Trehalolipids

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Contents

Abstract Trehalose-containing glycolipids are mainly produced by Gram-positive, high GC content bacteria of Actinomycetales. Their structures are quite diverse in hydrophobic moiety, varying from short simple to long complex fatty acids. Correspondingly, functions and physiochemical properties vary upon structures. From the view of practical use as a biosurfactant, the trehalose lipids from *Rhodococcus* and the genera other than *Mycobacterium* are of high potential in application. While, like other kinds of biosurfactants, their relative low productivity limits practical use. And yet, the biosynthesis mechanism of trehalose lipids has been less exploited and needs further investigations.

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1 Introduction

Trehalolipids, or trehalose lipids, are glycolipids containing trehalose hydrophilic moiety. They are among the best known biosurfactants but distinguished from rhamnolipids and sophorolipids in both composition and activity. They are active to lower water surface and interface tension. In addition, they function uniquely in many other ways.

The occurrence of trehalose in lipids was first noticed in 1933 in fat extracts from tubercle bacilli (Anderson and Newman 1933). At that time, the authors did not pay attention to the property as a biosurfactant but found intriguing that trehalose was in place of glycerol as the alcoholic component of a fat. However, the pure compound of trehalose lipid was first successfully purified in 1956 from the lipids of *Mycobacterium tuberculosis* and identified as 6, 6′-dimycoloyl-α′, α′-D-trehalose (cord factor). Later, several groups of trehalose-containing lipids were isolated, mainly from Mycobacteria, Nocardia, and Corynebacteria, as reviewed by Asselineau and Asselineau (Asselineau and Asselineau 1978).

Interest in trehalose lipids as a general surfactant can be traced back to the discovery that the emulsion layer of *Arthrobacter paraffineus* culture broths contained trehalose dimycolates when the cells were grown on hydrocarbon substrates (Suzuki et al. 1969). Various trehalose lipids varying in fatty acid part have been found. The glycolipids from mycobacteria usually have branched long-chain hydroxyaliphatic acids as their hydrophobic moiety; while some bacteria choose short-chain fatty acids, such as some cases of *Rhodococcus*. Trehalose lipids are quite diverse in chemical structure and attractive in applications as other biosurfactants.

2 Trehalolipid-Producing Bacteria

Production of trehalolipids mainly occurs in the following bacteria of Actinomycetales, including the genera of *Rhodococcus, Mycobacterium, Micrococcus, Nocardia, Gordonia, Corynebacterium, Brevibacteria*, *Arthrobacter,* etc. (Table 1). However, yeast and other fungus have seldom been reported as a producer of trehalose lipids.

Among the reported trehalolipid-producing bacteria, most are alkane-degrading bacteria. Surfactive compounds are generated only when bacteria feed on hydrophobic substrates such as *n*-hexadecane (listed in Table 1), such as *Rhodococcus erythropolis, Rhodococcus opacus*, *Rhodococcus wratislaviensis, Gordonia amarae, Arthrobacter parafineus, Micrococcus luteus, Mycobacterium paraffinicum,* Corynebacteria, etc. They have different isolation origins, some of which are from marine environments (Passeri et al. 1991; Schulz et al. 1991; Yakimov et al. 1999; Satpute et al. 2010); some are from alkaline soils or polar soils. These bacteria seem to be tolerant with high salt concentrations (up to 6% salinity).

