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Chemical and Physical Characterization of Interfacial-Active Lipids from *Rhodococcus erythropolis* Grown on *n*-Alkanes

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Lipophilic compounds of the culture suspension containing *Rhodococcus erythropolis* DSM43215 had surfactant properties when the bacteria were cultivated with *n*-alkanes as the sole carbon source. Thirteen main components from a dichloromethane-methanol extract of the *R. erythropolis* cultures were isolated and characterized to specify quantitatively their surfactant properties, e.g., minimum surface and interfacial tensions and critical micelle concentrations. The interfacial activity of the organic extract was dominated by α,α-trehalose-6,6′-dicorynomycolates which reduced interfacial tension from 44 to 18 mN/m. Phosphatidylethanolamines which were also present in the organic extract reduced the interfacial tension below 1 mN/m. The trehalose corynomycolates had extremely low critical micelle concentrations in high-salinity solutions, and the interfacial properties were stabile in solutions with a wide range of pH and ionic strength.

In recent years, nontoxic and easily biodegradable microbial surfactants have become of interest for different applications. These surfactants were produced with increased yields when eucaryotic or procaryotic microorganisms were cultivated in media containing low water-soluble lipophilic substrates as the sole carbon and energy sources. The production of extracellular sophorolipids by *Candida* sp. (18) and *Torulopsis* sp. (18) and rhamnolipids by *Pseudomonas* sp. (7, 23) were reported. These ionic glycolipids have emulsifying properties, and they are produced for specialized applications as humectants and emulsifiers (18). Nonionic and nonextracellular *n*-alkane-inducible α,α-trehalose-6,6′-dicorynomycolates were produced by *Rhodococcus erythropolis* DSM43215, facilitating the *n*-alkane uptake by the bacteria (15) (Fig. 1).

Interfacial-active emulsions of water and trehaloselipids from *R. erythropolis* have been useful in pilot-scale experiments for enhanced oil recovery from samples of northern German sandstone oil deposits (14). Emulsions of trehaloselipids were also used successfully to prevent devastation of flatlands on the northern German coast by oil pollution (Wagner et al., Federal Republic of Germany ([FRG] patent DE-OS 291106, 1979). The enhancement of oil recovery by using the culture filtrate or emulsions of a partially purified organic crude extract from *R. erythropolis* cultures required a detailed analysis and quantitative characterization of the isolated major components of the organic crude extract to specify the interfacial-active compounds.

After the isolation and characterization of the chemical structure of the components, a determination of the dependence of interfacial-active properties on the concentration of the pure surfactants, which has not been published so far, was possible.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. *R. erythropolis* DSM43215 was isolated from an oil-containing soil sample and identified by R. M. Kroppenstedt, Institut für Mikrobiologie, Technische Universität Darmstadt, Darmstadt, FRG. Bacteria were cultivated in a mineral salt medium (15), pH 6.8, containing (per liter) 1.0 g of yeast extract and 20 g of C_{14}-C_{15} or C_{13}-C_{18} *n*-alkanes or *n*-hexadecane as the sole carbon source. Batch cultivations were carried out in various bioreactors (Giovanola Frères, Monthey, Switzerland) equipped with either impeller turbine systems or intensor systems (types b10, b20, b50, b200, and b3000). Physiological activity was followed by the use of a pH electrode and oxygen and carbon dioxide gas analyzers (Unor and Oxygor, Fa. H. Maihak AG, Hamburg, FRG). The *n*-alkane concentration was monitored after extraction (20) by gas chromatography.

Preparation of the organic crude extract. After a cultivation time of 24 to 48 h, during which the *n*-alkane concentration in the culture decreased to 7%, the cells were precipitated, separated by centrifugation, and extracted twice with dichloromethane-methanol (2:1, vol/vol). The solvents were removed by

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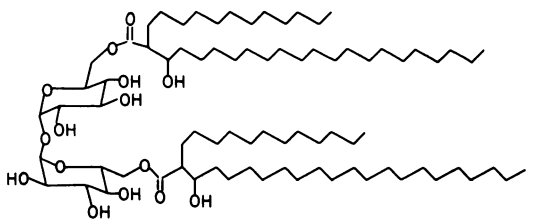


