this work. It was isolated from North German oil reservoirs at a depth of 1,500 m and was identified at the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) by physiological-biochemical characterization and by fatty acid methyl ester (FAME) analysis. The surfactin-producing strains *B. subtilis* DSM 3256 and DSM 3258 and the lichenysin B producer *Bacillus* sp. strain ATCC 39307 were obtained from the German Collection of Microorganisms and Cell Cultures and the American Type Culture Collection (Rockville, Md.). All bacteria were stored lyophilized and in frozen glycerol cultures at −70°C. The basic medium for cultivation of the strains was Cooper's minimal medium (8), which contained 2 to 4% glucose, 0.05 M $NH_4NO_3$, 0.03 M $KH_2PO_4$, 0.04 M $Na_2HPO_4$, $8.0 \times 10^{-4}$ M $MgSO_4$, $7.0 \times 10^{-6}$ M $CaCl_2$, and $4.0 \times 10^{-6}$ M $Na_2$ EDTA. The medium was supplemented with 1.0% (vol/vol) trace metals solution (7) designed to maintain the critical nitrogen-iron-manganese proportionality of 920:7.7:1.0 (molar basis) (33). The effects of salinity were determined by adding NaCl or condensed brine salts to the minimal Cooper's medium. Bacterial growth in minimal medium was monitored by measuring the optical density at 600 nm.

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Anaerobic cultures were prepared by the methods of Javaheri et al. (15). Cooper's medium was supplemented with 0.1% (wt/vol) $NaNO_3$ and 0.05% yeast extract (Difco, Detroit, Mich.). Subsequent manipulation of the anaerobic medium was performed in an anaerobic box (Coy, Ann Arbor, Mich.) by the Hungate technique, as modified by Bryant (6), under a 95% nitrogen–5% hydrogen atmosphere.

**Characterization of BAS50.** Strain BAS50 was tested for growth with different substrates in tubes of 10 ml of Cooper's medium with 10% NaCl and 0.1% $NaNO_3$–0.05% yeast extract. Each tube was inoculated with 0.1 ml of a late-log-phase culture of BAS50 and incubated at 50°C for 4 days. Tests for growth at different temperatures or NaCl concentrations were done in a similar manner, with glucose as the energy source.

FAMEs were prepared and extracted by the standard protocol of the Microbial Identification System (MIDI; Microbial ID, Inc., Newark, Del.).

The growth and surfactant production of aerobic and anaerobic cultures of BAS50 were compared by inoculating 300 ml of Cooper's medium enriched with 0.1% $NaNO_3$–0.05% yeast extract–2% glucose with 15 ml of an anaerobically grown culture. Anaerobic cultivation of flasks was carried out in a glove box.

**Surface activity assay.** The surface tension (ST) of spent medium and crude and purified surfactant was determined with a ring tensiometer (K6; Krüss, Hamburg, Germany). The critical micelle concentration value is defined as that point at which purified surface-active compound no longer aggregates to form micelles.

**Surfactant isolation.** Bacterial cells were removed from surfactant-containing medium by centrifugation (13,000 × *g*, 4°C, 15 min). The supernatant was subjected to acid precipitation by adding concentrated HCl to achieve a final pH of 2.0 and allowing the precipitate to form at 4°C overnight. The pellet was collected by centrifugation and washed several times with acidic water (pH 2.0 with concentrated HCl), dissolved in alkaline water (pH 8.0 with NaOH), and lyophilized overnight. The dried surfactant was extracted with tetrahydrofuran (THF), solvent was removed with the aid of a rotary evaporator under reduced pressure, and the solid was washed with 3 volumes of hexane to remove alkanes, free fatty acids, and alcohols. The crude material was collected for further purification by the chromatographic procedures described below.

Adsorption chromatography of surfactant was carried out on silica gel solid-phase extraction cartridges (B&J Inert SPE System; Burdick and Jackson, Muskegon, Mich.) with solvents of gradually increasing polarity: $CHCl_3$ > $CH_3COCH_3$ > $CHCl_3$:$CH_3OH$ (2:1) > $CH_3OH$ (21). The methanol-chloroform eluate from the cartridge demonstrated the highest surface activity, and the compounds in this eluate were further separated by thin-layer chromatography (TLC) on silica-coated glass plates (E. Merck, Darmstadt, Germany) in solvent system I (chloroform–methanol–28% ammonium hydroxide, 65:25:4, vol/vol/vol). The silica containing the separated spot was scraped from the glass plate and extracted with chloroform-methanol (2:1, vol/vol). The surfactant was dialyzed against sterile, double-distilled $H_2O$ overnight to remove the remaining cations and stored after lyophilization at −20°C.

Other TLC systems tested included solvent system II (chloroform-methanol-acetone-acetic acid, 90:10:6:1, vol/vol/vol/vol) and system III (chloroform-methanol-acetone, 90:10:6, vol/vol/vol) (22, 23). Two additional silica gel TLC solvent systems were used for fractionation of surfactants with different C-terminal amino acids: system IV (butanol-acetone-water, 32:48:8, vol/vol/vol) (30) and system V (chloroform–methanol–propan-1-ol–0.25% KCl–ethyl acetate, 25:13:25:9:25, by volume) (31).

**Hydrolysis and derivatization methods.** The method of hydrolysis-methanolysis (4, 22) was used to obtain the methyl esters without dehydration of the β-hydroxy fatty acids and to avoid the formation of the mixture of α- and β-monounsaturated fatty acids. Surfactant (5 mg) was methylated with 5% HCl-methanol at 90°C for 15 h in sealed tubes. After methanolysis, solvents were removed by evaporation, and the residue was extracted with petroleum ether. The extract was washed with $H_2O$, dried with anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The oily material was loaded onto a silica gel cartridge column. Chloroform and chloroform-methanol (5:1, vol/vol) were used to elute FAMEs and acyl amino acid methyl esters, respectively.

**Analysis of β-hydroxy FAMEs.** Trimethylsilyl (TMS) ether derivatives were prepared by combining 0.5 mg of fatty acid methyl esters and 0.1 ml of Tri-Sil reagent (Pierce, Rockford, Ill.). After the reaction was complete, the solvent and the reaction by-products were coevaporated after the addition of benzene. *n*-Octane was added to a final volume of 250 μl. The samples were analyzed by gas chromatography (GC) with flame ionization detection and by GC-mass spectrometry (MS) as described below.