Table 1 Trehalose lipids: producing bacteria and physiochemical properties

Bacteria	Trehalose lipids	Surface tension (mN m^{-1})	Interfacial tension (mN m^{-1})[a]	CMC (mg l^{-1})	Substrates	Production	Growth association	References
Mycobacterium fortuitum	Mycoside F	–	–	–	–	–	–	Gautier et al. (1992)
Mycobacterium tuberculosis H37Rv	2,3-di-*O*-acyltrehalose	–	–	–	–	–	–	Besra et al. (1992)
Mycobacterium smegmatis	Cord-factor (6,6′-diester of trehalose containing two moles of smegma mycolic acids)	–	–	–	–	–	–	(Mompon et al. 1978)
Corynebacterium matruchotii	Trehalose 6-mono- and 6,6′-di-corynomycolates	–	–	–	–	–	–	Datta and Takayama (1993)
Pseudomonas fluorescence	Trehaloselipid-*o*-dialkyl monoglycerides	–	–	–	Alkanes	High yield with gasoline; depending upon hydrocarbon source	–	Desai et al. (1988)
Arthrobacter parafineus KY4303	α-branched-β-hydroxy fatty acid trehalose ester	–	–	–	*n*-paraffin	1.3. Penicillin reduced the yield in emulsion layer; but stimulated extracellular accumulation	Yes	Suzuki et al. (1969)
Arthrobacter sp.4301; *Brevibacteria* sp.; *Corynebacterium* spp.; *Nocardia* spp.	Trehalose-containing lipids (not fully characterized)	–	–	–	*n*-paraffin	0.5–1.9	Yes	Suzuki et al. (1969)
Arthrobacter sp. EK 1	An anionic 2,3,4,2′-trehalose tetraester; containing succinate	–	–	–	Crude oil degrading	2 (crude extract)	–	Passeri et al. (1991), Schulz et al. (1991)
Rhodococcus isolate Q	A novel trisaccharide glycolipid; containing trehalose bears ester-linked hexanoate, succinate, and acyloxyacyl moieties	–	–	–	*n*-hexadecane; definitely not glucose	Significant amounts of cell-associated	Perhaps	Esch et al. (1999)
Rhodococcus fascians		32	–	–	*n*-alkanes		–	

(*continued*)

Table 1 (continued)

Bacteria	Trehalose lipids	Surface tension (mN m^{-1})	Interfacial tension (mN m^{-1})[a]	CMC (mg l^{-1})	Substrates	Production	Growth association	References
	a mixture of trehalose lipids					Both extracellular and cell-bound		Yakimov et al. (1999)
Rhodococcus erythropolis DSM43215	Trehalose dicorynomycolates	43	18	0.7	*n*-alkanes	–	Yes	Kretschmer et al. (1982), Kretschmer and Wagner (1983), Kim et al. (1990)
	Trehalose monocorynomycolates	32	14	4				
	Trehalose-2,2′,3,4-tetraester	26	<1	15	*n*-alkanes (best with C10)	32	No. nitrogen limited	Kim et al. (1990)
Rhodococcus sp. H13-A	A mixture with octaacyltrehalose as main component	–	0.02 (against decane)	1.5	*n*-alkanes	Extracellularly accumulated	No	Singer and Finnerty (1990), Singer et al. (1990)
Rhodococcus opacus 1CP	Novel trehalose dinocardiomycolates of unsaturated fatty acid chains	–	–	–	*n*-alkane	From 247 mg l^{-1} for *n*-decane up to 420 mg l^{-1} for *n*-dodecane Growth on C14 and C16 gave yields between 333 and 315 mg l^{-1}	Yes	Niescher et al. (2006)
Rhodococcus erythropolis 3C-9	A mixture of glucolipids and trehalolipids	33.4 (culture)	–	–	*n*-alkanes; definitely not glucose	–	Yes	Peng et al. (2007)
Rhodococcus erythropolis SD-74	Two main succinoyl trehalose lipids (STL-1 and STL-2); $MW_{STL\text{-}1} = 1{,}019$	19 (STL-1)	–	5 (STL-1)	*n*-hexadecane	Extracellular accumulation	–	Uchida et al. (1989), Tokumoto et al. (2009)
Rhodococcus wratislaviensis BN38	2,3,4,2′-trehalosetetraesters containing succinic acid	28.6 (mixture) 24.4 (purified)	5.3	5	C8–C17, best with *n*-hexadecane	3.1 g l^{-1}	Yes	Tuleva et al. (2008)

Rhodococcus erythropolis 51T7	A trehalose tetraester	27.9	5	37	Tetradecane (2%,V/V)	0.48–1.12	Yes	Marques et al. (2009)
Corynebacteria sp.51 T7 = *R. erythropolis* 51T7	Trehalose esters of the C8–C11 fatty acids (depending on the carbon source)	–	–	–	*n*-alkanes	In first 80 h, mainly associated with the cell wall; afterwards, mainly extracellular	–	Martin et al. (1991)
Micrococcus luteus BN56	Two main products of trehalose tetraesters, MW = 876, 848	24.1	1.7	25	*n*-hexadecane	–	Yes	Tuleva et al. (2009)

[a]Water against hexadecane

Among them, isolates of *Rhodococcus* are the most potential in biosurfactant production (Lang and Philp 1998).

