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Structure and bioconversion of trehalose lipids

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ABSTRACT

Cultivation of Coryneform bacteria on *n*-alkanes produced trehalose lipids which were isolated and identified by ^{1}H-n.m.r. spectroscopy and f.a.b.-mass spectrometry. Fatty acids were found as substituents at positions 2, 3, 4, and 2′ of the trehalose. On incubation up to 80 h, the trehalose lipids are associated mainly with the cell wall and only 10% are extracellular. After 80 h, the trehalose lipids are mainly extracellular.

INTRODUCTION

Many micro-organisms produce surface-active trehalose lipids when grown with a hydrocarbon as the carbon source, including trehalose mono-, di-, and tetra-esters[1–3] where the lipid moieties are long-chain α-branched-β-hydroxy fatty acids (corynomycolic acids)[4–6]. Interest in trehalose lipids as general surfactants can be traced to the discovery that trehalose dimycolates were present in the emulsion layer of culture broths of *Arthrobacter paraffineus* when the cells were grown on hydrophobic substrates[7].

The trehalose esters produced by *Rhodococcus erytropolis* have been studied extensively[8] with special reference to their interfacial activities and possible application in enhancing the recovery of oil. Under nitrogen limitation, *R. erytropolis* produced large amounts of non-ionic trehalose 2,3,4,2′-tetraesters[9]. Emulsions of trehalose lipids have been used to enhance the recovery of oil from sandstone oil deposits[9,10] and to diminish the effects of oil pollution[11].

We now report on the effect of different hydrophobic carbon sources on the structure of the trehalose lipids biosynthesised by a bacterium of the Corynebacteria group.

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EXPERIMENTAL

A strain from the Coryneform bacterium 51T7, isolated by enrichment culture techniques from a sample of earth, was used. The carbon sources were pure C_{10} and C_{13} *n*-alkanes, and mixtures of *n*-alkanes, namely, C_{14}, C_{15}, C_{16} (35, 56, 9%) and C_{10}, C_{11}, C_{12}, C_{13} (10, 32, 31, 27%), supplied by Petroquímica Española, S.A. (Cadiz, Spain).

Media and growth conditions. — The micro-organism was maintained on TSA (Trypticase Soy Agar) agar slants and transferred at monthly intervals. A sterilised mineral salts medium[12] (K_2HPO_4, 0.1%; KH_2PO_4, 0.056%; KCl, 0.01%; $MgSO_4 \cdot H_2O$, 0.05%; $CaCl_2$, 0.001%; $FeSO_4 \cdot 7H_2O$, 0.001%; yeast extract, 0.01%; and 0.05 mL of the following trace elements; B, 0.026%; Cu, 0.05%; Fe, 0.1%; Mn, 0.05%; Mo, 0.006%; and Zn, 0.07%; pH 6.8) was used. The inorganic nitrogen source was $NaNO_3$ (0.40%). The carbon sources were the above *n*-alkanes, D-glucose, D-fructose, D-mannose, and maltose, at 20g/L. During cultivation, the pH was not adjusted. Each culture was carried out in a 500-mL baffled Erlenmeyer flask with 100 mL of basal medium at 25° for 72 h with agitation at 200 r.p.m. When a growth study was carried out, the culture broth (400 mL) was incubated for 120 h.

Determination of biomass and residual carbon souce. — Whole culture broth (10 mL) was extracted with hexane (3 × 5 mL), the combined extracts were dried ($MgSO_4$), and the solvent was evaporated in a vacuum. The aqueous phase was centrifuged (4 × 30 min) at 5000 r.p.m. (Labofuge 5000). The resulting pellet was washed with water and dried at 105° to constant weight. The concentration of the residual hydrocarbon was determined by g.l.c. of the hexane extract, using tetradecane as standard, a Hewlett–Packard 5840 A gas chromatograph equipped with a flame-ionisation detector, and a fused-silica capillary column (12 m × 2 mm i.d.) coated with methyl silicone. The temperature program was 100° for 5 min, then to 200° at 8°/min. The detector and injection temperatures were 250° and 230°, respectively, the carrier gas was helium at 1 mL/min, and the chart speed was 5 cm/min.

