Marine Biosurfactants, II. Production and Characterization of an Anionic Trehalose Tetraester from the Marine Bacterium *Arthrobacter* sp. EK 1

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Within a screening for biosurfactants we could isolate various *n*-alkanes utilizing marine bacteria which were capable of synthesizing glycolipids. One strain was identified as *Arthrobacter* sp. EK 1 which produced trehalose lipids. After purification by column and thick layer chromatography the main fraction, an anionic 2,3,4,2′-trehalose tetraester, was obtained. The chain lengths of fatty acids ranged from 8 up to 14, furthermore succinate could be detected. Since the place of substitution of succinate has so far not been cited in literature, a definitive structural elucidation was carried out chemically by hydroboration and by ^{1}H, $2\,D^{1}H$, ^{13}C and $^{13}C-^{1}H$ correlation NMR measurements. All investigations confirmed the exact position of succinate at C 2 atom of trehalose. After improvement of growth conditions the production of the trehalose tetraester increased up to 4.8 g/l during a fermentation in a 20 l bioreactor under nitrogen limitation.

Introduction

In recent studies concerning oil pollution in the marine environment it has been shown that microbial biosurfactants from soil or fresh water bacteria were well suited for the elimination of polyaromatic hydrocarbons [1, 2].

In general the advantages of these interfacially active substances over synthetic detergents are their biodegrability and non-toxicity.

Concerning our own investigations we were interested in isolating biosurfactants from marine microorganisms because of an expected improved compatibility.

After a screening among *n*-alkane utilizing mixed populations the marine bacterium *Arthrobacter* sp. EK 1 was isolated. Introducing shake flask experiments and characterization methods indicated the formation of an anionic trehalose tetraester known from the fresh water bacterium *Rhodococcus erythropolis* [3].

As the positions of sugar acylations remained unsolved now we intensified structure elucidation studies. The results of these efforts as well as a bioreactor production of this glycolipid are to be presented in this paper.

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Materials and Methods

Microorganism

Arthrobacter sp. EK 1 was isolated from a sea water sample by enrichment culture techniques. Morphological and physiological characteristics indicated that the organism belongs to the genus of *Arthrobacter*.

Cultivation conditions

Arthrobacter sp. EK 1 was cultivated at first in a sea-water medium (sea-water/distilled water) (3/1), then in a mineral salt medium containing (g/l):

Yeast extract	1.0 (g/l)
Citrate	1.86 (g/l)
$NaNO_3$	3.5 (g/l)
KH_2PO_4	0.75 (g/l)
$Na_2HPO_4*2H_2O$	0.75 (g/l)
$CaCl_2*2H_2O$	0.07 (g/l)
$FeCl_3*6H_2O$	0.03 (g/l)
$MgSO_4*7H_2O$	1.65 (g/l)
$FeSO_4*7H_2O$	0.3 (g/l)
H_3PO_4	0.5 (g/l)
$C_{14,15}$-*n*-alkane	20.0 (g/l)

Batch cultivations were carried out in a 20 l bioreactor (Giovanola Frères, Switzerland) equipped with an intensor system. Physiological activity was monitored by the use of a pH electrode, a pO_2 electrode and oxygen and carbon dioxide gas ana-

lyzers. The pH value was titrated to pH 7 by addition of either 10% H_3PO_4 or 10% NaOH solution. The course of the cultivation was checked by measuring dry mass, trehalose lipid, alkane and nitrate ion concentration. The nitrate ion concentration in the supernatant was determined enzymatically (UV test, Boehringer) [4], the alkane concentration in the culture broth was monitored by gas chromatography after extraction. Biomass was determined by the method of Rapp *et al.* [5]. The presence of succinate in the water phase after saponification was detected with succinyl-CoA synthetase from Sigma GmbH, München.

Isolation and preparation of the glycolipid

After a cultivation period of 72 h cells were harvested by centrifugation. The culture broth was acidified with 10% H_2SO_4 to pH 2.5 and extracted twice with ethyl acetate. After solvent removal by rotary evaporation the residue was separated by medium pressure chromatography on silica gel 60 (280 mesh, Merck AG, Darmstadt, F.R.G.) and eluated with chloroform/methanol solvent mixtures varying in ratios from 98:2 to 60:40 (vol./vol.). The main fraction containing the trehalose tetraester was purified by preparative thick layer chromatography on silica gel 60 (No. 5717, Merck AG, Darmstadt, F.R.G.) in a solvent system containing chloroform, methanol and acetic acid (65/15/2) (vol./vol./vol.).