Rhodococcus bacteria usually produce trehalose lipids when they grow with alkanes as carbon sources (Espuny et al. 1995; Rapp et al. 1979; Singer et al. 1990). Among the bacteria of this genus, isolates of *R. erythropolis* are most frequently reported as a producer of trehalose lipids (Lang and Philp 1998; Peng et al. 2007). On the other hand, glycolipids other than trehalose lipids have been also found recently in bacteria of *Rhodococcus*, e.g., a glucolipid was found in *R. erythropolis* isolate 3C-9 in addition to a trehalolipid (Peng et al. 2007); for another example, rhamnose glycolipid was found as the main biosurfactant in the case of *Rhodococcus fascians* isolate A-3 grown with glucose or kerosene as a substrate (Gesheva et al. 2010).

R. erythropolis 51 T7 is a relatively intensively investigated bacterium. It was once named *Corynebacterium* sp. 51 T7 at the beginning (Martin et al. 1991), then renamed as *Rhodococcus* sp. 51 T7 (Espuny et al. 1996), and now named as *R. erythropolis* 51 T7 (Marques et al. 2009). This bacterium has been confirmed to produce tetraesters of trehalose with short fatty acid chains (C8–C11).

Mycobacterium is another group of trehalolipid-producing bacteria. But the trehalolipids produced by *Mycobacterium* are seldom reported as a biosurfactant, interests to them are mainly attributed to their role in pathogenicity and molecular immunology (Imasato et al. 1990; Ryll et al. 2001; Hunter et al. 2006; Ortiz et al. 2008, 2009; Zaragoza et al. 2009). Many species of this genus have been reported to produce trehalose-containing lipids, such as *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium paraffinicum, Mycobacterium phlei, Mycobacterium flavescens,* and *Mycobacterium avium* (Mompon et al. 1978; Batrakov et al. 1981; Fujita et al. 2005). In addition to their effects in health, bacteria of this genus also frequently reported as degraders of alkanes and polycyclic aromatic hydrocarbons (Hallas and Vestal 1978; Berekaa and Steinbuchel 2000; Kotani et al. 2006; Churchill et al. 2008; Vila and Grifoll 2009). The trehalolipids of mycobacteria might also involve in dissolving hydrophobic substrates. One example supporting this assumption is that a paraffin-oxidizing bacterium of *M. paraffinicum* can produce at least five trehalose lipids including the cord factor or two analogs (Batrakov et al. 1981).

3 Chemical Structures

Like other glycolipids, trehalose lipids are composed of a carbohydrate group in combination with fatty acids groups. Differently, their hydrophobic moieties are more diverse, including aliphatic acids and hydroxylated branched-chain fatty acids (mycolic acids) of varied chain lengths. The numbers of hydrophobic chain in each molecule of trehalose lipids are usually 1, 2, and 4, forming mono-, di-, and tetraesters, correspondingly. Triesters can also be the main glycolipid component in some cases, such as in *Mycobacteriurn fortuitum* (Gautier et al. 1992). Among

the trehalose lipids, the trehalose esters produced by *R. erytropolis* have been studied most extensively, in addition to the cord factors of *Mycobacteria*.

Trehalose is a nonreducing sugar formed from two glucose units joined by a 1–1 alpha bond, giving it the name of α-D-glucopyranosyl-(1 → 1)-α-D-glucopyranoside (Fig. 1). The bonding makes trehalose very resistant to acid hydrolysis, and therefore is stable in solution at high temperatures, even under acidic conditions. Trehalose is the carbohydrate group of cell wall glycolipids in Mycobacteria and Corynebacteria.