Determination of extracellular and cell-wall associated trehalose. — Samples (10 mL) of culture medium were centrifuged (4 × 30 min) at 8000 r.p.m., the supernatant solution was filtered through a 0.22 μm Millipore membrane, and the filtrate was extracted immediately with hexane (3 × 2 mL), then $CHCl_3$–MeOH (2:1, 3 × 2 mL). The latter extract was concentrated and the sugar content was determined by the phenol–sulfuric acid method[13], using trehalose as standard, to give the amount of extracellular trehalose.

The residual cell mass was washed with $CHCl_3$–MeOH (2:1, 3 × 10 mL), the combined extracts were dried ($MgSO_4$), the solvents were evaporated in a vacuum, and the carbohydrate content of the residue was determined, as described above, to give the amount of cell-wall associated trehalose.

Structure of the trehalose lipids. — The trehalose lipids were characterised by elemental and group analyses. Optical rotations were measured with a Perkin–Elmer model 141 polarimeter on 2.2% solutions in MeOH. N.m.r. spectra (CD_3OD, internal Me_4Si) were obtained with a Bruker AM-400 WB spectrometer, and 2D spectra (COSY and COSYDQF) were recorded using the standard Bruker software.

Saponification. — A solution of the trehalose lipid (20 mg) in $CHCl_3$ (1 mL) was treated with 0.5M NaOH in aqueous 90% methanol (4 mL) for 2 h at 60°, using tetradecane as the internal standard[2]. After the addition of water (2 mL), the organic layer was separated and the solvent was evaporated. The residue was acidified with aqueous HCl and extracted with $CHCl_3$ (3 × 2 mL). The aqueous solution was desalted by using a mixed bed of Amberlite IRA-400 (HO^-) and IR-120 (H^+) resins, filtered, and concentrated, and the residue was analysed by t.l.c. using *A*, ethyl methyl ketone–benzene–2-propanol–benzoic acid–H_2O (30:20:40:0.915g:15); and *B*, 2-propanol–H_2O (85:15)[14]; and detection[15] with α-naphthol–H_2SO_4.

Fatty acids from the organic extract were esterified with diazomethane and analysed by g.l.c. by comparison of the retention times with those of a homologous series of fatty acid methyl esters. Also, they were studied by g.l.c.–m.s. with a Hewlett–Packard 5890 instrument equipped with a capillary column (30 m × 2 mm i.d.) of SE-30 coupled with a Hewlett–Packard 5888 mass spectrometer[16].

Acid hydrolysis. — A solution of trehalose lipid (10 mg) in $CHCl_3$ (1 mL) was treated with 6M HCl (1 mL) at 90° under Ar for 4 h. The mixture was extracted with $CHCl_3$ (3 × 2 mL), the combined extracts were dried ($MgSO_4$), and the solvent was evaporated under vacuum. Fatty acids were methylated with diazomethane, and analysed by g.l.c. and g.l.c.–m.s. as described above. The aqueous solution (glucose) was desalted, as described above.

F.a.b.-mass spectra (positive ion) were obtained with an updated (VG) MS-9 instrument. Samples (<0.1 mg) were dispersed in diethanolamine used as the matrix.

Detection and isolation of trehalose lipids. — Culture broth (200 mL) was centrifuged (4 × 30 min) at 8000 r.p.m., and the supernatant solution was filtered through a 0.22-μm Millipore membrane. The pellet was washed with $CHCl_3$–MeOH (2:1, 3 × 25 mL). The combined extracts were dried ($MgSO_4$) and the solvent was evaporated under vacuum to give a mixture (~2 g) of crude trehalose lipids (TL-1/4). The crude product was eluted from a column (29 × 2.2 cm) of Silica Gel 60 (9385, Merck) with $CHCl_3$ (200 mL) then $CHCl_3$–MeOH (95:5 → 60:40, in 5% steps and 200 mL per step) to yield pure TL-1/4. The organic extracts were analysed by t.l.c. on silica gel (5553, Merck), using *C*, $CHCl_3$–MeOH–H_2O (65:25:4), and detection[15] with α-naphthol–H_2SO_4.