**Analysis of amino acids.** Lipopeptides were hydrolyzed in 6 M HCl at 110°C for 24 h in sealed tubes. The methanol-soluble products were dried after repeated evaporation with ethanol. The amino acid composition of the hydrolysate was monitored qualitatively by TLC with solvent system IV. Quantitative, automated amino acid analysis was done with an amino acid analyzer (420A; Applied Biosystems Foster City, Calif.). Additionally, GC and GC-MS analyses of the ester derivatives of the amino acid mixtures generated by the 6 M HCl hydrolysis were performed. The methanol-soluble hydrolysate was treated with 3 M HCl in *n*-butanol for 30 min at 120°C (3, 30). After elimination of solvents under a nitrogen stream, the residue was combined with a THF-MBTFA [*N*-methyl-*bis*(trifluoroacetamid); Pierce] mixture (1:1, vol/vol) and heated at 75°C for 30 min. The *N*-trifluoroacetyl (TFA) *C*-*n*-butyl esters of amino acids were analyzed after dilution with ethyl acetate by both GC and GC-MS.

**Reduction of lipopeptides with $LiBH_4$.** Surfactants were reduced by the methods of Hosono and Suzuki (13, 14) and Kakinuma et al. (18). Three forms of lipopeptide, native, opened ring, and permethylated derivative (diazomethane) of the native form, were reduced with lithium borohydride. A mild alkaline hydrolysis of the lipopeptides was used to open the lactone linkage of the cyclic peptide. The samples were dissolved in 1 M NaOH and allowed to stand for 12 h at room temperature. After acidification of the reaction medium, the linear form of the surfactant was obtained by extraction with chloroform. The respective samples were added to a solution of $LiBH_4$ in THF (Sigma), heated for 18 h at 80°C, and then cooled to room temperature. After addition of methanol, the solution was evaporated. The residue was adjusted to pH 2.0, extracted with ethyl acetate, dried under a nitrogen stream, and hydrolyzed with 6 M HCl at 110°C for 20 h. The hydrolysate was analyzed on an amino acid analyzer and by GC-MS as *N*-TFA *C*-*n*-butyl derivatives as described above.

**Hydrazinolysis.** The open form of surfactant (0.5 mg) was treated with 200 μl of hydrazine (Fluka) for 24 h at 80°C (30). The excess of hydrazine was eliminated under nitrogen, the residue was dissolved in 200 μl of $H_2O$, and the solution was shaken twice with an equal volume of benzaldehyde. The aqueous phase was kept for estimation of the C-terminal amino acid with the amino acid analyzer and by GC as *N*-TFA *C*-*n*-butyl derivatives.

**Permethylation.** The open form of surfactant was subjected to *O*,*N*-permethylation by the procedure of Hosono and Suzuki (14). Experimental conditions involved preparation of the reagent by heating 20 mg of NaH per oil dispersion (prerinsed three times with ether) in 0.2 ml of dimethyl sulfoxide (DMSO) at 100°C until hydrogen evolution ceased. The surfactant derivative is added to this reagent at room temperature, followed by 0.3 ml of methyl iodide. After 1 h, the product is diluted with water and extracted with chloroform.

**Enzymatic digestion.** The intact and open forms of surfactant were digested with carboxypeptidase Y (Boehringer, Mannheim, Germany) using the manufacturer's protocol. The products of the reaction were analyzed with the amino acid analyzer and by TLC with ninhydrin detection.

**IR spectrometric analyses.** Infrared (IR) absorbtion spectra were obtained with a Bio-Rad FTS-40 (Bio-Rad, Northeim, Germany), using a light bench equipped with a diffuse reflectance accessory in a dry atmosphere. Data were collected and processed with an SPC 3200 workstation running IDRIS IR analytical software. IR spectra were collected between 400 and 4,000 wave numbers (per centimeter), with a resolution of two measures per wave number. Sixteen spectra per sample were collected and averaged.

**Reverse-phase HPLC analysis.** The purified surfactant was characterized by reverse-phase high-pressure liquid chromatography (HPLC) with a System Gold chromatograph (Beckman, San Ramon, Calif.) with a $C_8$ 5-μm Lichospher (0.46 by 12.5 cm) column. The system was operated at a flow rate of 1.1 ml/min, with a linear gradient from 60% solvent A (0.1 M ammonium acetate [pH 4.8]) to 90% solvent B (100% methanol). Peaks eluting from the column were detected by their absorbance at 210 nm.

**GC.** A Hewlett-Packard model 5890 Series II gas chromatograph, equipped with a flame ionization detector and fused-silica capillary column (50 m by 0.32 mm inner diameter; CP-Sil 5; Chrompack, Middleburg, N.J.) was used throughout this work. Injections (1 μl) were made with a Hewlett-Packard autosampler in the on-column mode. The hydrogen carrier gas flow rate through the column was 40 cm/s. The oven temperature program ranged from 100 to 280°C. The injector was maintained in the overtracking mode. The temperature of the detector was 325°C.

**GC-MS.** GC positive-ion electron impact (70 eV)-MS was performed with an MS 5989A system (Hewlett-Packard, Waldbronn, Germany). The ion source was maintained at 200°C. A Hewlett-Packard gas chromatograph similar to that described above was used except that helium was used as carrier gas at a flow rate of 21 cm/s.

**SIMS and ESIMS.** Secondary ion mass spectrometry (SIMS) was performed on the mass spectrometer Kratos MS 50TC with a beam of cesium ions at an accelerating voltage of 8 kV. Glycerol was used as a matrix. Electrospray mass spectrometry (ESIMS) was performed on a Finnigan TSQ 700 (Bremen, Germany). The samples were dissolved in a mixture of methanol and water (50:50, vol/vol) supplemented with 1% acetic acid, and 10 pmol of samples was used.

**$FAB^+$ MS/MS.** Fast atom bombardment tandem mass spectrometry ($FAB^+$ MS/MS) measurements were carried out on a JEOL JMS-HX/HX110A tandem high-resolution mass spectrometer (Tokyo, Japan) in a setup of $E_1B_1E_2B_2$ configuration at 10 kV accelerating voltage (E and B represent electrostatic and magnetic sectors, respectively). The MS resolution was set to 1:1,000. The JEOL FAB gun was operated at 6 kV, with xenon as the FAB gas. Collision-induced fragmentation took place in the third field-free region. Helium served as the collision gas at a pressure sufficient to reduce the precursor ion signal to 30% of the original value. As a matrix, thioglycerol was used. The collision cell was operated at ground potential. $FAB^+$ MS/MS spectra linked at a constant B/E ratio were recorded at 100 Hz filtering with a JEOL DA7000 data system.