Mycolic acids were first identified in an unsaponifiable lipid extract isolated from *M. tuberculosis*. They are complex hydroxylated branched-chain fatty acids with 60–90 carbon atoms, while those from other species (*Corynobacterium, Nocardia*) are shorter and named corynomycolic (22–36 carbons) or nocardomycolic (44–60 carbons) acids. They may also contain diverse functional groups such as methoxy, keto, or epoxy ester group and cyclopropane ring (Fig. 2). The structure, physiological function, and biosynthesis of mycolic acids have been reviewed by Barry et al. (1998).

3.1 Cord Factor: A Trehalose Diester from Mycobacteria

Trehalose lipids in mycobacteria have been extensively exploited in chemical composition (Mompon et al. 1978; Batrakov et al. 1981; Fujita et al. 2005). Of all

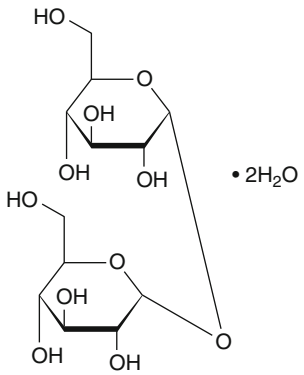


Fig. 1 Trehalose structure

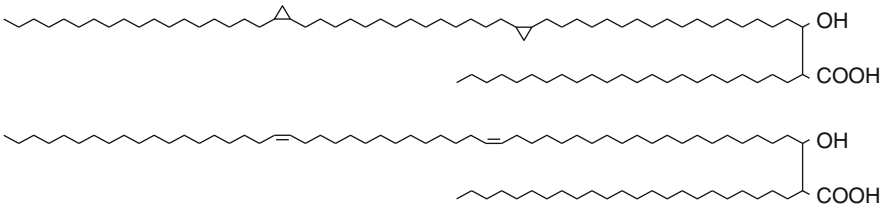


Fig. 2 Mycolic acids of the unsaturation and cyclopropane chains (cited from web page www.cyberlipid.org/fa/acid0006.htm)

trehalose lipids, cord factor is the best known. It has two mycolic acids of variable length, esterified to the 6-hydroxyl group of each glucose to give trehalose 6,6′-dimycolate (TMD) (Ryll et al. 2001) (Fig. 3). TDM is the most prominent and best-studied mycolic acid-containing glycolipid of mycobacteria. The structure varies greatly among mycobacterial species, and the mycolyl moiety is related with toxicity and antigenicity, thereby constituting potential virulence or immunostimulating mechanisms (Ryll et al. 2001; Hunter et al. 2006; Guidry et al. 2007; Ishikawa et al. 2009).

With MALDI-TOF mass spectrometry, cord factors were characterized by Fujita et al.(2005) from nine species of human-virulent and nonvirulent mycobacteria as follows, *M. tuberculosis* H_{37}Rv (ATCC 27294), *M. tuberculosis* Aoyama B (ATCC 31726), *M. bovis* BCG Tokyo 172 (ATCC 35737), *M. bovis* BCG Connaught (ATCC 35745), *Mycobacterium intracellulare* serotype 4 (ATCC 35767),

