APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1984, p. 610–617
0099-2240/84/090610-08$02.00/0

Vol. 48, No. 3

Formation and Identification of Interfacial-Active Glycolipids from Resting Microbial Cells

ZU-YI LI,[1] SIEGMUND LANG,[1] FRITZ WAGNER,[1]* LUDGER WITTE,[2] AND VICTOR WRAY[2]

Institute of Biochemistry and Biotechnology, Technical University,[1] and Gesellschaft für Biotechnologische Forschung,[2] D-3300 Braunschweig, Federal Republic of Germany

Received 9 March 1984/Accepted 30 May 1984

Resting cells of *Arthrobacter sp.* strain DSM2567 incubated in the presence of various mono-, di-, or trisaccharides biosynthesized different glycolipids. All eight glycolipids, containing the corresponding carbohydrate moiety and one, two, or three α-branched β-hydroxy fatty acids, were produced when mannose, glucose, cellobiose, maltose, and maltotriose were used as carbon sources in a simple phosphate buffer. The structures of the compounds were elucidated by means of ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy and by chemical ionization mass spectroscopy. In high-salinity solution, the substances showed different surfactant properties. Cellobiose and maltose monocorynomycolates reduced the interfacial tension from 42 to 1 mN/m at critical micelle concentrations below 20 mg/liter.

Arthrobacter paraffineus KY4303 produces lipids containing sucrose and α-branched β-hydroxy fatty acids when grown on an excess of sucrose as the sole carbon source (11). When the same strain is grown on fructose, a fructose 6-corynomycolate and a fructose-1,6-dicorynomycolate can be isolated (6). Glucose lipids have been identified as major components of the soluble lipids in several types of microorganisms grown in the presence of glucose (2). With corynebacteria, *Nocardia* spp., mycobacteria, and brevibacteria, the possibility of changing the carbohydrate moiety of glycolipids by manipulation of the carbon source in the culture medium is of great potential value. To investigate this possibility, we examined the effects of various carbon sources on the formation of glycolipids by an *Arthrobacter* sp. Initial experiments with the above-mentioned substrates confirmed previous results, but with cellobiose the method was unsuccessful because of the absence of growth and biomass. Therefore, we separated the cell growth and production phases. After growth on glucose, the cells were harvested and transferred into a simple buffer solution containing only the carbon source. This paper describes the isolation, the identification, and some properties of glycolipids produced by resting cells.

MATERIALS AND METHODS

Microorganisms and growth conditions. *Arthrobacter* sp. strain DSM2567 was incubated at 100 rpm (shaker RS 206; F. Braun, Melsungen, Federal Republic of Germany) and 30°C in a 500-ml Erlenmeyer flask containing 100 ml of medium. The composition of the medium was as follows (concentration per liter of water): 2 g of $(NH_4)_2SO_4$, 1.2 g of citric acid · $1H_2O$, 1 g of KH_2PO_4, 1 g of Na_2HPO_4 · $2H_2O$, 0.5 g of $MgSO_4$ · $7H_2O$, 1 g of yeast extract, 0.13 g of $FeCl_3$ · $6H_2O$, 0.22 g of $CaCl_2$ · $2H_2O$, 0.09 g of $MnSO_4$ · H_2O, 0.005 g of $ZnSO_4$ · $7H_2O$, 0.5 ml of 85% H_3PO_4, and 15 g of glucose, pH 6.8. After 24 h, 4 ml of this culture was inoculated into 200 ml of the same medium in a 1-liter shake flask. This mixture was incubated for 3 days.

Conditions for resting cells. After incubation the cells were harvested by centrifugation at 8,000 rpm (Varifuge S; F. Heraeus Christ, Osterode, Federal Republic of Germany) and washed twice with 0.1 M phosphate buffer, pH 6.5, under sterile conditions. A portion of the wet biomass, corresponding to 0.7 g of dry mass, was transferred into 1-liter shake flasks containing 200 ml of 0.1 M phosphate buffer, pH 6.5, and 2.5 g of carbohydrate. The reaction mixtures were incubated under sterile conditions at 100 rpm and 30°C for 4 or 5 days.

Isolation of glycolipids. After the appropriate incubation times, the reaction suspension was extracted twice with 400 ml of dichloromethane-methanol (2:1 [vol/vol]). With maltotriose as the carbon source, butan-1-ol was used for extraction. The solvents were removed by rotary evaporation, and the residue was separated by means of column chromatography on Silica Gel 60 (no. 7734; E. Merck AG, Darmstadt, Federal Republic of Germany) with $CHCl_3/CH_3OH$ ratios ranging from 20:1 to 1:2 (vol/vol). For measurement of the whole glycolipid content, the anthrone method (5) was used.