FIG. 1. α-D-Glucopyranosyl-α-D-glucopyranoside-6,6'-di-(2-alkyl-3-hydroxy)-carboxylic ester.

rotary evaporation, and the residue was separated from the remaining *n*-alkanes by column chromatography on Silica Gel 60 (no. 7734; E Merck AG, Darmstadt, FRG) with *n*-hexane. An organic crude extract (1,000 g) was prepared from a cultivation of *R. erythropolis* in a 3,000-liter fermentor (Giovanola type b3000). The components of the extract were isolated by repeated column chromatography with trichloromethane-methanol solvent mixtures or by preparative thin-layer chromatography on Silica Gel 60 (no. 7748, E. Merck AG) and were reprecipitated in dichloromethane-acetone mixtures.

Characterization of the components of the organic crude extract. The isolated lipids were identified by physical and chemical methods.

Melting points were determined with a Kofler heating-block microscope, and the specific optical rotation was measured with trichloromethane as the solvent in a Perkin-Elmer Polarimeter 241. Elementary analyses were carried out by the Mikroanalytisches Laboratorium, I. Beller, Göttingen, FRG.

Analytical thin-layer chromatography was conducted on high-performance thin-layer chromatography plates (no. 5642, E. Merck AG) which were horizontally developed. Different spray reagents were used to distinguish functional groups of the lipids. These reagents are presented in Table 1, together with the solvent systems which were used for isolation by repeated column chromatography and analytical high-performance thin-layer chromatography.

^{1}H and ^{13}C nuclear magnetic resonance spectra were measured with a Varian XL-100-12 spectrometer interfaced to a Varian 620-L computer.

Infrared spectra were detected by a Perkin-Elmer 521 double-beam lattice spectrometer; the compounds were dissolved in tetrachloromethane.

Mass spectra were recorded with an A.E.I. MS-30 instrument, and the fatty acid methyl esters were

TABLE 1. Chromatographic conditions for isolation and detection of the main components of organic crude extract from *R. erythropolis*

Lipid isolated	Solvent system (ratio by volume)		Spray reagent (19) (color after reaction)
	Column chromatography	High-performance thin-layer chromatography	
n-Alkanes	*n*-Hexane	*n*-Hexane	Concentrated H_2SO_4 (brown)
Unsaturated hydrocarbons	*n*-Hexane-$CHCl_3$ (3:1)	*n*-Hexane-diethylether (9:1)	4-Methoxy-benzaldehyde (red)
Triglycerides	$CHCl_3$	*n*-Hexane-$CHCl_3$-acetic acid (20:80:0.5)	Hydroxylamine-$FeCl_3$ (brown)
3-Keto-2-alkyl fatty acids	$CHCl_3$-CH_3OH (10:1)	$CHCl_3$-CH_3OH (95:5)	*o*-Dianisidine (brown); 2,4-nitrophenylhydrazine (yellow)
Fatty alcohols	$CHCl_3$-CH_3OH (10:1)	$CHCl_3$-CH_3OH (95:5)	4-Methoxy-benzaldehyde (violet)
Fatty acids	$CHCl_3$-CH_3OH-concentrated HCl (5:1:0.01)	*n*-Hexane-$CHCl_3$-acetic acid (20:80:0.5)	2',7'-Dichlorofluorescein-$AlCl_3$-$FeCl_3$ (red)
3-Hydroxy-2-alkyl fatty acids	$CHCl_3$-CH_3OH-concentrated HCl (5:1:0.01)	$CHCl_3$-isopropanol-acetic acid (70:5:1)	2',7'-Dichlorofluorescein-$AlCl_3$-$FeCl_3$ (red)
Trehaloselipids Dicorynomycolates Monocorynomycolates	$CHCl_3$-CH_3OH (5:1.5) (5:2)	$CHCl_3$-CH_3OH-H_2O (65:15:2) (65:25:4)	4-Methoxy-benzaldehyde (green)
Phosphatidylethanolamines	$CHCl_3$-CH_3OH (5:2)	$CHCl_3$-CH_3OH-H_2O (65:25:4)	Ninhydrin-reagent (violet); Hanes reagent (blue)
Phosphatidic acids	$CHCl_3$-CH_3OH (5:2)	$CHCl_3$-CH_3OH-H_2O (65:25:4)	Hanes reagent (blue)