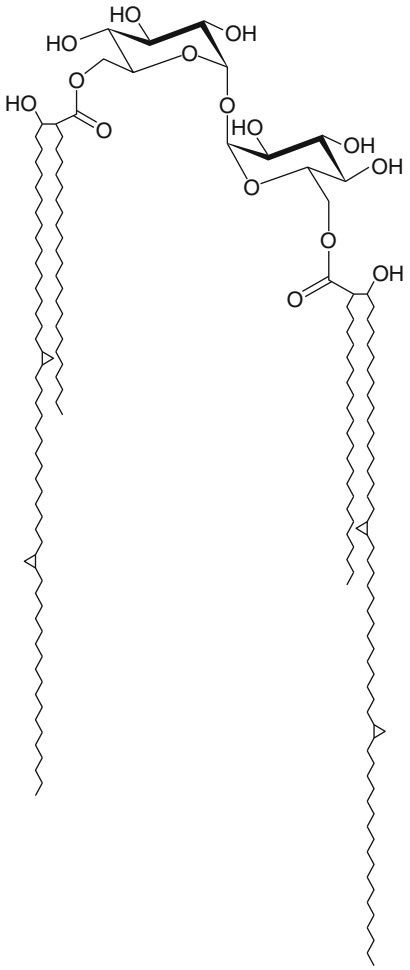


Fig. 3 Trehalose 6,6′-dimycolate(TDM) from Isolated from *M. tuberculosis*

M. intracellulare serotype 16 (ATCC 13950), *Mycobacterium kansasii* (ATCC 12478), *Mycobacterium phlei* (ATCC 11758), and *Mycobacterium flavescens* (ATCC 14474). They found that α-mycolic acid was a ubiquitous component among the mycobacterial species, with carbon numbers ranging from C74 to C88 and with two cyclopropane rings or equivalent double bonds. Ketomycolates were also widely distributed, with carbon numbers ranging from C76 to C91, but the ranges of the carbon number of ketomycolates differed greatly among the species (Fujita et al. 2005).

3.2 *Trehalose Lipids from* Rhodococcus

The surface-active lipids in rhodococci have been reviewed by Lang S (Lang 1999). The biosurfactants produced by members of the genus *Rhodococcus* is dominated by trehalose-containing glycolipids. They are quite diverse in the fatty acid moiety, such as dicorynomycolates, monocorynomycolates, and 2, 2′, 3, 4-tetraester (Kim et al. 1990; Espuny et al. 1996; Rapp et al. 1979; Kretschmer et al. 1982; Kretschmer and Wagner 1983). Additionally, succinoyl-trehalose and octaacyl-trehalose have also been detected in *R. erythropolis* (Uchida et al. 1989; Singer and Finnerty 1990; Tokumoto et al. 2009).

3.2.1 Trehalose Diesters

Trehalose Dimycolates from *R. erythropolis* DSM 43215

The trehalose lipids produced by *R. erythropolis* DSM 43215 were characterized as α-D-Glucopyranosyl-α-D-glucopyranoside-6,6′-di-(2-alkyl-3-hydroxy)-carboxylic ester(Rapp et al. 1979; Kretschmer et al. 1982) (Fig. 4). The bacterium was grown on 2% (w/v) *n*-alkanes (chain length C12–C18). The glycolipid was extracted from the biomass with *n*-hexane and purified by repeated chromatography on silica gel.

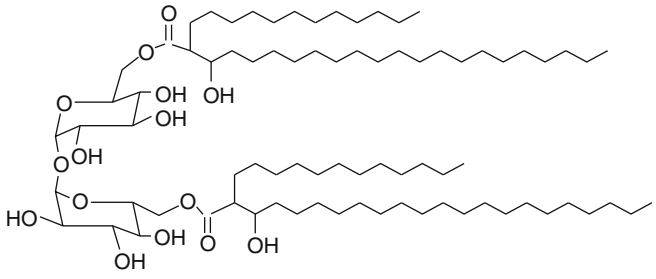


Fig. 4 α, α-trehalose-6,6′-dicorynomycolates from *R. erythropolis* DSM 43215 (Rapp et al. 1979). The mycolic acids range from $C_{32}H_{64}O_3$ to $C_{38}H_{76}O_3$.

NMR revealed that its lipid moiety was consisted of saturated long-chain α-branched β-hydroxy fatty acids (mycolic acids) ranging from $C_{32}H_{64}O_3$ to $C_{38}H_{76}O_3$, of which $C_{34}H_{38}O_3$ and $C_{35}H_{70}O_3$ predominated. Prior to this study and afterwards, similar trehalose dimycolates were found in *Rhodococcus, Nocardia*, and *Rhodochrous* grown on glycerol.

Novel Trehalose Dimycolate from *Rhodococcus opacus* 1CP

Glycolipids were purified from culture of *R. opacus* 1CP growing with *n*-decane as sole carbon source. They are characterized to be trehalose dimycolate with ^{1}H-NMR spectroscopy. The fatty acids are nocardiomycolic acids of total chain lengths between 48 and 54 carbons (Fig. 5). In addition, in each mycolic acid, two double bonds exist (Niescher et al. 2006). These features (longer chain and unsaturation) distinguish the trehalose dimycolates from those of *R. erythropolis* DSM43215 (Rapp et al. 1979; Kretschmer et al. 1982) and *Rhodococcus ruber* IEGM231 (Philp et al. 2002).