Characterization of the glycolipids. The isolated glycolipids were identified by chemical and physical methods. Analytical thin-layer chromatography was conducted on plates (no. 5554; E. Merck AG) with chloroform-CH_3OH-water (65:15:2 [vol/vol/vol]) as the solvent system. 4-Methoxy-benzaldehyde in acetic acid and H_2SO_4 (0.5:50:1 [vol/vol/vol]) served as the spray reagent (green color after reaction). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were measured at room temperature on a Bruker WM-400 NMR spectrometer at 400 and 100 MHz, respectively. Chemical shifts are reported with respect to tetramethylsilane. Chemical ionization (CI) mass spectra were recorded with an AEI MS-30 instrument operating at 4 kV accelerating voltage with isobutane as the reagent gas. Samples were inserted directly into the ion source. Elementary analyses were carried out by the Mikroanalytisches Laboratorium, I. Beetz, Kronach, Federal Republic of Germany. The surface and interfacial tensions of the pure glycolipids were determined by the ring method with a Lauda Autotensiomat (Fa. Lauda-Wobser KG, Königshofen, Federal Republic of Germany) (7). For this method, the substances were emulsified in synthetic deposit water containing (g/liter) NaCl (100), $CaCl_2$ (28), and $MgCl_2$ (10).

Chemical methods. Alkaline hydrolysis of the glycolipids, for isolation of the corynomycolic acids, was carried out by refluxing with 0.5 N ethanolic sodium hydroxide solution containing 10% water. Methyl esters of the carboxylic acids were prepared with diazomethane.

* Corresponding author.

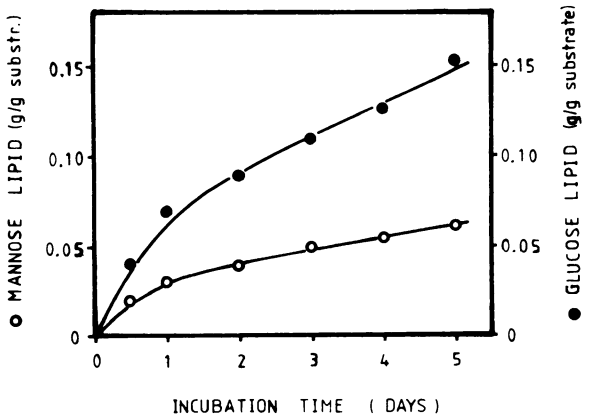


FIG. 1. Specific glycolipid production by resting cells of *Arthrobacter* sp. Conditions: 0.2 liter of 0.1 M phosphate buffer, pH 6.5; 0.7 g of biomass (dry weight); 2.5 g of mannose; and 2.5 g of glucose. Temperature, 30°C.

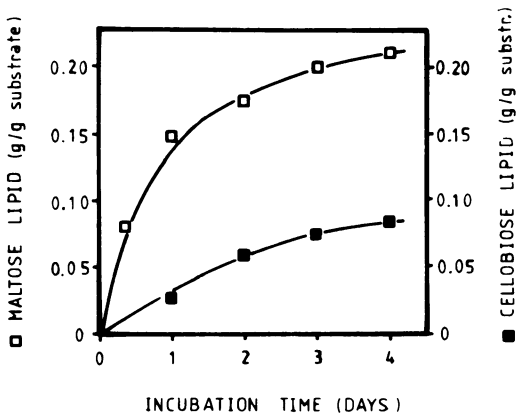
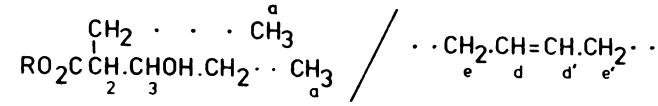


FIG. 2. Specific glycolipid production by resting cells of *Arthrobacter* sp. The conditions are the same as described in the legend to Fig. 1. Maltose and cellobiose concentrations, 2.5 g per 0.2 liter.

RESULTS

Effect of the carbon source on glycolipid formation by *Arthrobacter* sp. Various carbohydrates were used in resting-cell experiments to study the effect on the sugar moiety of the glycolipids produced by *Arthrobacter* sp. After growing on glucose under nitrogen saturation conditions, the cells were harvested and then incubated with 2.5 g each of glucose, mannose, cellobiose, maltose, and maltotriose in a phosphate buffer. The corresponding glycolipids were synthesized with incorporation of the appropriate sugar residue. When the monosaccharides glucose and mannose were used as carbon sources, 380 mg of a glucose lipid (R_f, 0.42 in thin-layer chromatography) and 140 mg of a mannose lipid (R_f, 0.47), respectively, could be isolated after extraction and purification of 5-day-old cultures. The corresponding specific-production curves (grams per gram of substrate) are shown in Fig. 1. Incubation with disaccharides led to two different types of spots on thin-layer plates. The more polar ones seemed to be derived from monoesters, and the lipophilic ones seemed to be derived from diesters. Thus, cellobiose and maltose are esterified at one and two posi-

TABLE 1. ^{1}H chemical shifts and coupling constants of the monosaccharide glycolipids