identified by gas chromatography and mass spectrometry. The mass spectrometer (A.E.I. MS-30) was used with a combined calculator (A.E.I. data system DS 50). This was connected to a gas-liquid chromatograph (Perkin-Elmer F22). A capillary column (15 m by 0.2 mm; WCOT SE 30; J. u. W. 2) was used. Gas-liquid chromatography of *n*-alkanes was carried out with a Packard 427 gas chromatograph equipped with a glass column OV-1 (1 m by 2 mm) and a flame ionization detector.

Triglycerides were determined by the spectroscopic methods described above, and after alkaline hydrolysis (2 h at 70°C in 1 N NaOH-ethanol with 10% water), the amount of the fatty acids was gravimetrically determined. The distribution of homologous fatty acids was measured by gas-liquid chromatography. The glycerol was quantitatively determined by an enzymatic method (5).

Interfacial tensions of aqueous samples containing lipids of the organic crude extract were determined with the ring method by a Lauda Autotensiomat (Fa. Lauda-Wobser KG, Königshofen, FRG) equipped with a thermostatically controlled shade. The samples were emulsified ultrasonically with a Branson Sonifier cell disruptor B30 (Branson Sonic Power Co., Danbury, Conn.) and then were measured automatically until a constant interfacial tension was recorded.

The glycolipid content of the culture suspension or cell mass was determined colorimetrically in the organic extract of samples by the anthrone method (6). Before the test, the organic extracts were filtered through a membrane filter (0.25-μm pore size; Millipore Corp., Bedford, Mass.), and then the solvent was evaporated from the test tubes. The residue was mixed ultrasonically with the reagent, and after the color reaction was read, any remaining *n*-alkanes were extracted by *n*-hexane.

Chemical methods. Alkaline hydrolysis of the phospholipids and the glycolipids for separating the fatty acids or the corynomycolic acids was carried out by refluxing with 1 N ethanolic sodium hydroxide solution containing 5% water. Methyl esters of the carboxylic acids were prepared by diazomethane.

Synthesis of reference compounds. 3-Keto-2-alkyl fatty acid methyl esters were prepared by a Claisen condensation of fatty acid methyl esters in dioxane with equimolar amounts of sodium alcoholate as base and fractional distillation at 120°C for 12 h. The reaction products were separated, dried, and isolated by column chromatography with Silica Gel 60 (*n*-hexane–diethylether; 20:1, vol/vol). 3-Keto-2-alkyl fatty acid methyl esters were also obtained from native corynomycolic acid methyl esters by oxidation of the 3-hydroxyl group with aluminum tri-*tert*-butylate in acetone-benzene (1:1, vol/vol) by refluxing for 10 h.

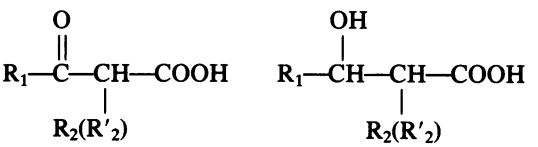
Synthetic corynomycolic acids were prepared by reducing synthetic 3-keto-2-alkyl fatty acid methyl esters with twice the molar amount of $NaBH_4$, refluxing in ethanol for 5 h, and performing alkaline hydrolysis of the ester as described above.

Synthetic esters of α,α-trehalose and corynomycolic acids or other fatty acids were obtained by transesterification. The methyl esters with α,α-trehalose were dissolved in *N*,*N*-dimethylformamide and refluxed with equimolar amounts of K_2CO_3 for 15 h. Since the molar ratio of the fatty acids and the trehalose influenced the yield of mono-, di-, or higher esters markedly, mainly C-6 and C-6′ esters were formed when equimolar amounts of α,α-trehalose and methyl esters were used.