Characterization of the anionic THL-tetraester

This glycolipid was identified by physical and chemical methods. Analytical thin layer chromatography was carried out on silica gel plates (No. 5554, Merck AG, Darmstadt, F.R.G.) using the following solvent systems:

(A) $CHCl_3$/MeOH/CH_3COOH (65/12/2) or
(B) $CHCl_3$/MeOH/H_2O (65/15/2)

In order to determine the functional groups of the glycolipids various spray reagents were used after vertical development. Sugar was determined by 4-methoxy-benzaldehyde. Lipids were identified by 2,7-dichlorofluorescine (No. 9677, Merck) and for the recognition of free acid groups bromocresol green reagent was used (No. 1998, Merck) [6].

Chemical methods: Acid methanolysis of the TL-4 intended for obtaining the fatty acid methyl esters was performed by refluxing with methanolic hydrochloric acid solution for 2.5 h. After extracting the methyl esters twice with *n*-hexane, GC/MS measurements were performed. Alkaline hydrolysis resulted from refluxing with 0.5 m ethanolic sodium hydroxide solution at 60 °C; the sugar moiety was identified by HPLC. In order to determine succinic acid substitution position, hydroboration [7] coupled with acid hydrolysis was performed.

The IR spectrum of trehalose tetraester was recorded on a Perkin-Elmer 1600 FTIR spectrometer.

NMR: 1 D (^1H and ^{13}C) and 2 D (COSY, ^1H-detected one-bond [8] and multiple-bond ^{13}C multiple-quantum coherence spectra [9], HMQC and HMBC respectively) spectra were recorded at 300 °K on a Bruker AM 600 NMR spectrometer locked to the major deuterium resonance of the mixed solvent, $CDCl_3/CD_3OD$ (7:3). The value of delay to optimize one-bond correlations in the HMQC spectrum and suppress them in the HMBC spectrum was 3.45 ms and the evolution delay for long range couplings in the latter was set to 70 ms. All spectra were recorded using the standard Bruker software package and data manipulation of the 2 D spectra were performed on a Bruker Aspect X 32 Data station equipped with the Aurelia software [10]. All chemical shifts are given in ppm relative to TMS and couplings in Hz.

GC/MS measurements of fatty acid methyl esters were carried out with a Finnigan MAT 4514 spectrometer. HPLC measurements of the sugar were carried out with an Aminex HPX-87 C column.

Results and Discussion

After careful screening of biosurfactants by the following methods of

- hemolysis of red blood cells [11]
- direct TLC of bacterial cell mass [12] and
- enrichment culture technique coupled with ethyl acetate extraction

we were able to isolate the marine bacterium *Arthrobacter* sp. EK 1, which produces trehalose lipids (Fig. 1) during its growth on hydrocarbons.

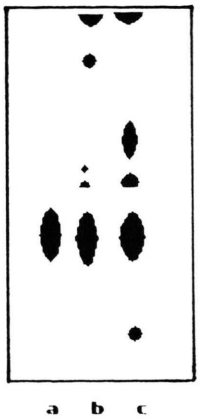

Fig. 1. TLC analysis of ethyl acetate extracts from the whole culture broth. a) Anionic trehalose lipid from *Rhodococcus erythropolis;* b) single strain *Arthrobacter* sp. EK 1; c) marine mixed culture.

During cultivation in a 20 l bioreactor the trehalose tetraester, which has already been described in literature [3, 13], was mainly produced as nitrogen became limiting after 30 h. It was possible to isolate 4.8 g/l of this glycolipid after a cultivation period of 70 h. At this point in time bio dry mass amounted to 9.2 g/l resulting in a specific production of 0.52 g trehalose tetraester per g biomass. Figure 2 shows the growth parameters and the typical course of glycolipid production.

The trehalose tetraester, purified by repeated column and thick layer chromatography was obtained as a light yellow wax that was homogeneous by TLC.

Structural elucidation

In order to identify the sugar moiety the glycolipid was saponified and the aqueous fraction analyzed with regard to its sugar content. No reducing sugar was detected upon spraying with aniline phthalate reagent and after comparing the R_f value of 0.1 in butanol/acetic acid/water (50/25/25) with literature data indicated trehalose. This was confirmed by HPLC; D(+) trehalose (Sigma, Boehringer) served as a reference. Retention times obtained for purchased trehalose (5.338) and the isolated sugar (5.331) showed a great degree of correspondence.