Glucose 6'-monoester (CD_3OD)			Mannose 6'-monoester (CD_3OD)		
Proton[a]	Chemical shift	Coupling constant (J) (Hz)	Proton[a]	Chemical shift	Coupling constant (J) (Hz)
Lipid			Lipid		
2	2.475		2	2.435	
3	3.725		3	3.723	
e/e'	2.07		e/e'	2.076	
d/d'	5.387		d/d'	5.386	
a	0.94		a	0.942	
$(CH_2)_n$	1.2–1.7		$(CH_2)_n$	1.3–1.7	
Sugar			Sugar		
1'α	5.118	3.5 (1'α-2'α)	1'α[b]	5.091	1.5 (1'α-2'α)
1'β	4.514	7.8 (1'β-2'β)	2'α }	3.78–3.86	NA[c] (2'α-3'α)
2'β	3.177	9.0 (2'β-3'β)	3'α }		9.4 (3'β-4'α)
3'α	3.717	9.2 (2'α-3'α)	4'α	3.672	9.7 (4'α-5'α)
5'α	4.000	9.2 (3'α-4'α)	5'α	3.972	2.3 (5'α-6'$_A$α)
5'β	3.522	NA[c] (3'β-4'β)	6'$_A$α	4.473	6.2 (5'α-6'$_B$α)
6'$_A$α	4.517	10 (4'α-5'α)	6'$_B$α	4.262	−11.6 (6'$_A$α-6'$_B$α)
6'$_A$β	4.487	9.5 (4'β-5'β)			
6'$_B$α	4.197	2.3 (5'α-6'$_A$α)	1'β	4.779	
6'$_B$β	4.226	2.3 (5'β-6'$_A$β)	2'β }		
2'α }		5.3 (5'α-6'$_B$α)	3'β }	NA[c]	
3'β }	3.3–3.4	5.3 (5'β-6'$_B$β)	4'β }		
4'α }		−12.0 (6'$_A$α-6'$_B$α)	5'β }		
4'β }		−12.0 (6'$_A$β-6'$_B$β)	6'$_A$β	4.535	
			6'$_B$β	4.210	

[a] Numbering of the sugar protons is as normal with 1' being the anomeric proton.
[b] The α-anomer was ca. 76% of the total.
[c] NA, Not available.

TABLE 2. ^1H chemical shifts and coupling constants of the disaccharide glycolipids

Cellobiose monoester (CD_3OD)			Cellobiose diester (CD_3OD)		Maltose monoester (CD_3OD)			Maltose diester ($CD_3OD/CDCl_3$ [1:1]		
Proton[a]	Chemical shift	Coupling constant (J) (Hz)	Proton[a]	Chemical shift	Proton[a]	Chemical shift	Coupling constant (J) (Hz)	Proton[a]	Chemical shift	Coupling constant (J) (Hz)
Lipid			Lipid		Lipid			Lipid		
2	2.53		2	2.46	2	2.53		2	2.50, 2.41	
3	3.733		3	3.70	3	3.73		3	3.69	
e/e′	2.11		e/e′	2.05	e/e′	2.08		e/e′	2.03	
d/d′	5.385		d/d′	5.36	d/d′	5.39		d/d′	5.37	
a	0.95		a	0.91	a	0.94		a	0.90	
$(CH_2)_n$	1.3–1.7		$(CH_2)_n$	1.3–1.7	$(CH_2)_n$	1.3–1.7		$(CH_2)_n$	1.2–1.7	
Sugar			Sugar		Sugar			Sugar		
1′α	5.143	3.6 (1′α-2′α)	1′α	5.14	1′α	5.153	~3 (1′α-2′α)	1″	5.12	
1′β	4.543	6.9 (1′β-2′β)	1′β	NA	1′β	4.533	7.5 (1′β-2′β)	Rest of sugar protons	4.6–3.25	3.8 (1″-2″)
1″αβ	4.443	7.8 (1″αβ-2″αβ)	1″αβ, 1″ββ	4.52	1″αβ, 1″ββ	5.143	~3(1″-2″)			
1″ββ	4.431	7.8 (1″ββ-2″ββ)								
$6''_A(6'_A)$	4.760		$6'_A$	4.50	$6''_A$(or $6'_A$)	4.548				
$6''_B(6'_B)$	4.124		$6''_A$	4.65	$6''_B(6'_B)$	4.163				
2″	3.268		$6'_B$	4.26	Rest of sugar protons	4.05–3.20				
3″	3.436		$6''_B$	4.23						
4″	3.378		Rest of sugar protons	4.05–3.15						
5″	3.610									
Rest of sugar protons	3.98–3.32									

[a] Numbering for the lipid is as in Table 1. Single-primed (′) number, the sugar adjacent to the lipid; double-primed (″) number, the second sugar ring.