**NMR.** All one- and two-dimensional (1D and 2D) $^1H$ nuclear magnetic resonance spectroscopy (NMR) spectra were recorded at 299 K on an AM 600 NMR spectrometer (Briker, Karlsruhe, Germany) locked to the deuterium resonance of the solvent, DMSO-$d_6$, without spinning. All 2D phase-sensitive total correction (TOCSY) (mixing time, 70 ms) and Overhauser enhancement

(NOESY) (mixing time, 200 ms) spectra were recorded and processed as described previously (38).

**Antibiotic assay.** The antibiotic activities of surfactin and intact and opened forms of lichenysin A were tested against 12 microorganisms by the agar diffusion method (17). A filter paper disk with 15 μg of surfactant was assayed on the surface of an agar medium containing a soft agar culture of microorganisms. Growth inhibition was measured by the diameters of the halos around the paper disks.

## RESULTS

**Characterization of BAS50.** BAS50 was isolated from a sample taken aseptically from an oil storage cavern from a depth of 1,480 m. BAS50 is a facultatively anaerobic, gram-positive, motile, rod-shaped bacterium with a length of 1.9 to 2.9 μm and a width of 0.6 to 0.8 μm. Colonies were round and mucoid and flattened out with longer incubation times. The sporangium was not swollen, whereas the spores were oval and subterminally positioned. Spores were only observed after initial isolation and during aerobic cultivation; they were not produced when BAS50 was repeatedly subcultured anaerobically. BAS50 used a variety of carbon sources for growth, including arabinose, fructose, galactose, glucose, inulin, mannitol, methyl-D-glucoside, salicin, sorbitol, starch, and xylose but not lactose, melibiose, raffinose, or rhamnose. BAS50 hydrolyzed gelatin, casein, esculin, urea, and Tween 80; used citrate, propionate, and gluconate; and reduced nitrate, but did not produce indole or hydrogen sulfide. Arginine dihydrolase and β-galactosidase activities were observed, but no activities of lysine decarboxylase, ornithine decarboxylase, phenylalanine desaminase, or tryptophan desaminase were detected. BAS50 grew in medium with a range of 0 to 13% (wt/vol) NaCl, at temperatures of 25 to 55°C, and at pHs of 5.4 to 8.5. Identification of the FAME fingerprints and generation and validation of the database were performed by using the MIDI software package. The calculated index revealed a very high similarity to *B. licheniformis* (0.948).

**Growth and surfactant production.** In comparison to anaerobic cultivation, aerobic cultivation of BAS50 was characterized by a shorter lag phase of growth and higher biomass concentration. The ST of the aerobic medium decreased during early exponential growth phase, reaching a minimum of 28.3 mN/m coincidently with the transition to the stationary growth phase (Fig. 1). During the different growth phases, the ST of the anaerobic cultures was similar to that of aerobic cultures, but its minimum value was approximately 35 mN/m. The biomass and surfactant yields in anaerobic culture were comparatively low and influenced by the temperature and salt concentration. Optimal growth and surfactant production under anaerobic conditions occurred in Cooper's medium with 5% NaCl at 40 to 45°C.

Glucose and sucrose but not arabinose, fructose, or maltose supported the best surfactant production. The amount of lichenysin A isolated from stationary-phase cells was approximately 160 mg/liter by cultivation in glucose- or sucrose-containing medium and up to 70 mg/liter by cultivation in Cooper's medium supplemented with other sugars. Increasing the glucose or sucrose concentration above 2% (wt/vol) did not affect surfactant production. Solutions of molasses with concentrations of up to 4% (vol/vol) in Cooper's medium or water injection brine (130 g of NaCl per liter) supplemented with 0.1% $NaNO_3$ and 0.05% yeast extract supported the growth of BAS50 and surfactant production at temperatures of up to 50°C (data not shown).

**Surfactant isolation.** The surface-active compounds were isolated from the culture supernatant of *B. licheniformis* BAS50 as described in Materials and Methods. Adsorption chromatography of the crude product obtained after HCl precipitation and THF extraction on a silica gel cartridge column by stepwise elution with solvents of increasing polarities resulted in the separation of several products. The compounds of the acetone eluate were analyzed by GC and GC-MS and were identified as saturated long-chain carboxy acids and alcohols, namely, palmitic acid, stearic acid, and dodecanol. The methanol-chloroform eluate, the main component of the crude product and the most surface-active one, was analyzed by different procedures after further purification by TLC (see Materials and Methods), and the active product, lichenysin A, was isolated.

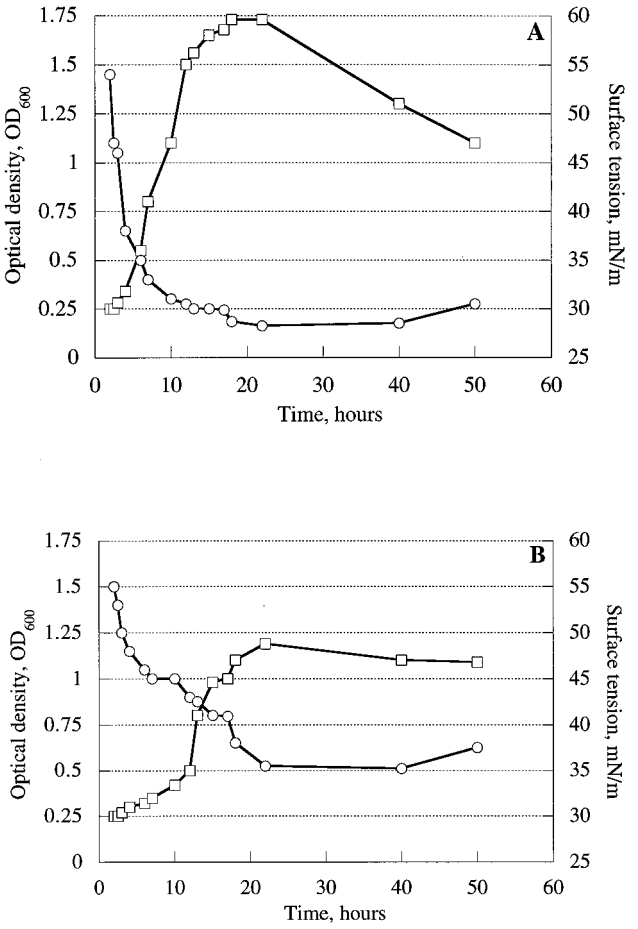


FIG. 1. Growth and ST decreases by aerobic (A) and anaerobic (B) cultures of BAS50. Each culture of strain BAS50 was grown at 45°C in Cooper's medium supplemented with 4% sucrose, 0.1% $NaNO_3$, 0.05% yeast extract. Symbols: □, absorbance; ○, ST. Values are averages for three cultures.