The presence of a free carboxyl group in the intact glycolipid was detected by a strong band at 1580 cm^{-1} in the IR spectrum and also by an acidic reaction with bromocresol green. Further an enzymatic investigation of the water phase with succinyl-CoA synthetase after saponification allowed unambiguous identification of succinate.

Since the position of the succinate has not as yet been determined [3, 13], its structural elucidation was undertaken. The free acid group, which distinguishes succinate from the other fatty acids present in the molecule, was modified chemically in order to elucidate the substituent position. The free acid group was reduced to the alcohol by hydroboration. After ring formation the substituent was cleaved from the molecule in the form of butyrolactone by acidic hydrolysis with *p*-toluene sulphonic acid. Fig. 3 shows the possible reaction mechanism. ^1H, 1D and 2D COSY NMR spectra of the modified glycolipid (R_f 0.58, of native glycolipid 0.33 in the mobile solvent B) indicated the

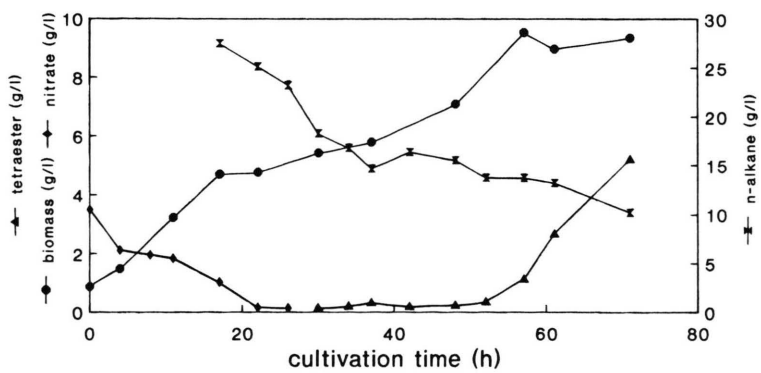

Fig. 2. Cultivation of the marine bacterium *Arthrobacter* sp. EK 1 in a 20 l bioreactor. Conditions: pH 7.0, T = 21 °C, $C_{14,15}$-*n*-alkane, N/P/Fe/YE supplementation.

R = 3,4,2'-triacyl trehalose

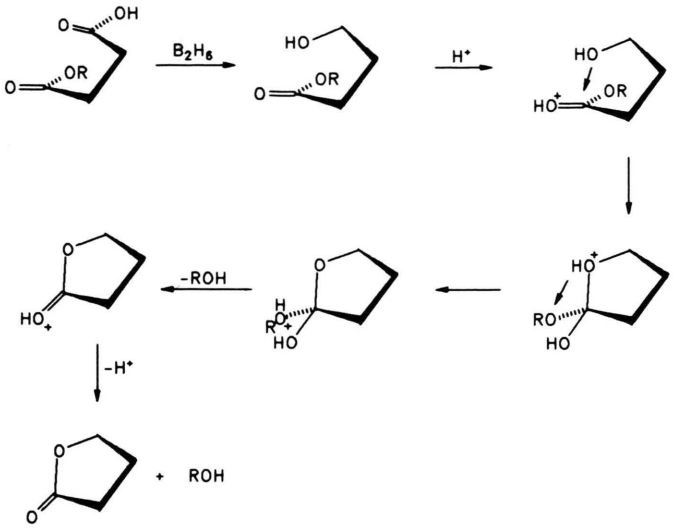

Fig. 3. Reaction mechanism of hydroboration following by acidic hydrolysis.

absence of the succinate moiety and the high field shift of H-2 compared to the native compound suggested the succinate moiety in the latter was attached to C-2. A definitive proof of the structure of the native trehalose lipid was afforded by a high field NMR study. The structure of the trehalose tetraester (Fig. 4) was deduced from the NMR data given on Tables I–III. The magnitude of the coupling constants in the 1 D ^{1}H spectrum, Table I, indicated the presence of two α-D-glucose moieties

Table I. ^{1}H data of tetraacyl-2,3,4,2'α,α-trehalose in $CHCl_3/CD_3OD$ (7:3).