RESULTS

Thirteen major lipids from the organic crude extract of *R. erythropolis* culture suspensions were isolated by repeated column and thin-layer chromatography. Their purification was checked by the spectroscopic methods described above. The analyses of the purified components revealed nonpolar and polar lipids, from which nonionic triglycerides and α,α-trehalose corynomycolates were the most abundant lipids. The structures of the isolated lipids were determined by the spectroscopic methods described above. The data obtained were compared with published data or with data from synthesized reference compounds as follows: lipids 1.1 to 2.3 (C_{13}-C_{18} *n*-alkanes, isoprenoids, triglycerides, 3-keto-2-alkyl fatty acids, fatty alcohols) (2, 13); corynomycolic acids (14, 21, 22); trehalose corynomycolates and trehalose fatty acid esters (15; A. Kretschmer, Ph.D. thesis, Technische Universität Braunschweig, Braunschweig, FRG, 1981); phosphatidylethanolamines and phosphatidic acids (2, 8). A thin-layer chromatogram of the organic crude extract and the determined structures of the isolated lipids (fractions 1.1 to 2.10) are presented in Fig. 2.

Fractions 2.1 and 2.4 contained homologous 2-alkyl alkanoic acid derivatives with either an oxo group (3-keto-2-alkyl alkanoic acids) or a hydroxy group (corynomycolic acids) at C-3. The distribution of homologs is given in the general structure:



R_1: $C_{16}H_{33}$-$C_{25}H_{51}$; R_2: C_8H_{17}-$C_{13}H_{27}$; R'_2: C_8H_{15}-$C_{13}H_{25}$ (one *cis*-configured carbon double bond).

Fractions 2.5 to 2.7 and 2.9 contained the acids of fractions 2.1, 2.3, and 2.4 and 2-alkylalkanoic acids (with the same R_1 and R_2 homologs mentioned in fractions 2.1 and 2.4) esterified to C-6 and C-6′ of α,α-trehalose. More than 90% by weight of these trehaloselipids were α,α-trehalose-6,6′-dicorynomycolates.

Fractions 2.8 and 2.10 consisted of phospholipids which contained 27% by weight methyl-branched acyl moieties. Iso- or anteisoacyl residues were not detected. Dominant homologs were 1,2-ditetradecanoyl-*sn*-glycero-3-phosphates or 1,2-tetradecanoyl-(15-methyl-nonadecanoyl)-*sn*-glycero-3-phosphates.

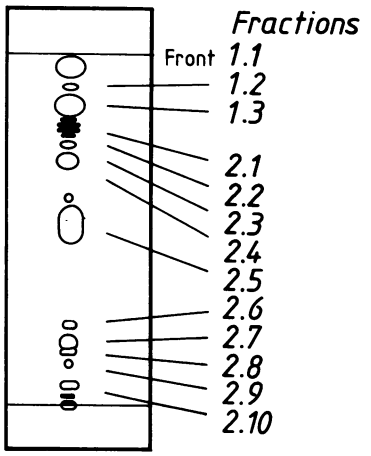


FIG. 2. Thin-layer chromatogram of the organic crude extract and structures of the determined compounds when *R. erythropolis* was grown on 85% *n*-tetradecane and 15% *n*-pentadecane. Solvent system: trichloromethane-methanol-water, 65:15:2 (vol/vol/vol). 1.1, *n*-alkanes; 1.2, methyl-branched unsaturated hydrocarbons; 1.3, triacylglycerols (1,2,3-propantriol-di-tetradecanoate-pentadecanoate); 2.1, 3-keto-2-alkyl fatty acids; 2.2, C_{13}-C_{18} *n*-alkanols; 2.3, C_{11}-C_{22} *n*-alkanoic and C_{13}-C_{18} *cis-n*-alkenoic acids; 2.4, corynomycolic acids; 2.5, trehalose-6,6'-dicorynomycolates; 2.6, trehalose-6,6'-diacylates; 2.7, trehalose-6-corynomycolates; 2.8, phosphatidylethanolamines; 2.9, trehalose-6-acylates; 2.10, phosphatidic acids.