**Initial structural characterization.** Surfactin (Sigma, St. Louis, Mo.), lichenysin B (the surface-active lipopeptide from *Bacillus* sp. strain ATCC 39307), viscosin (Sigma), and lichenysin A were analyzed by TLC with different solvent systems. TLC analysis with neutral solvent systems showed lichenysin A as an elongated spot, suggesting the presence of an ionic compound, whereas in the acidic and alkaline solvent systems, the spot was not elongated, suggesting that the compound is amphoteric (zwitterion). The retention index ($R_f$) of lichenysin A in the TLC systems employed is distinctly different from those of the known surface-active lipopeptides viscosin, lichenysin B, and surfactin. The $R_f$ values of lichenysin A and surfactin were similar in the neutral and acidic solvent systems but different in

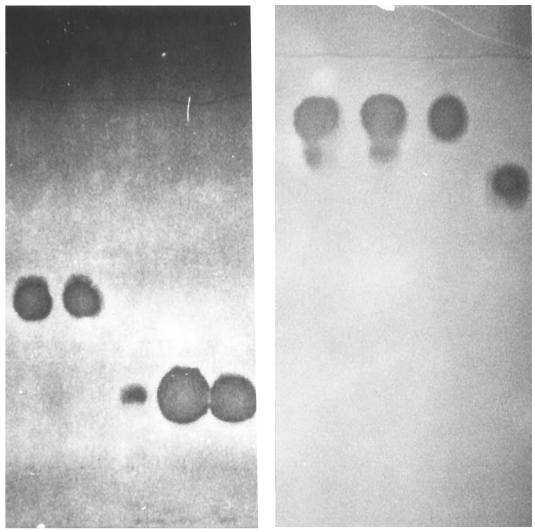


FIG. 2. TLC of surface-active lipopeptides in alkaline system III (lanes 1 to 5) and in solvent system IV (lanes 6 to 9). Lanes 1 and 2, lichenysin A produced by aerobic and anaerobic cultures of BAS50, respectively; lane 3, lichenysin B from *Bacillus* sp. strain ATCC 39307; lanes 4 and 6, surfactin from *B. subtilis* DSM3256; lanes 5 and 7, surfactin from *B. subtilis* DSM3258; lanes 8 and 9, native and open forms of lichenysin A, respectively.

the alkaline system (Fig. 2), indicating that lichenysin A has basic groups. It is known that in solvent systems IV and V, surfactins were resolved into two spots, $Leu^7$- and $Val^7$-surfactins (30, 31). Only one spot was obtained for lichenysin A with these systems (Fig. 2).

**IR analysis.** The IR spectrum of lichenysin A from *B. licheniformis* BAS50 in KBr showed strong bands, indicating the presence of a peptide component at 3,300 $cm^{-1}$ resulting from the N-H stretching mode, at 1,655 $cm^{-1}$ resulting from the stretching mode of the CO-N bond, and at 1,535 $cm^{-1}$ resulting from the deformation mode of the N-H bond combined with the C-N stretching mode. The bands at 2960 to 2860 and 1470 to 1370 $cm^{-1}$ resulting from the C-H stretching mode suggest the presence of an aliphatic chain. These results were strong evidence that lichenysin A contains aliphatic and peptide-like moieties. The band at 1,735 $cm^{-1}$ was due to lactone carbonyl absorption. These patterns were similar to those of surfactin and lichenysin B (21).

**Reverse-phase HPLC analysis.** Reverse-phase HPLC analysis of purified lichenysin A revealed at least 10 peaks, arranged as singlets, doublets, and a triplet. Compared with that of lichenysin B, the HPLC pattern of lichenysin A has quite different retention times, indicating that it is less polar (data not shown).

**SIMS and ESIMS analysis.** The molecular weight of the various components of the surfactants was determined by negative- and positive-ion SIMS and by ESIMS analyses. The mass spectra of surfactin showed a series of ions at *m/z* 992 to 1,034 in the negative-ion mode and at *m/z* 994 to 1,036 with positive-ion SIMS and ESIMS analyses (Fig. 3A). The negative-ion SIMS spectra of lichenysin A showed abundant molecular ions $(M-H)^-$ at *m/z* 1,005, 1,019, and 1,033 that indicated the molecular weights of the most abundant components (data not shown). In ESIMS and positive-ion SIMS, the corresponding ions $(M+H)^+$ were clearly observed at *m/z* 1,007, 1,021, and 1,035 (Fig. 3B). Thus, lichenysin A is a novel lipopeptide whose abundant structural analogs range in even-numbered molecular weights ranging from 1006 to 1034. In contrast, surfactin (1), lichenysin B (21), and lichenysin C (17) have odd-numbered molecular weights in the range from 1007 to 1035. The molecular weight difference of 1 Da may arise from the presence of the amide form of either glutamic or aspartic acid. The pattern of structural analogs with mass shifts of 14 Da is due to a mixture of closely related molecules varying both in their fatty acid residues and in replacement of leucine or isoleucine by valine that has already been found in surfactant BL86 and surfactin (12, 30).

Mild alkaline hydrolysis cleaved the lactone ring of native lichenysin A and yielded linear lipopeptide. TLC of the hydrolysate with neutral solvent system I produced tailing spots with an increased polarity (a shift in $R_f$ values from 0.72 to 0.43),