Sugar							
	Shift	Coupling		Shift		Coupling*	
H-1	5.414	(1–2)	3.7	H-1'	5.274	(1'–2')	3.7
H-2	4.916	(2–3)	9.9	H-2'	4.746	(2'–3')	9.8
H-3	5.539	(3–4)	9.8	H-3'	3.991	(3'–4')	9.5
H-4	5.079	(4–5)	9.9	H-4'	3.386	(4'–5')	9.5
H-5	3.762	(5–6A)	2.4	H-5'	3.71–3.66		
H-6A	3.574	(5–6B)	4.9	H-6'A	3.808		
H-6B	3.505	(6A–6B)	12.5	H-6'B	3.71–3.66		

Acyl group**			Shifts		
	b	c	d	e, f, g	h
R2	2.65–2.48	2.65–2.48			
R3/R4	2.31–2.22	1.563	1.29	1.281	0.882
R2'A	2.450	1.653	1.35	1.281	0.882

* H-5'/H-6'AB is a complex spin system in which the couplings J(5–6'), J(5'–6'B) and J(6'A–6'B) were not measurable.

** The measurable couplings in the R 2' substituent are as follows: J(bA–c)=J(bB–c) 7.8, J(bA–bB) 15.8, J(c–d) 7.5.

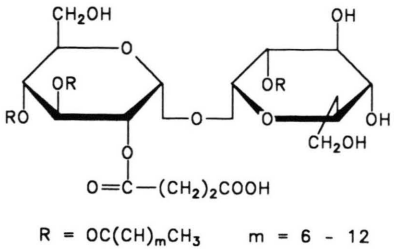

Fig. 4. Structure of the anionic trehalose tetraester produced by *Arthrobacter* sp. EK 1 (GC, MS, NMR measurements, chemical derivatization).

Table II. ^{13}C chemical shifts for tetraacyl-2,3,4,2′-α,α-trehalose in $CHCl_3/CD_3OD$ (7:3).

Sugar				
C-1	90.21		C-1′	90.80
C-2	70.65		C-2′	72.31
C-3	69.51		C-3′	70.53
C-4	68.17		C-4′	70.53
C-5	70.07		C-5′	72.36
C-6	60.06		C-6′	61.09
Acyl groups				
	R 2	R 3	R 4	R 2′
a	172.28	172.85	172.53	173.63
b	29.43*	33.76	33.76	33.76
c	29.21*	24.48/24.46		24.56
d	176.49	29.08/29.01/28.93/28.78/28.60		
e				
f		31.55/31.51/31.31		
g		22.28/22.23		
h		13.54		

* The assignments are interchangeable.

Table III. Long range $^{13}C-^{1}H$ correlations of acyl group carbonyl carbons.

Acyl group	Carbon	Hydrogen
R 2	C-a	H-2, R 2-b, R 2-c
	C-d	R 2-b, R 2-c
R 3	C-a	H-3, R 3-b, R 3-c
R 4	C-a	H-4, R 4-b, R 4-c
R 2′	C-a	H-2′, R-2′-b_{AB}, R 2′-c

which were present as trehalose unit from the characteristic ^{1}H and ^{13}C chemical shifts (and see below). The presence and position of four acyl substituents were evident from the cross peaks in the 2D ^{1}H COSY spectrum where low field shifts of glucose ring protons, in the region 4.5 to 5.7 ppm, adjacent to the substituents were observed. The latter spectrum also showed that the substituents consisted of one succinic acid and three straight-chain fatty acid derivatives. The ^{13}C signals were assigned from a 2D ^{1}H-detected one-bond $^{13}C-^{1}H$ correlation (Table II). A 2D ^{1}H-detected long range $^{13}C-^{1}H$ correlation, where correlations over two and three bonds were observed, gave the exact position of the substituents by the observation of cross peaks from the trehalose ring protons and from the methylene protons to the various carbonyl carbons of the substituents (Table III). The trehalose ring junction was similarly confirmed in the latter 2D spectrum from the observation of cross peaks between H-1 and C-1′, and between H-1′ and C-1.

The fatty acids were identified by coupled GC/MS measurements after being transformed to methyl esters. The results are shown in Table IV.

Acknowledgements

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Table IV. Quantification of the fatty acid methyl ester mixture after acidic methanolysis of the trehalose tetraester from *Arthrobacter* sp. EK 1.

Octanoic acid	37.82%
Nonanoic acid	5.73%
Decanoic acid	53.29%
Undecanoic acid, Lauric acid, Myristic acid	3.0%

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