TABLE 3. ^{1}H chemical shifts of the trisaccharide glycolipids

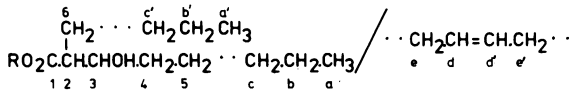
Maltotriose monoester (CD_3OD)		Maltotriose triester (CD_3OD)	
Proton[a]	Chemical shift	Proton[a]	Chemical shift
Lipid		Lipid	
2	2.53	2	2.50
e/e′	2.08	e/e′	2.04
d/d′	5.39	d/d′	5.35
a	0.95	a	0.90
$(CH_2)_n$	1.2–1.7	$(CH_2)_n$	1.2–1.7
3	5.15–3.15	3	4.78–3.15
Sugar		Sugar	
1″ or 1‴	5.20	1″, 1‴	5.17, 5.09
Rest of sugar protons	5.15–3.15	Rest of sugar protons	4.78–3.15

[a] See footnote to Table 2.

tions, respectively, with fatty acids. The corresponding glycolipid yields are shown in Fig. 2. With cellobiose, 125 mg of a monoester (R_f, 0.23) and 25 mg of a diester (R_f, 0.52) were separated by column chromatography after 2 days. On treatment of the cells with maltose, the yield of the diester (R_f, 0.60) was greater (345 mg) than for the monoester (R_f, 0.21; 25 mg) after 1 day. After 2 days almost all of the monoester had disappeared. With maltotriose, 13 mg of a monoester (R_f, 0.10) and 25 mg of a triester (R_f, 0.58) were isolated after 27 h.

Characterization of the glycolipids. The glycolipids were characterized by ^{1}H and ^{13}C NMR spectroscopy, mass spectrometry, and elementary analysis. The ^{1}H NMR spectra (Tables 1 to 3) allowed the structure of the lipid moiety to be determined and showed the presence of the sugar. In all cases comparison with literature data (3, 14) indicated that the lipid moiety was the ester of high-molecular-weight β-hydroxy fatty acids (the corynomycolic acids), some of which contained a double bond in the alkyl chain. The relative proportions of lipid and sugar were determined by integration of the methyl-group signal with respect to specific sugar signals. In those cases where ^{13}C NMR spectra (Table 4) were recorded, the position of attachment of the lipid to the sugar was directly identified. Integration of the

TABLE 4. ^{13}C chemical shifts of the glycolipids



Carbon	Glucose 6′-monoester (CD_3OD)	Mannose 6′-monoester (CD_3OD)	Maltose 6′,6″-diester (CD_3OD/$CDCl_3$[1:1])[a]	Maltotriose monoester[b] (CD_3OD)	Maltotriose triester (CD_3OD)[c]
Aglycon					
1	176.20	176.53	157.87/175.77	177.68	176.21
2	54.15	54.17	53.45/53.38	54.07	53.60/53.62
3	73.53	73.53	73.22/73.18	—[d]	—[d]
4	35.64	35.67	35.40/35.31	35.65	35.45/35.24
5	26.50	26.46	25.94/25.89	26.47	25.99/25.83
6	29.73[e]	29.79[e]	29.56[e]/29.52[e]	29.79[e]	29.59[e]
a/a′	14.41	14.42	14.25	14.30	14.27
b/b′	23.70	23.70	23.11	23.60	23.20
c/c′	33.05	33.05	32.40	32.98	32.50
d/d′	130.87	130.87	130.36	130.90	130.45
e/e′	28.54[e]/28.10[e]	28.54[e]/28.10[e]	27.94[e]/27.64[e]	28.47[e]/28.08[e]	28.02[e]/27.73[e]
$(CH_2)_n$	30.73, 30.45, 30.26	30.72, 30.45, 30.25	30.01, 29.89, 29.80	30.64	30.24, 29.98, 29.90
Sugar					
1′α	94.03	95.95		93.89	
1′β	98.30	95.91	97.62	98.28	
2′α	77.99[f]	72.83[h]			
2′β	76.28[f]	73.28			
3′α	75.47[f]	72.25[h]			
3′β	74.80[f]	75.92[i]			
4′α	73.90[f]	69.09			
4′β	72.03[f]	68.91	80.18		
5′α	71.93[f]	71.73[h]			
5′β	70.75[f]	75.51[i]			
6′α	64.87[g]	65.20[j]			
6′β	64.62[g]	65.20[j]			
1″			101.66	102.63[f]	
1‴				103.12[f]	
6′			64.43[k]	64.82[m]	64.46
6″			64.32[k]	62.79[m]	
6‴				62.32[m]	
Broad					

[a] Only signals of the predominant β anomer were observed. In addition to the sugar proton signals shown, other signals were observed at 77.83, 74.25, 72.51, 71.34, 70.99, 69.79, and 68.49.

[b] In addition to the sugar proton signals shown, other signals were observed between 82.3 and 71.8.

[c] In addition to the sugar proton signals shown, other signals were observed between 102.6 and 68.9.

[d] —, Signal hidden in with sugar signal.

[e–m] Signal assignments interchangeable.