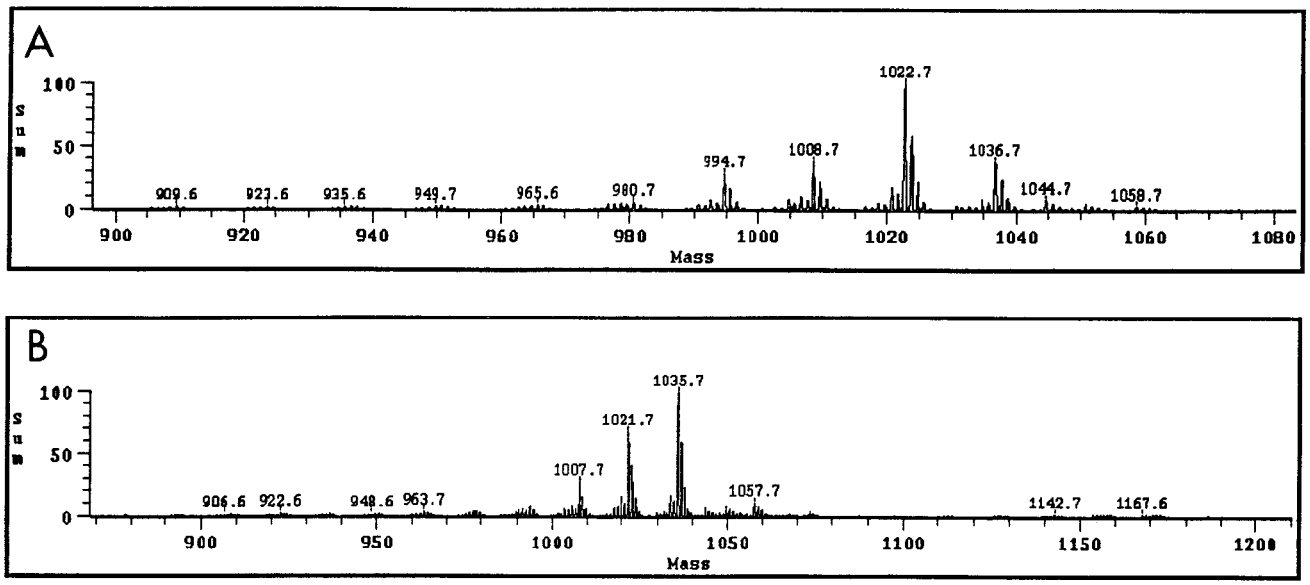


FIG. 3. Positive-ion SIMS analyses of surfactin (A) and lichenysin A (B).

TABLE 1. Lipophilic moieties of lichenysin A, lichenysin B, and surfactin[a]

| β-Hydroxy fatty acid | % of total (SD) | | |
|---|---|---|---|
| | Lichenysin A | Lichenysin B | Surfactin |
| *iso* $C_{12}$ | 0.3 (0.0) | ND[b] | ND |
| *n* $C_{12}$ | 0.80 (0.2) | ND | 2.05 (0.0) |
| *iso* $C_{13}$ | 3.8 (0.7) | 3.8 (0.1) | 3.0 (0.1) |
| *anteiso* $C_{13}$ | 2.4 (0.5) | 2.5 (0.1) | 3.1 (0.1) |
| *n* $C_{13}$ | 1.1 (0.1) | ND | 0.65 (0.0) |
| *iso* $C_{14}$ | 8.7 (0.2) | 30.5 (0.4) | 16.8 (0.3) |
| *n* $C_{14}$ | 21.2 (0.8) | 6.3 (0.1) | 40.8 (0.7) |
| *iso* $C_{15}$ | 38.6 (0.6) | 18.2 (0.2) | 11.1 (0.2) |
| *anteiso* $C_{15}$ | 19.5 (0.4) | 36.4 (0.3) | 19.4 (0.6) |
| *n* $C_{15}$ | 1.20 (0.0) | ND | 0.3 (0.0) |
| *iso* $C_{16}$ | 0.35 (0.0) | 2.3 (0.1) | 0.8 (0.1) |
| *n* $C_{16}$ | 1.20 (0.1) | ND | 2.0 (0.1) |
| *iso* $C_{17}$ | 0.55 (0.0) | ND | ND |
| *anteiso* $C_{17}$ | 0.3 (0.1) | ND | ND |

[a] Values are means of GC data obtained from three independent series of surfactants. Cooper's medium supplemented with 2% glucose was used. Values are given as a percentage of the total amount of β-hydroxy fatty acids isolated from the lipophilic moiety of the surfactants.

[b] ND, not detectable by the methods of analysis.

suggesting the generation of an additional free carboxyl group. Additionally, the ester linkage absorption band (wave number 1735) disappeared from an IR spectrum of lichenysin A, indicating the hydrolytic opening of an intramolecular lactone ring.

The lipophilic moiety of lichenysin A was released by hydrolysis and analyzed after appropriate derivatization. A method (22) was used to obtain the β-hydroxy fatty acid methyl esters without the formation of unsaturated fatty acids by reaction of dehydration. TMS derivatives were formed quantitatively after reaction at 80°C for 15 min. The yields were determined by comparing the peak areas of the methyl esters with those of the free and TMS-substituted hydroxyl groups.

The methyl ester (Me)/TMS derivatives of fatty acids were analyzed by combined GC-MS. The retention times of these fatty acid derivatives were compared with those of reference β-hydroxy-*n* $C_{12}$ and β-hydroxy-*n* $C_{14}$ acid Me/TMS derivatives under the same conditions. β-Hydroxy fatty acids were identified by characteristic molecular ions *m/z* (M-Me) and the *m/z* 175 ion caused by β,γ fragmentation (3, 26). Ions corresponding to (M-73) [loss of $(CH_3)_3Si$] were also produced. An abundance of *m/z* 57 and *m/z* (M-57) ions was detected in spectra of the β-hydroxy-*anteiso* fatty acid derivatives and *m/z* 43 and *m/z* (M-43) for derivatives of the *iso* fatty acids. The resulting GC spectrum showed the presence of four main peaks (β-hydroxy-*iso* $C_{14}$, β-hydroxy-*n* $C_{14}$, β-hydroxy-*iso* $C_{15}$, and β-hydroxy-*anteiso* $C_{15}$ acid derivatives) and 10 minor peaks (Table 1).

Lichenysin A was hydrolyzed under acidic conditions for characterization of the hydrophilic moiety, and the water-soluble products were analyzed directly by TLC with system IV on an amino acid analyzer and by GC-MS as the *N*-TFA *C*-*n*-butyl esters. In each case, the following amino acid composition was obtained: aspartic acid, glutamic acid, valine, isoleucine, and leucine, 1.1:1.1:1.0:1.0:2.8, respectively. Glutamine and asparagine could not be found under these conditions, as the initial hydrolysis conditions would also cause hydrolysis of these amino acids.

**$FAB^+$ MS/MS analysis.** The sequence of amino acids was determined by the application of tandem MS. The surfactant was changed into an *O*,*N*-permethylated derivate. In Fig. 4, the mass spectrum of the $C_{15}$ fraction of lichenysin A is shown. In this spectrum, the molecular ion peak (M, *m/e* 1,207) is not recognized. The fragment peak (M-32, *m/e* 1,175) at the high mass end is due to the loss of methanol from the fatty acid moiety of the lipopeptide. Specific fragment ions resulting from degradation at each peptide linkage were recognizable, and sequential losses of the C-terminal amino acid were observed. By referring to the mass unit of the respective amino acids, the amino acid sequence Glx-Leu-Leu-Val-Asx-Leu-Ile seemed to be the most probable.