The relative composition of the organic crude extract was determined gravimetrically after the separation of fractions 1.1 to 2.10 by the chromatographic methods mentioned above. Unwashed dry cell mass of the early stationary growth phase contained 24% by weight extractable lipids. These lipids consisted of 5.5% *n*-alkanes, 0.8% methyl-branched unsaturated hydrocarbons, 70.3% triacylglycerols, 1.3% *n*-alkanols, 2.1% *n*-alkanoic and *n*-alkenoic acids, 10.2% trehalose dicorynomycolates, 0.9% trehalose monocorynomycolates, 0.4% phosphatidylethanolamines, 0.4% phosphatidic acids, and 6.0% residue. The residue contained different nonpolar and polar lipids which were not characterized because of their small amounts.

The analyses of the isolated lipids showed that terminally oxidized derivatives of the *n*-alkanes used as substrates were dominant constituents of the triglycerides, fatty alcohols, free fatty acids, corynomycolates, and trehalose fatty acid esters (F. Wagner et al. *in* J. E. Zajic et al., ed., *Microbial Enhanced Oil Recovery*, in press).

Interfacial-active properties of the isolated lipids. The highly lipophilic lipids of fraction 1 (*n*-alkanes, isoprenoids, and fatty acid esters, including the triglycerides) were separated by column chromatography from the organic crude extract. The remaining amphiphilic lipids (fractions 2.1 to 2.10) contained 50% by weight trehaloselipids. This partially purified crude extract was emulsified in distilled water or electrolytes, and the surface tension and interfacial tension of the emulsion were reduced from 72 to 30 mN/m and from 40 to 18 mN/m, respectively (Fig. 3). These emulsions were applied with success in pilot tests for enhanced oil recovery (14). Since fraction 2 is a mixture of different ionic and nonionic compounds (fractions 2.1 to 2.10), the main components of the organic crude extract were isolated to determine quantitatively the relation between the interfacial tension and the concentrations (in milligrams per liter) of each of the pure components. The surface and interfacial tensions presented in Fig. 3 through 6 and Table 2 were measured with stabile emulsions of the components in distilled water or electrolytes at 40°C until a constant tension was registered. In the range of the critical micelle concentration, a constant interfacial tension was often obtained only after several hours of measurement.

The isolated compounds of fraction 2 can be classified into two groups of surfactants: ionic

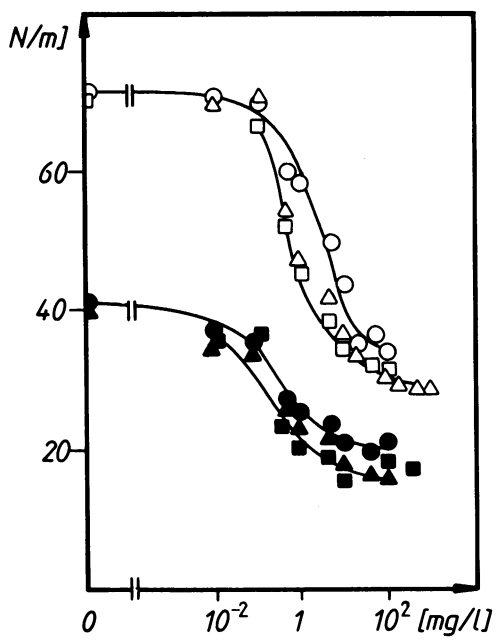


FIG. 3. Surface and interfacial tensions of fraction 2, containing about 50% trehaloselipids, emulsified in distilled water or in different electrolytes at 40°C. Open symbols show surface tension with distilled water (○), 5% NaCl solution (△), and synthetic deposit water (□; 100 g of NaCl, 28 g of $CaCl_2$, and 10 g of $MgCl_2$ per liter of water). Solid symbols show the corresponding interfacial tensions against *n*-hexadecane.

surfactants like corynomycolates, phosphatidylethanolamines, and phosphatidic acids, and nonionic surfactants like the different trehaloselipids.

Ionic surfactants of *R. erythropolis* cultures. The isolated ionic surfactants of *R. erythropolis* showed different interfacial-active properties, especially the phosphatidylethanolamines, which acted as the most powerful surfactants by lowering the interfacial tension in a water-hexadecane system below 1 mN/m (Fig. 4).

The interfacial-active properties of phosphatidic acids are considerably different from those of phosphatidylethanolamines (Table 2).