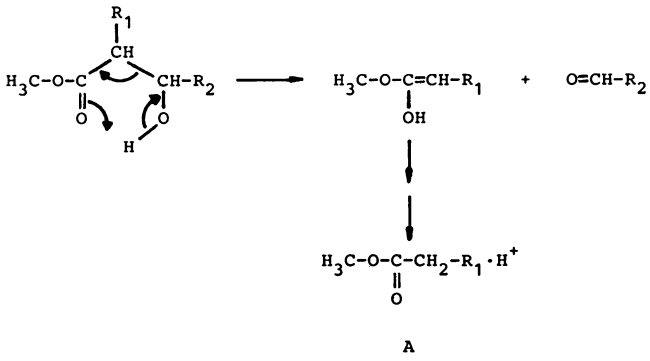
TABLE 5. Percentage of corynomycolic acids estimated by the heights of the $(M-17)^+$ peaks

Corynomycolic acid, RCO_2H	Mol wt of corynomycolic acid methylester, RCO_2CH_3	Glucose monoester (%)	Mannose monoester (%)	Maltose diester (%)	Maltotriose triester (%)
$C_{32}H_{64}O_3$	510	4	1.5	5	2.5
$C_{33}H_{64}O_3$	522[a]	2	1	2	2
$C_{33}H_{66}O_3$	524	5	2.5	7	4.5
$C_{34}H_{66}O_3$	536[a]	4	1.5	3.5	3
$C_{34}H_{68}O_3$	538	13	5.5	13	7.5
$C_{35}H_{68}O_3$	550[a]	4	2	4	4
$C_{35}H_{70}O_3$	552	6	4.5	9.5	7.5
$C_{36}H_{70}O_3$	564[a]	8	4	6	5
$C_{36}H_{72}O_3$	566	13	9.5	14	12.5
$C_{37}H_{72}O_3$	578[a]	4.5	3.5	4.5	5
$C_{37}H_{74}O_3$	580	4	5	5	7
$C_{38}H_{74}O_3$	592[a]	8	6	5.5	7
$C_{38}H_{76}O_3$	594	6	8.5	6	8.5
$C_{39}H_{76}O_3$	606[a]	3	4	3	5
$C_{39}H_{78}O_3$	608	1.5	2.5	1.5	2.5
$C_{40}H_{78}O_3$	620[a]	5.5	7	4	6
$C_{40}H_{80}O_3$	622		3	2	2.5
$C_{41}H_{80}O_3$	634[a]	2	4.5	2	3
$C_{41}H_{82}O_3$	636		1		
$C_{42}H_{82}O_3$	648[a]	4	7.5	2.5	5
$C_{42}H_{84}O_3$	650		1		
$C_{43}H_{84}O_3$	662[a]	1	4		
$C_{42}H_{86}O_3$	664				
$C_{44}H_{86}O_3$	676[a]	1.5	5.5		
$C_{44}H_{88}O_3$	678				
$C_{45}H_{88}O_3$	690[a]		2.5		
$C_{45}H_{90}O_3$	692				
$C_{46}H_{90}O_3$	704[a]		2.5		

[a] Double bond containing corynomycolic acids.

^1H spectra in all cases indicated that the lipid moiety was heterogeneous with the proportions of the double bond in the side chain varying from compound to compound. The composition and character of these lipid moieties were estimated by CI mass spectrometry.

For the monosaccharide corynomycolates, the high-field ^1H NMR spectra, together with homonuclear decoupling experiments, allowed an almost complete assignment of the spectra (Table 1). The presence of both anomers, in proportions found for the free sugars, indicated the presence of a free hydroxyl group at C-1, whereas the low-field shift of the two protons of C-6 ($6'_A$ and $6'_B$) relative to the free sugars characterized the attachment of the lipid moiety at this position. For the mannose derivative, several of the ring proton signals of the minor β anomer could not be observed as these were hidden under those of the α anomer. The signals in the ^{13}C NMR spectra were assigned from their multiplicity in the single-frequency off-resonance proton-decoupled spectra and from comparisons with literature data (1, 9). The ^{13}C spectra of the free sugars in CD_3OD had the signals of C-6 at ca. 62 ppm. The shift in the monoesters for C-6 of ca. 65 ppm is regarded as direct evidence for the attachment of the lipid moiety at this position.



A

FIG. 3. Formation of the ion A during isobutane CI mass spectrometry.

The shift of the olefinic carbons and the adjacent methylene carbons of the lipid is characterisitic of a *cis*-alkene. The symmetrical environment of the alkene, indicated by the observation of only one signal at 130.87 ppm in the ^{13}C spectra and one multiplet at 5.39 ppm in the ^1H spectra, suggests that the double bond is positioned centrally in the alkyl chain.

For the disaccharide corynomycolates, the ^1H (Table 2) spectra allowed characterization of the aglycon. The number and position of the lipid substituents were deduced by partial analysis of the sugar signals of the cellobiose monoester and diester and of the maltose monoester. For the cellobiose monoester the signals of H-2″, H-3″, and H-4″ of the second sugar ring could be tentatively assigned as these were furthest away from the anomeric center and hence showed the fewest chemical-shift differences for the two anomeric forms. Homonuclear double-irradiation experiments indicated that H-4″ was coupled to a proton (H-5″) that was also coupled to the CH_2 group carrying the aglycon. Thus, the lipid moiety was attached to C-6″ of the second sugar ring.