**NMR analysis.** The amino acid composition and sequence were also confirmed by 2D NMR spectroscopy. The complete amino acid spin systems were identified from a 2D $^1H$ phase-sensitive TOCSY spectrum starting from the backbone amide protons in the region from 10 to 7 ppm and confirmed by correlations of the $H_\alpha$s in the region from 4.4 to 4.0 ppm. Sequence-specific assignments were then readily determined from the cross peaks in the 2D $^1H$ phase-sensitive NOESY spectrum, with a mixing time of 200 ms, where nuclear Overhauser effect (NOE) signals were observed between $H_N$, $H_\alpha$, and $H_\beta$ of amino acid *i* and $H_N$ of amino acid *i* + 1 (Table 2). The following amino acid sequence was proposed: Glx-Leu-Leu-Val-Asx-Leu-Ile. These data agreed completely with the sequence determined by $FAB^+$ MS/MS analysis. Although only one primary amide side chain grouping was found, it was not possible to determine whether this belonged to asparagine or glutamine, as the appropriate NOEs between the amide protons and the $H_\beta$s were not observed in the 2D NOESY spectrum. The 2D TOCSY spectrum allowed unambiguous identification and assignment of the β-hydroxy fatty acid signals; $CH_2\alpha$, 2.84 and 2.28 ppm; CHβ, 4.90 ppm; $CH_2\gamma$, 1.61 and 1.54 ppm; $CH_2\gamma$, 1.32 ppm; other $CH_2$, 1.20 ppm; and $CH_3$, 0.83 ppm.

**Clarification of the C-terminal amino acid, the position of the lactone linkage, and the presence of asparagine.** The C-terminal amino acid was first identified. Purified lichenysin A gave a single spot by TLC on silica gel plates in solvent systems IV and V, which were used for fractionation of the $Leu^7$- and $Val^7$-surfactins (Fig. 2). Hydrazinolysis and quantitative estimation of the C-terminal amino acids of the linear lipopeptide showed that isoleucine was the principal C-terminal amino acid.

The location of the lactone ring was determined by digestion with carboxypeptidase Y and by reduction with $LiBH_4$. The carboxypeptidase specifically cleaves peptides possessing a free C-terminal residue and has already been used to clarify the lactone ring position in the lipopeptide from *B. licheniformis* (17). The native lichenysin A did not show any reaction after a treatment for 24 h with the enzyme, but the linear form reacted within 45 min, and the products were ninhydrin positive.

Compounds containing a lactone or ester carbonyl are reduced to primary alcohols with $LiBH_4$. The native, linear, ring-opened esterified form (from reaction with diazomethane) was reduced with lithium borohydride. When lichenysin A (native form) was reduced without any pretreatment, isoleucine could not be detected in the amino acid composition of the hydrolyzed surfactant. When the lipopeptide was esterified prior to reduction, glutamic acid and isoleucine could not be detected. When the lactone linkage was previously opened by mild alkaline hydrolysis, no change in the amino acid composition occurred. However, no glutamic acid or isoleucine residues were detected after esterification and reduction of the opened form. From these results, it was concluded that a lactone ring is formed between the carboxyl group of the C-terminal isoleucine and the β-hydroxyl group of the fatty acid. The heptapeptide chain contains glutamic acid as the N-terminal amino acid, asparagine, valine, three leucine residues, and isoleucine as the C-terminal amino acid. The proposed