Physico-chemical measurements. — Surface tension (γ_L) was measured for aqueous solutions at ~23° and interfacial tension ($\gamma_{L/L'}$) against kerosene was measured with a Lauda Automatic Tensiometer TE-1 by the De Nouy ring method[17].

*2,3,4-Tri-*O*-decanoyl-*α*-*D*-glucopyranosyl-2'-*O*-decanoyl-*α*-*D*-glucopyranoside (2, 3,4,2'-tetra-*O*-decanoyl-α,α-trehalose, TL-1).* — With decane (20 g) as the carbon source and the media (1 L), growth conditions, and the isolation and purification procedures described above, TL-1 (1.99 g) was obtained (10% conversion) as an oil, R_f 0.70 (solvent *C*), $[\alpha]_D$ +67°; γ_L 24 mN/m, c.m.c. (critical micelle concentration) 170 p.p.m.; ν_{max} 3600 (OH), 1745 cm^{-1} (ester). ^{1}H-N.m.r. data (400 MHz): δ 0.82, (t, 12 H, J 7.4 Hz, 4 CH_3CH_2), 1.20–1.60 (m, 56 H, 28 CH_2), 2.13–2.54 (m, 8 H, 4 CH_2CO_2), 3.32 (dd, 1 H, $J_{4',5'}$ 10, $J_{3',4'}$ 9.1 Hz, H-4'), 3.43 (dd, 1 H, J_{gem} 12.5, $J_{5,6a}$ 4.8 Hz, H-6a), 3.48 (dd, 1 H, $J_{5,6b}$ 2.5 Hz, H-6b), 3.60 (dd, 1 H, J_{gem} 11.7, $J_{5',6'a}$ 5.4 Hz, H-6'a), 3.68 (m, 1 H, H-5'), 3.75 (dd, 1 H,

$J_{5',6'b}$ 2.0 Hz, H-6'b), 3.82 (m, 1 H, H-5), 3.91 (dd, 1 H, $J_{2',3'}$ 9.9 Hz, H-3'), 4.67 (dd, 1 H, $J_{1',2'}$ 3.5 Hz, H-2'), 4.90 (dd, 1 H, $J_{1,2}$ 3.5, $J_{2,3}$ 9.3 Hz, H-2), 5.02 (dd, 1 H, $J_{3,4}$ 9.3, $J_{4,5}$ 9.5 Hz, H-4), 5.20 (d, 1 H, H-1'), 5.29 (d, 1 H, H-1), 5.44 (t, 1 H, H-3). F.a.b.-mass spectrum (positive ion): *m/z* 981 $(M + Na)^+$, 959 $(M + H)^+$.

Anal. Calc. for $C_{52}H_{94}O_{15}$: C, 65.11; H, 9.88. Found: C, 65.21; H, 9.90.

Saponification of TL-1, as described above, gave trehalose in the aqueous phase [t.l.c., R_F 0.1 (solvent *A*), 0.25 (soivent *B*), and methyl decanoate in the organic phase (identified by g.l.c. and g.l.c.–m.s.).

Acid hydolysis of TL-1, as described above, gave glucose [t.l.c., R_F 0.2 (solvent *A*) and 0.35 (solvent *B*)] and methyl decanoate.