The critical micelle concentration of corynomycolates exceeds 600 mg/liter in the electrolyte emulsion of pH 3.0 and 200 mg/liter in the electrolyte emulsion of pH 9.0 (Fig. 5). From Fig. 5, graphic extrapolation can be used to show that the critical micelle concentration of the corynomycolate anions is smaller than that of the free acids, though it is expected that the charged molecule is more soluble.

Nonionic surfactants of *R. erythropolis* cultures. Compared with the ionic surfactants, the nonionic surfactants showed more similar interfacial activity concerning the minimum interfacial tensions, although different compounds, like mono- and diesters of trehalose corynomycolates or mixtures of the trehaloselipids, have been measured.

A very similar dependence of surfactant concentration and interfacial activity as presented for α,α-trehalose-6-corynomycolates (Fig. 6) was measured when α,α-trehalose-6,6′-dicorynomycolates were used as surfactants (Table 2). The trehalose monocorynomycolates showed an increased critical micelle concentration in distilled water owing to their solubility compared with the more lipophilic trehalose dicorynomycolates. The interfacial activity of emulsions of

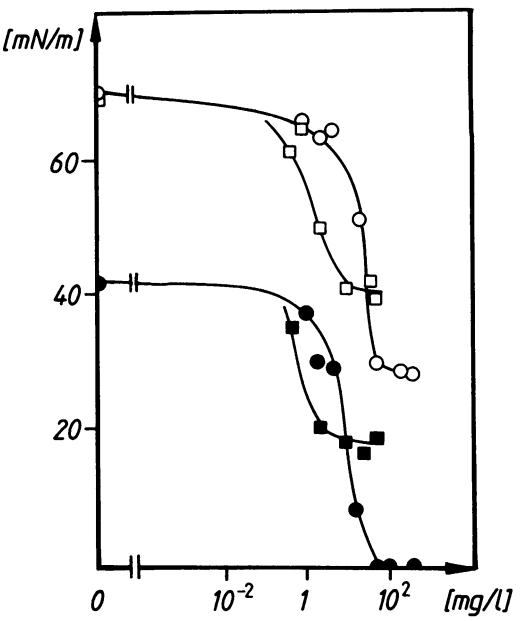


FIG. 4. Surface and interfacial tensions of phosphatidylethanolamines from *R. erythropolis* emulsified in distilled water or synthetic deposit water at 40°C. Symbols are defined in the legend to Fig. 3.

the trehaloselipids and of fraction 2 was also insensitive against electrolyte pH variations in the range of 3.0 to 9.0. At low pH values of 1.0 to 3.0, the trehaloselipids were not hydrolyzed within 96 h. Though phosphatidylethanolamines from fraction 2 exhibited a much lower interfacial tension compared with the trehaloselipids (Table 2), their small amounts in the organic crude extract did not give rise to more interfacial activity of fraction 2 as compared with the pure trehaloselipids.

The surfactant properties of the other amphiphilic components of fraction 2, such as fatty

TABLE 2. Surfactant properties of amphiphilic compounds from *R. erythropolis* grown on *n*-alkanes

Surfactant	Minimum surface tension (mN/m)		Minimum interfacial tension (mN/m)		Critical micelle concn (mg/liter)	
	Water	Electrolyte	Water	Electrolyte	Water	Electrolyte
Organic crude extract	35	30	22	22	15	20
Corynomycolic acids						
pH 3				9		600
pH 9				6		200
Trehalose dicorynomycolates	43	36	18	17	0.7	1.7
Trehalose monocorynomycolates	32	32	14	16	165	2
Phosphatidylethanolamines	29	38	1	15	30	5
Phosphatidic acids	33	40	17	14	70	0.01

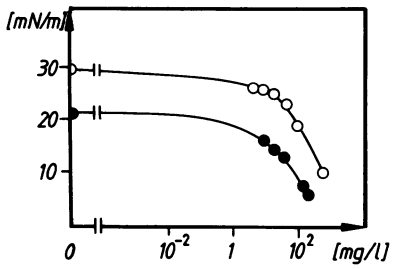


FIG. 5. Interfacial tensions of corynomycolic acid emulsions in a K_2HPO_4 (158 g/liter) electrolyte against *n*-hexadecane at 40°C at pH 3.0 (○) and 9.0 (●).

alcohols and fatty acids, have already been characterized (11). In Table 2 the surfactant properties of components of the organic crude extract of *R. erythropolis* are characterized by the minimum surface tension, the minimum interfacial tension, and the critical micelle concentration.