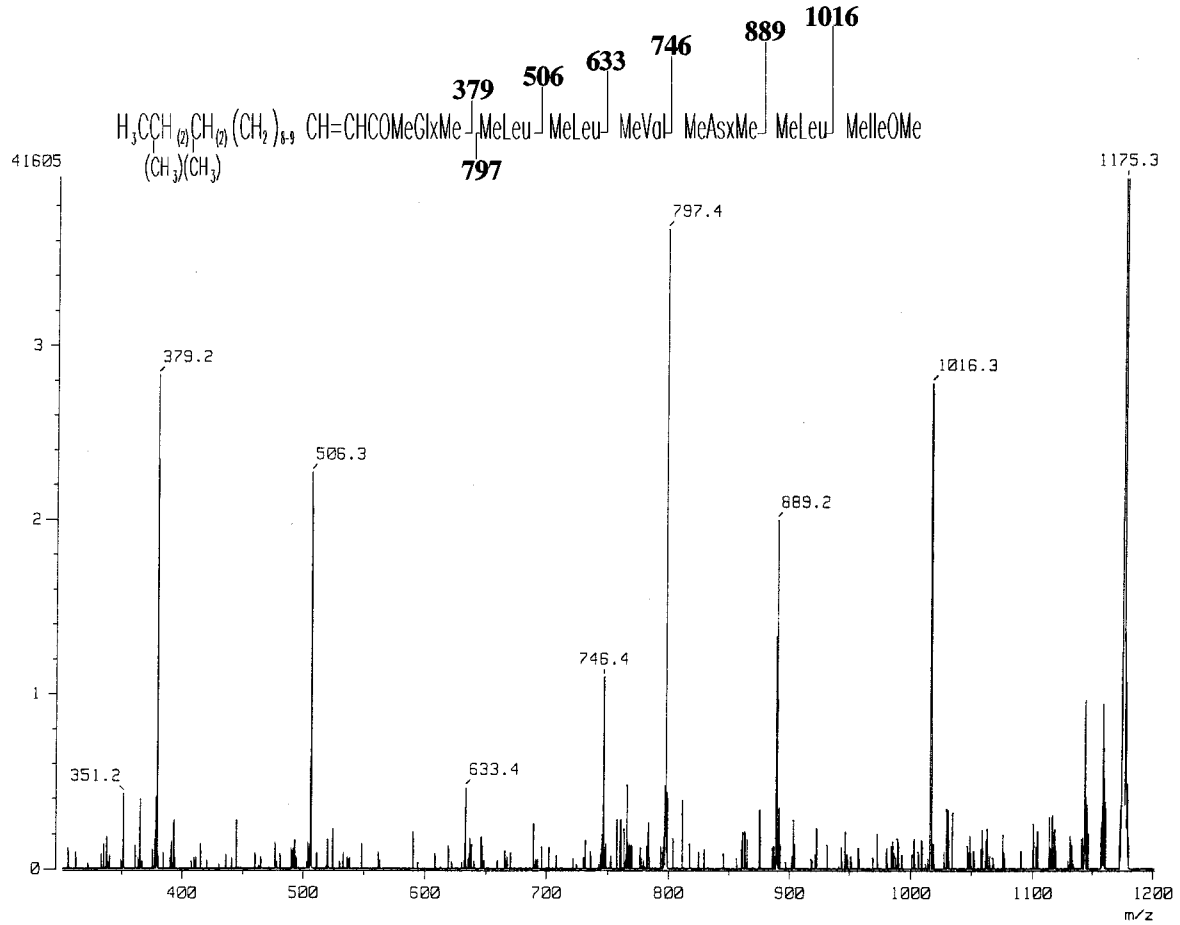


FIG. 4. Tandem FAB$^+$ MS/MS spectrum of permethylated lichenysin A. Proposed cleavage sites and fragments are shown by indicating the *m/z* values of the corresponding main peaks.

structure of the new variant of lichenysin, lichenysin A, was elucidated, as shown in Fig. 5.

**Physicochemical and biological properties of lichenysin A.** Lichenysin A revealed surface activity at very low concentrations, reaching a critical micelle concentration of 12 μg/ml. As shown in Fig. 6, the lowering of ST effected by a purified lichenysin A is not substantially inhibited by NaCl concentrations of up to 10% but is slightly inhibited by higher NaCl concentrations (up to 30%). NaCl concentrations of more than 30% were not tested.

**Antimicrobial characterization.** The antibiotic activities of surfactin and lichenysin A were compared. A survey of the

TABLE 2. $^1$H NMR assignments and chemical shifts for the peptide moiety of lichenysin A in DMSO-$d_6$ at 299 K

| Residue | Assignment (ppm) | | | |
|---|---|---|---|---|
| | $H_N$ | $H_\alpha$ | $H_\beta$ | Others |
| Glu-1 | 9.80 | 4.28 | 2.01; 1.75 | |
| Leu-2 | 9.64 | 4.23 | 1.48 | γ, 1.48; δ, 0.82 |
| Leu-3 | 7.40 | 4.38 | 1.45 | γ, 1.34; δ, 0.86; 0.78 |
| Val-4 | 8.24 | 4.05 | 2.18 | 0.90; 0.82 |
| Asn-5 | 8.50 | 4.25 | 2.69; 2.12 | 7.21; 6.69 |
| Leu-6 | 7.08 | 4.29 | 1.53 | γ, 1.43; δ, 0.84 |
| Ile-7 | 8.24 | 4.07 | 1.82 | γ, 1.39; γ, δ (Me), 1.07; 0.79 |

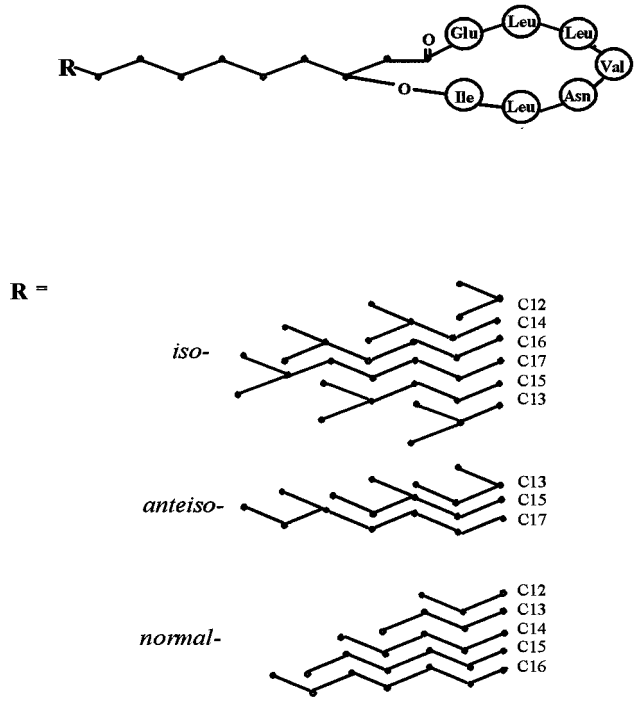


FIG. 5. Proposed structure of lichenysin A.

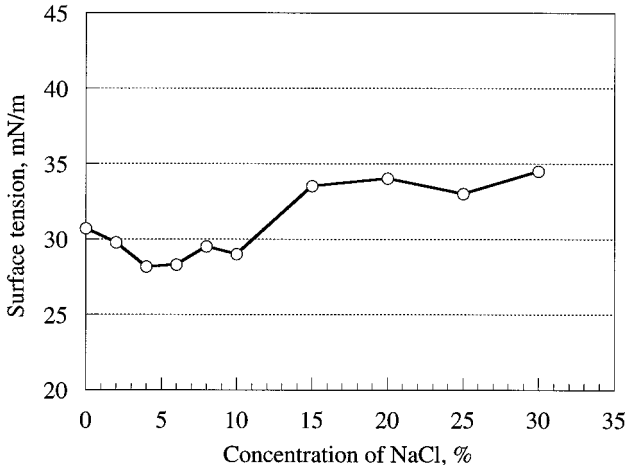


FIG. 6. ST characteristics of lichenysin A as a function of NaCl concentration.

results is given in Table 3. The lipopeptide lichenysin A inhibited the growth of most of the bacteria tested on nutrient agar plates, but this inhibition was less than that observed with surfactin. *Bacillus* sp. strain ATCC 39307, a lichenysin B-producing strain, showed higher susceptibility to lichenysin A than to surfactin. No growth inhibition by lichenysin A was detected for *B. licheniformis* BAS50 itself, *B. subtilis*, or *Rhodococcus globerulus*. To clarify the role of the polar groups on the antimicrobial activity, lichenysin A was treated with alkali to open the lactone linkage and derivatized with diazomethane or a silylation reagent. The open form of lichenysin A has approximately the same antimicrobial activity as the closed form (Table 3), but when the free polar groups were esterified, the activity disappeared (data not shown).