*2,3,4,2'-(Di-*O*-decanoyl-di-*O*-nonanoyl-)*α-D-*glucopyranosyl* α-D-*glucopyranoside [2,3,4,2'-(di-*O*-decanoyl-di-*O*-nonanoyl)-α,α-trehalose, TL-2].* — With tridecane (20 g) as the carbon source and 1 L of media as for TL-1, TL-2 (1.08 g) was obtained (5.4% conversion). Recrystallisation from ether–pentane afforded a solid, m.p. 180–185°, $[\alpha]_D$ +100.5°, R_F 0.70 (solvent *C*); γ_L 25 mN/m, c.m.c. 20 p.p.m., $\gamma_{L/L'}$ 3.9 mN/m; ν_{max} 3600 (OH), 1745 cm^{-1} (ester). ^{1}H-N.m.r. data (400 MHz): δ 0.86 (t, 12 H, *J* 7.4 Hz, 4 CH_3CH_2), 1.25–1.70 (m, 52 H, 26 CH_2), 2.15–2.63 (m, 8 H, 4 CH_2CO_2), 3.34 (t, 1 H, $J_{4',5'} = J_{3',4'} = 9.5$ Hz, H-4'), 3.46 (dd, 1 H, J_{gem} 12.2, $J_{5,6a}$ 4.8 Hz, H-6a), 3.52 (dd, 1 H, $J_{5,6b}$ 2.3 Hz, H-6b), 3.63 (dd, 1 H, J_{gem} 11.7, $J_{5',6'a}$ 5.7 Hz, H-6'a), 3.71 (m, 1 H, H-5'), 3.78 (dd, 1 H, $J_{5',6'b}$ 1.7 Hz, H-6'b), 3.84 (m, 1 H, H-5), 3.93 (t, 1 H, $J_{2',3'}$ 9.5 Hz, H-3'), 4.69 (dd, 1 H, $J_{1',2'}$ 3.6 Hz, H-2'), 4.91 (dd, 1 H, $J_{1,2}$ 3.6, $J_{2,3}$ 10.3 Hz, H-2), 5.05 (t, 1 H, $J_{4,5} = J_{3,4} = 10.3$ Hz, H-4), 5.23 (d, 1 H, H-1'), 5.33 (d, 1 H, H-1), 5.48 (t, 1 H, H-3). F.a.b.-mass spectrum (positive ion): *m/z* 981 $(M + Na)^+$, 959 $(M + H)^+$.

Anal. Calc. for $C_{50}H_{90}O_{15}$: C, 65.11; H, 9.88. Found: C, 65.34; H, 9.95.

Saponification of TL-2, as described above, gave trehalose, methyl nonanoate, and methyl undecanoate.

Acid hydrolysis of TL-2, as described above, gave glucose, methyl nonanoate, and methyl undecanoate.

*2,3,4,2'-(Di-O-decanoyl-*O*-octanoyl-*O*-undecanoyl)-α-D-glucopyranosyl* α-D-*glucopyranoside [2,3,4,2'-(di-*O*-decanoyl-*O*-octanoyl-*O*-undecanoyl)-α,α-trehalose, TL-3].* — With a mixture of tetradecane, pentadecane, and hexadecane (20 g) as carbon source and 1 L of media as for TL-1, TL-3 (1.8 g) was obtained (9% conversion). Recrystallisation from ether–pentane mixtures afforded a solid, m.p. 182–187°, $[\alpha]_D$ +98°, R_F 0.70 (solvent *C*); ν_{max} 3600 (OH), 1745 cm^{-1} (ester). ^{1}H-N.m.r. data (400 MHz): δ 0.85 (t, 12 H, *J* 7.4 Hz, 4 CH_3CH_2), 1.22–1.70 (m, 54 H, 27 CH_2), 2.18–2.65 (m, 8 H, 4 CH_2CO_2), 3.30 (t, 1 H, $J_{4',5'} = J_{3',4'} = 9.8$ Hz, H-4'), 3.48 (dd, 1 H, J_{gem} 12.1, $J_{5,6a}$ 4.7 Hz, H-6a), 3.50 (dd, 1 H, $J_{5,6b}$ 2.7 Hz, H-6b), 3.60 (dd, 1 H, J_{gem} 11.8, $J_{5',6'a}$ 5.7 Hz, H-6'a), 3.70 (m, 1 H, H-5'), 3.78 (dd, 1 H, $J_{5',6'b}$ 1.8 Hz, H-6'b), 3.84 (m, 1 H, H-5), 3.94 (t, 1 H, $J_{2',3'}$ 9.8 Hz, H-3'), 4.70 (dd, 1 H, $J_{1',2'}$ 3.6 Hz, H-2'), 4.90 (dd, 1 H, $J_{2,3}$ 9.3, $J_{1,2}$ 3.6 Hz, H-2), 5.05 (t, 1 H, $J_{4,5} = J_{3,4} = 10$ Hz, H-4), 5.21 (d, 1 H, H-1'), 5.22 (d, 1 H, H-1), 5.45 (t, 1 H, H-3). F.a.b.-mass spectrum (positive ion): *m/z* 967 $(M + Na)^+$, 945 $(M + H)^+$.