Trehalose Lipids of *Rhodococcus ruber* IEGM231

Three kinds of glycolipids were purified from *R. ruber* culture, namely C1, C2, and C3. Each has analogs with varied chain lengths (Philp et al. 2002). Their structures were determined by NMR and ESI-MS (Fig. 6). In common, they share trehalose as the hydrophilic moiety. Among them, C1 was a typical trehalose dicorynomycolate with chain α-branched-β-hydroxy fatty acids (the total carbon number ranging from 34 to 46, with C40 as a main component), which is longer than that in

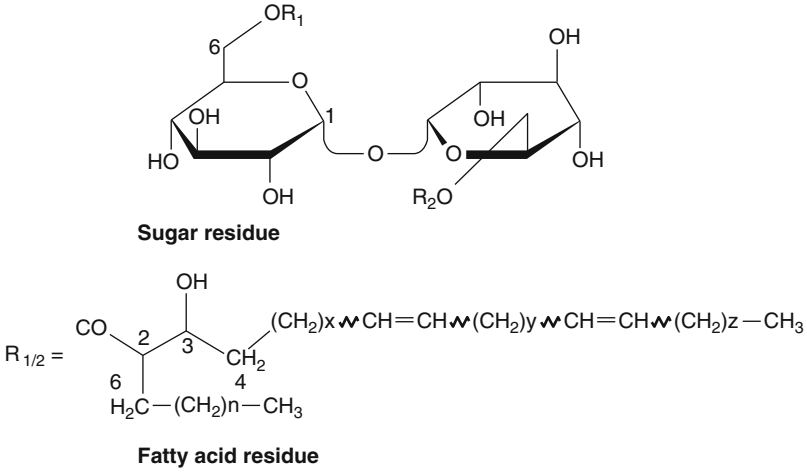
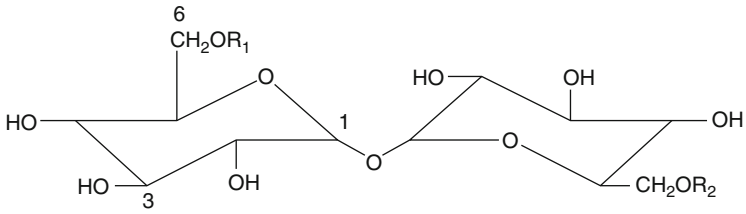


Fig. 5 Structure of the trehalose dinocardiomycolate of *R. opacus* 1CP (Niescher et al. 2006). The mycolic acid components of carbon number ranging from C-48 to C-54 (n + x + y + z from 37 to 43)



C1 $R_1 / R_2 =$ — C(=O) — CH(α) — CH(OH, β) — $(CH_2)m$ — CH_3; CH(β) — $(CH_2)n$ — CH_3

m + n = 29 – 41 (centered at 35)

C2 $R_1 / R_2 =$ — C(=O) — $(CH_2)m$ — CH_3

m = 13 – 15 (i.e. probably 14 + 12 and 14 + 16 with main component 14 + 14)

C3 $R_1 =$ — C(=O) — $(CH_2)m$ — CH_3

$R_2 = H$

m = 10 – 14 (main component 12)

Fig. 6 Trehalose lipids of *Rhodococcus ruber* IEGM231 (Philp et al. 2002)

R. erythropolis DSM 43215 (Rapp et al. 1979). In the case of C2 component, the major component was equivalent to a molecule carrying two C16-fatty acid units and other analogs of the C11–C14 acids. The major C3 component is C14-glycolipids containing fatty acids of both the saturated and unsaturated.

Succinoyl Trehalose Lipids from *R. erythropolis* SD-74

The bacterium strain SD-74 can abundantly produce extracellular trehalose lipids from *n*-alkanes. The characteristic of this trehalose lipid is succinic acid containing. Two main components named STL-1 and STL-2 have been characterized from culture of strain SD-74 (Uchida et al. 1989; Tokumoto et al. 2009). They are not only powerful surfactants, but they also show versatile biochemical actions, such as induction of human cell differentiation (Isoda et al. 1995; Sudo et al. 2000).