The attachment of both lipid moieties at the two methylene carbons, C-6′ and C-6″, in cellobiose diester was indicated by the low-field shift of the respective protons. Similarly, the lipid moiety of maltose monoester was attached to one of the methylene groups although distinction between the two eventualities was not possible.

TABLE 6. Comparison of the calculated and found data for the elemental composition of four glycolipids

Glycolipid	Elemental composition (%)					
	Calculated			Found		
	C	H	O	C	H	O
Glucose monoester $C_{43}H_{82}O_8$[a]	71.07	11.29	17.63	71.72	11.02	17.47
Mannose monoester $C_{44}H_{86}O_8$[a]	71.16	11.59	17.25	70.97	11.26	17.90
Maltose diester $C_{84}H_{158}O_{15}$[a]	71.69	11.23	17.07	72.03	10.98	16.96
Maltotriose triester $C_{129}H_{248}O_{22}$[a]	72.06	11.54	16.39	71.03	11.58	16.76

[a] Average value, derived from percentage of corynomycolic acids (see Table 5).

As the ^1H spectrum of maltose diester was complex, the position of attachment of the lipid moieties at C-6′ and C-6″ was calculated from the observation of two methylene carbon signals at ca. 64 ppm in the ^{13}C NMR spectrum.

The two isolatable esters of the trisaccharide maltotriose were identified as the mono- and triester from integration of the ^1H NMR spectra (Table 3) and from the relative intensities of the sugar and aglycon carbon signals in the ^{13}C NMR spectra. The observation of ^{13}C signals at 62.3, 62.8, and 64.8 ppm in the monoester indicated that the lipid was present on one of the C-6 carbons, whereas the observation of only one broad signal at 64.5 ppm for the triester indicated that the lipid was present on all C-6 carbons.

The molecular weights and approximate percentages of the individual components of the corynomycolic acids from glucose and mannose monoester, maltose diester, and maltotriose triester were determined by isobutane CI mass spectrometry with the methylester derivatives. The most intense peaks in the molecular-ion region of the CI mass spectra were the $(M-17)^+$ peaks. The peak heights of these ions were used for the calculation of their relative proportions shown in Table 5. The intensities of the protonated ester ions were ca. 10% of $(M-17)^+$. The composition of the corynomycolic acids is represented by two homologous series, one ranging from $C_{32}H_{64}O_3$ to $C_{45}H_{90}O_3$ and the second, containing a double bond, ranging from $C_{33}H_{64}O_3$ to $C_{46}H_{90}O_3$. Components with even-numbered carbons predominated (60%).

Three further, more intense peaks in the CI mass spectra were observed. These ions at *m/e* 215, 243, and 271 with intensity proportions of 7:10:7 can be explained by analogy to the results of Wong et al. (14) by a McLafferty rearrangement (Fig. 3) and corresponded to the protonated ion A. Therefore, R_1 seems to be a mixture of alkyl chains $C_{10}H_{21}$, $C_{12}H_{25}$, and $C_{14}H_{29}$. Other homologous ions of this rearrangement could not be observed. The composition of R_1 indicated that the double bond is located in the same chain as the hydroxyl group. R_2 consisted of homologous series ranging from $C_{15}H_{31}$ to $C_{32}H_{65}$ and from $C_{16}H_{31}$ to $C_{32}H_{63}$ for the series with the double bond. Using the data from Table 5, we calculated the average values of the molecular weights of the whole glycolipids, including both the corynomycolic and carbohydrate moieties. Taking this into consideration, the values of elemental analysis were in good agreement with the theoretical ones (Table 6).

Surfactant properties of the glycolipids. The isolated nonionic surfactants of *Arthrobacter* sp. showed different surface- and interfacial-active properties after emulsification in synthetic deposit water, the composition of which is similar to that of the water of north German wells. The results are comparable with that of the nonionic trehalose-corynomycolates produced by *Rhodococcus erythropolis* (7) and fructose or saccharose corynomycolates from *A. paraffineus* (8). The combination of two carbohydrate moieties and one fatty acid always led to stronger reduction in surface and interfacial tension than did the combination of two sugars with two fatty acids or of one sugar with one fatty acid. This fact is illustrated by Fig. 4 to 6, where the surface and interfacial tension curves of the monosaccharide lipids, disaccharide lipids, and trisaccharide lipids are presented. The mannose and glucose lipids reduced the surface tension of synthetic deposit water from 72 to 40 mN/m and the interfacial tension against *n*-hexadecane from 41 to 19 and 9 mN/m, respectively (Fig. 4). With the maltose monoester, the corresponding values decreased to 33 and 1 mN/m, respectively, and with the diester only to 46 and 13 mN/m, respectively (Fig. 5). The monoesters of maltose and cellobiose possessed the best surfactant behavior (minimum surface and interfacial tensions, 35 and 1 mN/m, respectively) of the glycolipids studied. Also the values for critical micelle concentrations, ranging from 1 to 50 mg/liter, confirmed that the combination of two sugars with one corynomycolic acid was superior to