## DISCUSSION

In situ application of microbial emulsification for lowering of interfacial tension requires that microorganisms grow and produce surfactants under the environmental conditions that exist in the reservoirs. Aerobic microorganisms are known to produce biosurfactants, but these are not well suited for in situ microbially enhanced oil recovery (many petroleum reservoirs are anaerobic and have high salinities and temperatures [7]). Biosurfactant production by anaerobic organisms has not been well studied. Only one microorganism, *B. licheniformis* JF-2, has been shown to produce, under strictly anaerobic conditions, a biosurfactant that significantly lowers the ST of medium (<30 mN/m) (24). Field studies on *B. licheniformis* JF-2 demonstrated that this strain can grow and produce its surfactant throughout an oil reservoir, and an increase in oil recovery of 13% was obtained (25). Our studies on the production of the lichenysin A surfactant by *B. licheniformis* BAS50 show that this strain also has the potential for enhanced oil recovery. The conditions found in many oil reservoirs are optimal for the growth of this *B. licheniformis* strain. Lichenysin A shows a lowering of ST at high NaCl concentrations, up to 30% (wt/vol). The very low critical micelle concentration (12 μg/ml) shows that lichenysin A is effective at dilute concentrations. BAS50 is currently in use in a field trial of microbially enhanced oil recovery.

TABLE 3. Antimicrobial activity of surfactin and lichenysin A in agar diffusion tests[a]

| Microorganism | Halo diam | | |
|---|---|---|---|
| | Surfactin | Lichenysin A | |
| | | Native form | Open form |
| *Acinetobacter calcoaceticus* | +++ | ++ | + |
| *Alcaligenes eutrophus* | +++ | ++ | + |
| *Bacillus cereus* | +++ | − | − |
| *Bacillus licheniformis* BAS50 | ++++ | − | − |
| *Bacillus* sp. strain ATCC 39307 | − | + | − |
| *Bacillus subtilis* | ++ | ++ | + |
| *Escherichia coli* | +++ | ++ | ++ |
| *Enterobacter* sp. strain 306 | ++ | + | + |
| *Pseudomonas fluorescens* | ++++ | ++ | ++ |
| *Pseudomonas proteofaciens* | ++ | + | ++ |
| *Rhodococcus globerulus* | ++++ | − | − |
| *Staphylococcus aureus* | ++++ | + | + |

[a] Maximum diameter of halos: −, <5 mm; +, 6 to 7 mm; ++, 8 to 9 mm; +++, 10 to 11 mm; ++++, >11 mm.

The structure and composition of the lipopeptide surfactant lichenysin A have been studied by a variety of analytical techniques. The NMR data indicated that the peptide moiety contained seven amino acids per molecule. The complete amino acid spin systems and amino acid sequence were identified from a 2D $^1H$ phase. The data are in agreement with those obtained by the GC-MS, $FAB^+$ MS/MS, and amino acid analyses, wherein Glx, Asx, valine, isoleucine, and leucine were found in a molar ratio of 1.1:1.1:1.0:1.0:2.8. In order to clarify the presence of amino acid amides, native and esterified forms of lichenysin A were reduced with $LiBH_4$. Amino acid analysis of the products did not detect glutamic acid in the esterified form, as this was the methylated amino acid, while asparagine was confirmed. The presence of a lactone ring in lichenysin A was detected by IR and NMR spectra, and its location was determined by different biochemical methods. The native form of lichenysin A was ninhydrin negative, indicating that the peptide has a blocked N terminus. TLC analysis, reduction with $LiBH_4$, hydrazinolysis, and reaction with carboxypeptidase Y showed that isoleucine was the C-terminal amino acid and that its carboxy group formed the lactone ring.

The IR and NMR spectra indicated the presence of a long-chain fatty acid, which, from the cross peaks in the 2D NMR spectra, always contained a β-hydroxyl group. This was supported by data from reverse-phase HPLC, ESIMS, and SIMS, which also confirmed that lichenysin A is a mixture of structurally similar β-hydroxy, $C_{12}$ to $C_{17}$ fatty acid components. The latter were shown by GC-MS to be a mixture of 14 different β-hydroxy fatty acids. No differences in the amino acid composition were found.

Hence, lichenysin A is a new lipopeptide surfactant, different from surfactin (1, 2), lichenysin B (21, 24), lichenysin C (17), and surfactant BL86 (11, 12) in the composition of both the peptide and lipophilic moieties. Lichenysin A has an isoleucine as the C-terminal amino acid instead of the leucine of surfactin and lichenysin B and an asparagine residue instead of the aspartic acid residue of surfactin, lichenysin B, and lichenysin C. Wilkinson has isolated a surface-active compound from *Pseudomonas rubescens* that has both a free carboxyl group and a free amine group, but this lipid has only one amino acid, ornithine, in the polar part of molecule (37). The presence of basic amino acid residues in the peptide part of lipopeptide surfactants has not been reported. The main β-hydroxy fatty acids in the lipophilic parts of lichenysin B are *iso* $C_{14}$ (30%) and *anteiso* $C_{15}$ (36%). Surfactin contains 40% *n* $C_{14}$, whereas lichenysin A contains an *iso* $C_{15}$ residue in 39% of the mole-

cules. Lipopeptide BL86 has the same molecular weight distribution as lichenysin A, but it contains valine instead of isoleucine as the C-terminal amino acid in 40% of the molecules. The presence of amino acid amides, the lactone ring position, and the heterogeneity of the lipid moiety in this surfactant were not determined for BL86 (12).

Lichenysin A can be added to the group of lipopeptides which constitute the most effective biosurfactants known. The elucidation of the structure-function relationship of lipopeptides is possible by analyzing the surface activities of structure-characterized compounds. The critical micelle concentration of lichenysin A (12 mg/liter) was approximately twofold lower than that of surfactin (25 mg/liter) (8) and lichenysin B (20 mg/liter) (24) and similar to that of arthrofactin (13.5 mg/liter) (27), surfactant BL86 (10 mg/liter) (12), and the fraction of lichenysin B containing $C_{15}$ β-hydroxy fatty acid residues (10 mg/liter) (21). The less polar peptide moiety and the presence of a longer β-hydroxy fatty acid in the lichenysin A molecules appear to have an important influence on the surface activity of this lipopeptide. This may be due to the delicate hydrophile-lipophile balance. As recently shown, surfactin-Glu-γ-methyl ester obtained by selective esterification of surfactin exhibits a higher surfactant power than surfactin because of the disappearance of one carboxyl group in the methyl monoester and an increase in the hydrophobic character of this derivative (34).

It is well known that the production of most lipopeptides is dependent on the composition of the culture medium. Catabolite regulation and induction appear to be general regulatory mechanisms that control the onset of lipopeptide synthesis (5, 19, 36). Currently, the regulatory effects on lichenysin A synthesis are being studied.

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