DISCUSSION

A quantitative characterization of interfacial-active properties of isolated and pure microbial surfactants related to certain concentrations has not been published so far. The surface activities of undefined mixtures of dissolved or emulsified metabolites were estimated by Cooper et al. and Macdonald et al. (3, 4, 10) by a method in which the surface tension of the supernatants of culture media or of the whole broth was measured. The critical micelle concentration was derived from surface tension measurements by dilution of the samples of the whole broth with water. The remaining hydrocarbon phase in the samples interfered with the determination of the equilibrium surface tension, and the derived experimental data could not be related to discrete compounds or their concentrations.

For the determination of the surface-active properties of the surfactants produced by *R. erythropolis*, the use of the analytical procedure described here was necessary to characterize quantitatively the single components of the organic crude extract of the culture suspension. It was found that only the trehaloselipids showed surfactant properties which corresponded to the results of experiments for enhanced oil recovery. Therefore, an optimization of surfactant production related to trehaloselipids could be established.

In general, the trehaloselipids of *R. erythropolis* are of interest as surfactants because they have advantages in comparison with commercial surfactants like sucrose fatty acid esters. The trehaloselipids emulsified in aqueous solutions are much more stable against hydrolysis at extreme pH values, e.g., pH 1.0 or 14.0 at 40°C,

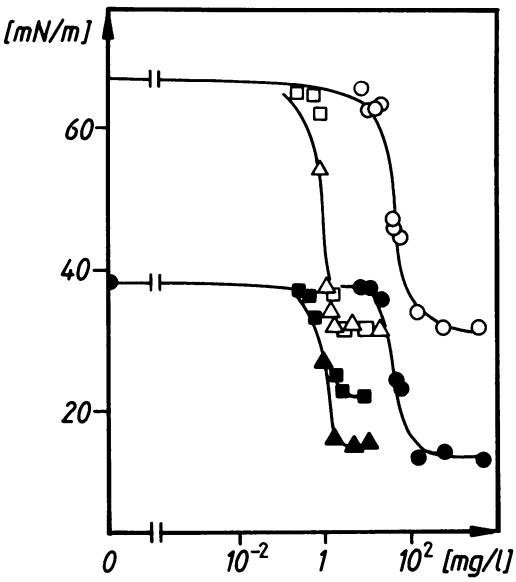


FIG. 6. Surface and interfacial tensions of α,α-trehalose-6-corynomycolates emulsified in distilled water or in different electrolytes at 40°C. Symbols are defined in the legend to Fig. 3.

than are sucrose fatty acid esters, and their surface activity is not reduced at temperatures exceeding 90°C, in contrast to ionic surfactants (14). In addition, a very low critical micelle concentration (ca. 2 mg/liter) led to the minimum surface and interfacial tension in electrolytes at low concentrations of the trehalose corynomycolates. The experimental data presented in Fig. 3 and 6 also show that the minimum surface and interfacial tensions are constant within a wide range of electrolyte concentrations, in contrast to phosphatidylethanolamines, which did not show constant surfactant properties under the different conditions. The surfactant properties of trehaloselipids are less sensitive to salt concentrations than are common synthetic surfactants (12), which makes them even more attractive for special application in enhanced oil recovery.

Besides their importance as surfactants, the function of trehalose corynomycolates and trehalose mycolates as a cell envelope constituent is of interest (1, 16). Trehalose dicorynomycolates have also been produced in the absence of *n*-alkanes or other lipophilic carbon sources by *Brevibacterium vitarumen* 12143 (9), *Corynebacterium diphtheriae* (17), and different pathogenic *Mycobacteriaceae* (1). Therefore, it can be concluded that structurally identical molecules, α,α-trehalose-6,6′-dicorynomycolates, have different physiological significance. This might be taken into account to specify actinomycetes on the basis of taxonomy.

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