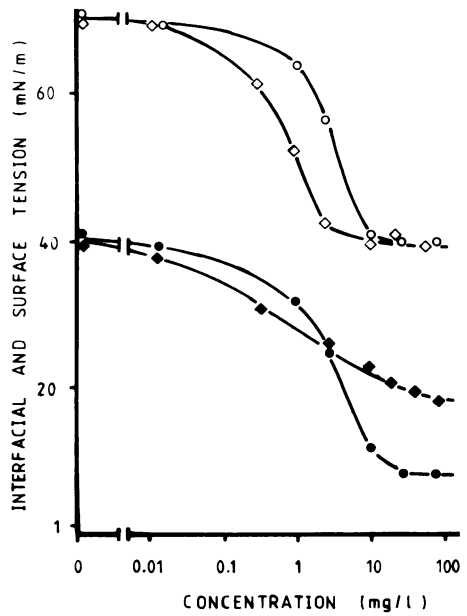


FIG. 4. Surface (open symbols) and corresponding interfacial (closed symbols) tensions against *n*-hexadecane of glucose monoester (○, ●) and mannose monoester (◇, ◆) in synthetic deposit water (composition [grams per liter]: NaCl, 100; $CaCl_2$, 28; $MgCl_2$, 10) at 40°C.

TABLE 7. Surfactant properties of the glycolipids from *Arthrobacter* sp.

Glycolipid	Minimum surface tension (mN/m)	Critical micelle concn (mg/liter)	Minimum interfacial tension (mN/m)	Critical micelle concn (mg/liter)
Mannose monoester	40	5	19	50
Glucose monoester	40	10	9	20
Maltose monoester	33	1	1	20
Maltose diester	46	10	13	10
Cellobiose monoester	35	3	1	4
Maltotriose triester	44	20	19	10

the other combinations including the trisaccharide triester. The surfactant properties of the glycolipids are summarized in Table 7.

DISCUSSION

Most biosurfactants are synthesized by bacteria or yeasts during growth on lipophilic substrates such as hydrocarbons. Trehalose (9, 12) and rhamnose lipids (4, 13) have been reported in the literature. The *Arthrobacter* sp. studied in this work also produces surface- and interfacial-active compounds when grown on a mixture of C_{14}-C_{15} *n*-alkanes. A new ionic α,α-trehalose-tetraester was the main component (10; E. Ristau, S. Lang, Z. Y. Li, and F. Wagner, 29th IUPAC Congress, Cologne, Federal Republic of Germany, 1983, p. 436). Suzuki and co-workers (6, 11) also found glycolipids when arthrobacteria, corynebacteria, or nocardiae were cultivated in excess sucrose or fructose. In all of these cases, the carbon source determined the type of sugar moiety, whereas the fatty acid remained constant.

As expected, our study with mannose and glucose led to the corresponding glycolipids esterified at the C-6 position with an α-branched β-hydroxy fatty acid. Somewhat surprisingly, cellobiose corynomycolates were also synthesized,

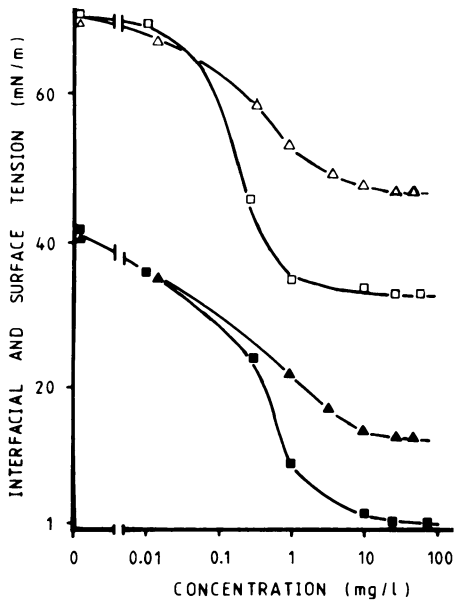


FIG. 5. Surface (open symbols) and corresponding interfacial (closed symbols) tensions against *n*-hexadecane of maltose monoester (□, ■) and maltose diester (△, ▲) in synthetic deposit water at 40°C.

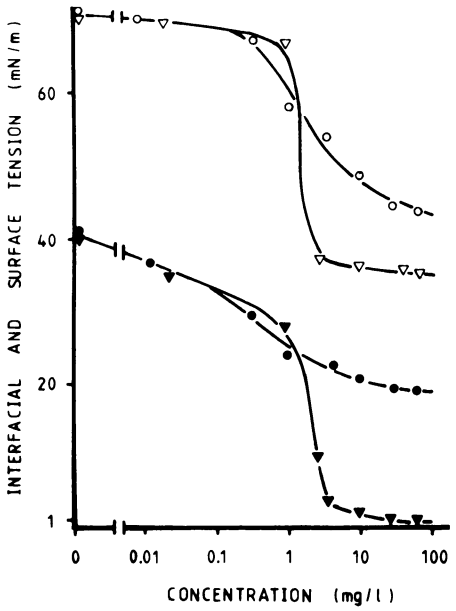


FIG. 6. Surface (open symbols) and corresponding interfacial (closed symbols) tensions against *n*-hexadecane of cellobiose monoester (▽, ▼) and maltotriose triester (○, ●) in synthetic deposit water at 40°C.

although initial experiments with growing cultures had been negative. Similarly, for maltose and maltotriose the yields of glycolipids were very low in studies with growing cultures. After separation of the cell growth and production phases, the yields rose when resting cells in a phosphate buffer were used. The energy gain by degradation of the carbohydrates was high enough to permit synthesis of the lipids over several days.