Anal. Calc. for $C_{51}H_{92}O_{15}$: C, 64.80; H, 9.74. Found: C, 64.89; H, 9.94.

Saponification of TL-3, as described above, gave trehalose, methyl octanoate, methyl decanoate (× 2), and methyl undecanoate.

Acid hydrolysis of TL-3, as described above, gave glucose, methyl octanoate, methyl decanoate (× 2), and methyl undecanoate.

2,3,4,2'-(O-Decanoyl-O-nonanoyl-O-octanoyl-O-undecanoyl)-α-D-glucopyranosyl α-D-glucopyranoside [2,3,4,2'-(O-decanoyl-O-nonanoyl-O-octanoyl-O-undecanoyl)-α,α-trehalose, TL-4]. — With a mixture of decane, undecane, dodecane, and tridecane (20 g) as the carbon source and 1 L of media as for TL-1, TL-4 (0.6 g) was obtained (4% conversion). Recrystallisation from ether–pentane afforded a solid, m.p. 155–160°, $[\alpha]_D$ +92°, R_F 0.70 (solvent *C*); γ_L 25 mN/m, c.m.c. 1200 p.p.m.; ν_{max} 3600 (OH), 1745 cm^{-1} (ester). ^{1}H-N.m.r. data (400 MHz): δ 0.88 (t, 12 H, *J* 7.4 Hz, 4 CH_3CH_2), 1.32–1.70 (m, 52 H, 26 CH_2), 2.21–2.60 (m, 8 H, 4 CH_2CO_2), 3.36 (t, 1 H, $J_{4',5'} = J_{3',4'}$ = 9.8 Hz, H-4'), 3.47 (dd, 1 H, J_{gem} 12.1, $J_{5,6a}$ 4.7 Hz, H-6a), 3.51 (dd, 1 H, $J_{5,6b}$ 2.4 Hz, H-6b), 3.62 (dd, 1 H, J_{gem} 11.6, $J_{5',6'a}$ 5.6 Hz, H-6'a), 3.70 (m, 1 H, H-5'), 3.78 (dd, 1 H, $J_{5',6b'}$ 1.9 Hz, H-6'b), 3.85 (m, 1 H, H-5), 3.93 (t, 1 H, $J_{3',4'} = J_{2',3'}$ = 9.8 Hz, H-3'), 4.72 (dd, 1 H, $J_{1',2'}$ 3.8 Hz, H-2'), 4.93 (dd, 1 H, $J_{2,3}$ 9.5, $J_{1,2}$ 3.8 Hz, H-2), 5.05 (t, 1 H, $J_{4,5} = J_{3,4}$ = 9.5 Hz, H-4), 5.23 (d, 1 H, H-1'), 5.32 (d, 1 H, H-1), 5.49 (t, 1 H, $J_{3,4}$ and $J_{2,3}$ 9.5 Hz, H-3). F.a.b.-mass spectrum (positive ion): *m/z* 953 $(M + Na)^+$, 931 $(M + H)^+$.

Anal. Calc. for $C_{50}H_{90}O_{15}$: C, 64.49; H, 9.74. Found: C, 64.61; H, 9.88.

Saponification of TL-4, as described above, gave trehalose, methyl octanoate, methyl nonanoate, methyl decanoate, and methyl undecanoate.

Acid hydrolysis of TL-4, as described above, gave glucose, methyl octanoate, methyl nonanoate, methyl decanoate, and methyl undecanoate.

RESULTS AND DISCUSSION

Growth of the Corynebacterium strain and production of biosurfactants is not satisfactory in the presence of hydrophilic substrates like glucose, fructose, and mannose, but the production of non-ionic trehalose glycolipids from hydrophobic carbon sources is well known[2,18]. With the Corynebacterium at an incubation temperature of 25–30°, and C_{10} or C_{13} *n*-alkanes or mixtures of C_{14}–C_{16} or C_{10}–C_{13} *n*-alkanes as the carbon sources, growth and the production of glycolipids were promoted. Clumping of the bacteria occurred when the agitation was not vigorous, thereby retarding growth and the biosynthesis of trehalose lipids. Figure 1 shows the production of extracellular and cell-wall associated glycolipid with decane as the carbon source.

The trehalose lipid formed by the 51T7 bacterium was probably located first in the periphery of the cells, because it was removed easily by extraction with solvents. Up to an incubation time of 80 h, the trehalose lipids were mainly cell-wall associated and only ~10% was extracellular. After 80 h, extracellular trehalose lipid was the main conversion product. These results do not accord with the usual pattern of production of non-ionic trehalose glycolipids which are generally cell-wall associated[18–23]. When the carbon source was depleted after incubation for 80 h, the maximum yield (0.85 g/L) of extracellular trehalose lipid (TL-1) was reached.

The trehalose lipids TL-1/4 were obtained when the C_{10}, C_{13}, C_{10}–C_{13}, and C_{14}–C_{16} *n*-alkanes, respectively, were used as carbon sources.

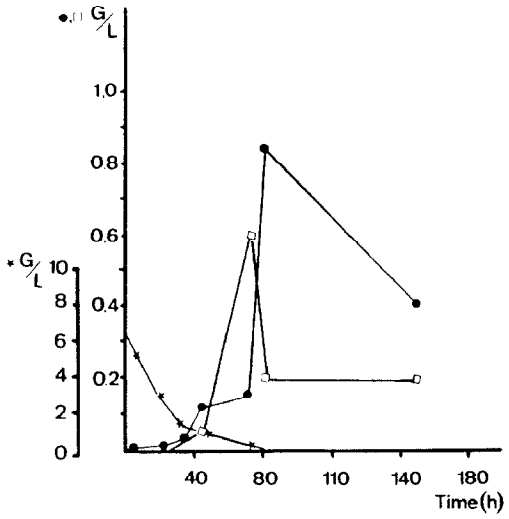

Fig. 1. Production of TL-1 from decane: *, residual decane; □, cell-associated TL-1 ●, extracellular TL-1; (α,α-trehalose 2,3,4,2′-tetradecanoate).

Elemental analysis and f.a.b.-mass spectral data were compatible with the molecular formula assigned to the trehalose lipids, and the i.r. spectra contained bands for hydroxyl (3600 cm^{-1}) and ester groups (1745 cm^{-1}).

Saponification of each trehalose lipid afforded α,α-trehalose as the water-soluble product and, after reaction with diazomethane, the methyl esters of the C_8–C_{11} fatty acids (depending on the carbon source) were identified by g.l.c. and g.l.c.–m.s. Thus, the bacterial strain used synthesised short-chain fatty acids, but not corynomycolic acids. This strain is regulated by a de novo synthesis of the carbohydrate moiety and the synthesis of the lipid moiety is dependent on the chain length of the hydrocarbon substrate. Oxidation of the alkyl chain and β-degradation of the alkane substrate[20] followed by esterification of the trehalose occurred.

The locations of the ester groups in TL-1/4 were determined by 400-MHz ^{1}H-n.m.r. spectroscopy. The protons of glycoside rings resonated in the ranges δ 3.2–4.0 (*H*COH) and 4.5–5.5 (*H*COAcyl), so that the locations of the acyl groups could be determined after assignment of the ^{1}H resonances. The assignment of ^{1}H of the carbohydrate moieties of TL-1/4 was possible with the aid of the correlation spectroscopy. Starting with the resonances of H-1 and H-1′, all the other resonances could be assigned on the basis of the cross-peaks in the 2D-COSY ^{1}H-n.m.r. spectra, as illustrated for TL-1 in Fig. 2. In this way, it was concluded that the ester groups in TL-1/4 were located at positions 2, 3, 4, and 2′. However, from the evidence available, it was not possible to identify the substituents at these positions in the mixed esters TL-2/4.