As far as biosynthesis is concerned, the hydrophilic moiety is determined by the nature of the carbon source, whereas the lipophilic moiety is independent of this and consists of α-branched β-hydroxy fatty acids.

As well as in their actual application as wetting, foaming, or emulsifying agents, surfactants seem to be of interest in enhanced oil recovery. To simulate the high salinity in north German wells, we have emulsified the glycolipids with synthetic deposit water. The experimental data show that the strongest reductions in surface and interfacial tension down to around 35 and 1 mN/m, respectively, were obtained with the more hydrophilic substances such as cellobiose and maltose monocorynomycolates (Fig. 4 to 6).

These low values, which were unchanged up to 90°C, and critical micelle concentrations below 50 mg/liter in high-salinity solution are good presuppositions for the use of these glycolipids in tertiary oil recovery.

LITERATURE CITED

1. **Bremser, W., L. Ernst, B. Franke, R. Gerhards, and A. Hardt.** 1981. Carbon-13 NMR spectral data, 3rd ed. Verlag Chemie GmbH, Weinheim, Federal Republic of Germany.
2. **Brennan, P. J., D. P. Lehane, and D. W. Thomas.** 1970. Acylglucoses of corynebacteria and mycobacteria. Eur. J. Biochem. **13**:117–123.
3. **Danielson, S. J., and G. R. Gray.** 1982. Structures of the two homologous series of dialkene mycolic acids from *Mycobacterium smegmatis*. J. Biol. Chem. **257**:12196–12203.
4. **Hisatsuka, K., T. Nakahara, N. Sano, and K. Yamada.** 1971. Formation of rhamnolipid by *Pseudomonas aeruginosa* and its function in hydrocarbon fermentation. Agric. Biol. Chem.

35:686–692.

5. **Hodge, J. E., and B. T. Hofreiter.** 1962. Determination of reducing sugars and carbohydrates. Methods Carbohydr. Chem. **1**:380–394.
6. **Itoh, S., and T. Suzuki.** 1974. Fructose-lipids of *Arthrobacter*, *Corynobacteria*, *Nocardia* and *Mycobacteria* grown on fructose. Agric. Biol. Chem. **38**:1443–1449.
7. **Kretschmer, A., H. Bock, and F. Wagner.** 1982. Chemical and physical characterization of interfacial-active lipids from *Rhodococcus erythropolis* grown on *n*-alkanes. Appl. Environ. Microbiol. **44**:864–870.
8. **Lang, S., A. Gilbon, C. Syldatk, and F. Wagner.** 1984. Comparison of interfacial active properties of glycolipids from microorganisms, p. 1365–1376. *In* K. L. Mittal and B. Lindman (ed.), Surfactants in solution, vol. 2. Plenum Publishing Corp., New York.
9. **Rapp, P., H. Bock, V. Wray, and F. Wagner.** 1979. Formation, isolation and characterization of trehalose dimycolates from *Rhodococcus erythropolis* grown on *n*-alkanes. J. Gen. Microbiol. **115**:491–503.
10. **Ristau, E., and F. Wagner.** 1983. Formation of novel anionic trehalosetetraester from *Rhodococcus erythropolis* under growth limiting conditions. Biotechnol. Lett. **5**:95–100.
11. **Suzuki, T., H. Tanaka, and S. Itoh.** 1974. Sucrose lipids of *Arthrobacteria*, *Corynebacteria* and *Nocardia* grown on sucrose. Agric. Biol. Chem. **38**:557–563.
12. **Suzuki, T., K. Tanaka, I. Matsubara, and S. Kinoshita.** 1969. Trehalose lipid and α-branched-β-hydroxy fatty acid formed by bacteria grown on *n*-alkanes. Agric. Biol. Chem. **33**:1619–1627.
13. **Wa ,ner, F., U. Behrendt, H. Bock, A. Kretschmer, S. Lang, and C. Syldatk.** 1983. Production and chemical characterization of surfactants from *Rhodococcus erythropolis* and *Pseudomonas* sp. MUB grown on hydrocarbons, p. 55–60. *In* J. E. Zajic, D. G. Cooper, T. R. Jack, and N. Kosaric (ed.), Microbial enhanced oil recovery. Pennwell Publishing Co., Tulsa, Okla.
14. **Wong, M. Y. H., P. A. Steck, and G. R. Gray.** 1979. The major mycolic acids of *Mycobacterium smegmatis*. J. Biol. Chem. **254**:5734–5740.