The trehalose tetraesters were soluble in chloroform and chloroform–methanol mixtures, but were only slightly soluble in water. The surface tension of TL-1/4 measured in aqueous solutions at ~23° and the interfacial tensions against kerosene were < 30 and 5mN/m, respectively, with a low value of the critical micelle concentra-

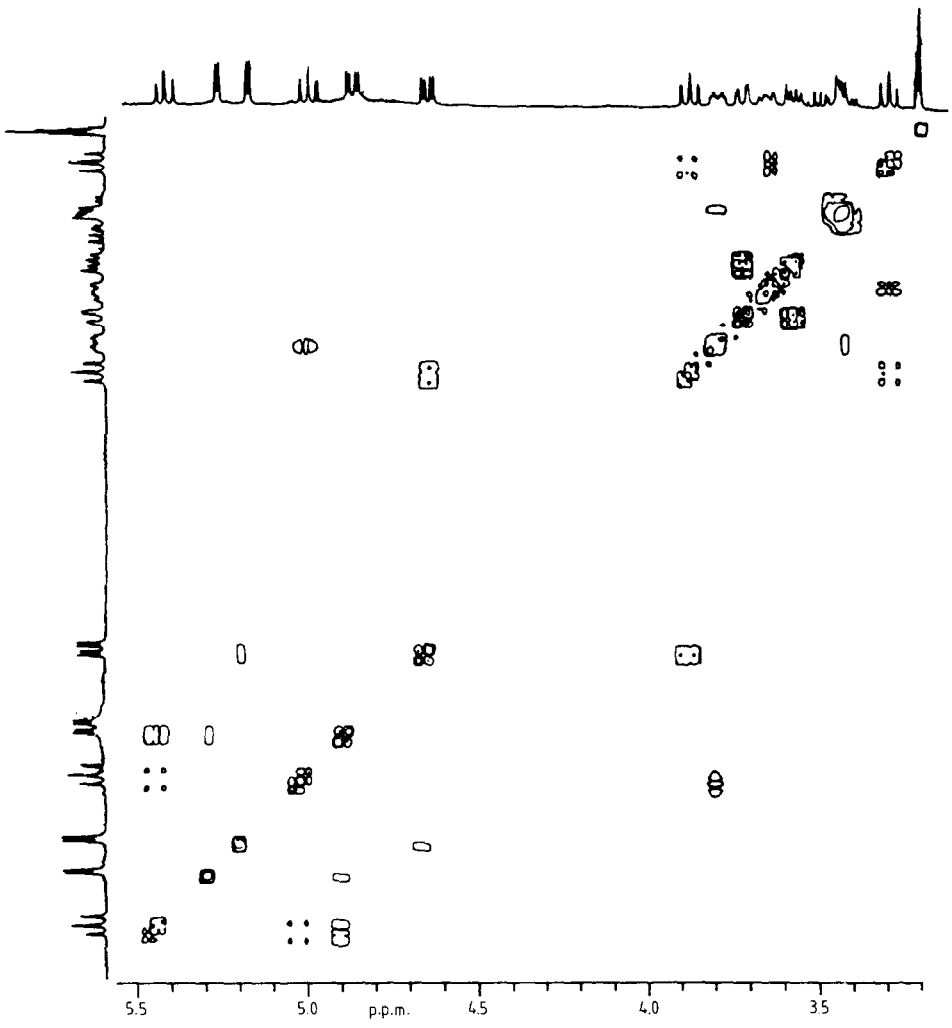

Fig. 2. 2D-COSY ^{1}H-n.m.r. spectrum of TL-1 (α,α-trehalose 2,3,4,2'-tetradecanoate).

tion (c.m.c.) in the range 20–170 p.p.m. Trehalose lipids can drastically decrease the interfacial tension between *n*-alkane and water, even at a low concentration[11]. This fact and the probable induction of the formation of trehalose lipids by hydrophobic substrates[7] suggest that trehalose tetraesters may play a role in the uptake of *n*-alkanes by this strain.

These preliminary data suggest that biosurfactants may be produced from relatively inexpensive carbon sources and are effective at low concentrations. There is a wide range of commercially important surfactant functions[24], ranging from flooding, spreading, wetting, solubilisation, detergency, emulsification, de-emulsification, foaming, and antifoaming, and the production of *in situ* microbial biosurfactants is gaining interest in the context of better protection of the environment.

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