The STL structure is primarily determined by NMR and GC-MS. STL1 and STL2 were 2,3,4,2″-di-*O*-succinoyl-di-*O*-alkanoyl-α,α-trehalose and 2,3,4-mono-

O-succinoyl-di-*O*-alkanoyl-α,α-trehalose, respectively(Uchida et al. 1989). This is the first report of succinoyl trehalose lipids (STLs) (Uchida et al. 1989).

Recently, the structure of STL1 produced by *Rhodococcus* sp. SD-74 was subjected to further characterization (Tokumoto et al. 2009). The exact structure of STL-1 was depictured on the basis of NMR, MALDI-TOF/MS, and GC-MS analyses. The STL-1 in this report was produced from *n*-hexadecane and was the main component. It was identified to be 3,4-di-*O*-alkanoyl-2-*O*-succinoyl-α-D-glucopyranosyl-2′-*O*-succinoyl-α-D-glucopyranoside (Fig. 7). The major fatty acid of STL-1 was C16, indicating that *n*-hexadecane as the carbon source is possibly directly incorporated into the carbohydrate moiety via terminal oxidation.

Trehalose Diesters and Triesters of *Mycobacteriurn fortuitum*

M. fortuitum produced a family of trehalose-containing glycolipids named mycoside F (Gautier et al. 1992). Three main glycoconjugates were detected and their structures established as 2, 3-diacyl, 2, 3, 4- and 2, 3, 6-triacyl trehalose. The nature of the fatty acyl substituents identified primarily as 2-methyl-octadecen-2-oyl.

In another case, a trehalose-containing glycolipid was detected in several strains of *M. fortuitum* and characterized as 2,3-di-*O*-acyltrehalose (DAT) (Ariza et al. 1994). In the report, lipid constituents were identified as a mixture of straight-chain (14–18 carbon atoms) and methyl-branched-chain (17–21 carbon atoms) fatty acyl groups. DAT was further fractionated by reverse phase TLC into four fractions that were designated DAT- I to IV. DAT-I contained 70–75% straight-chain acyl substituents (hexadecanoyl and octadecanoyl predominating) and 25–30% 2-methyl branched substituents (mainly 2-methyl octadecadienoyl). DAT-II was composed of a mixture in which the acyl groups were almost exclusively 2-methyl branched, with 2-methyl octadecadienoyl and 2-methyl octadecen-2-oyl predominating. DAT-III, which was the major isolated fraction, consisted of compounds in which the ratio linear to branched acyl groups varied between 0.8 and 0.9, 2-methyl octadecen-2-oyl, hexadecanoyl and octadecanoyl being the most abundant. Finally,

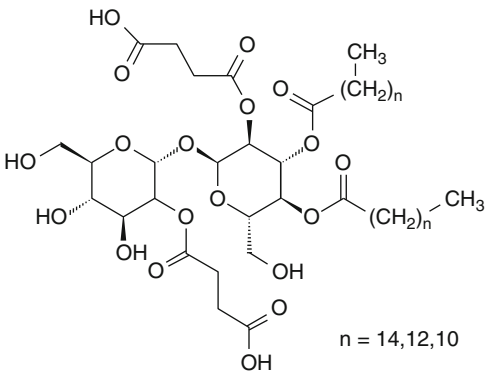


Fig. 7 Succinoyl trehalose lipids (STL-1) from *R. erythropolis* SD-74

DAT-IV comprised a mixture of DAT molecules containing mostly 2-methyl octadecadienoyl, 2-methyl octadecen-2-oyl, 2-methyl eicosadienoyl, and 2-methyl eicosen-2-oyl groups. Actually, early in 1964, trehalose lipids of *M. fortuitum* had been described having hydrophobia moiety like above described, i.e., C16:0 and sometimes with C19:0 and C20:0 in fatty acids moiety (Vilkas and Rojas 1964).

3.2.2 Trehalose Tetraesters Produced by *Rhodococcus* and Related Bacteria

In this section, the structure of the trehalose lipids produced by *Arthrobacter*, *Micrococcus,* and *Rhodococcus* is presented.

Arthrobacter sp. EK 1 (Passeri et al. 1991)

Arthrobacter sp. EK 1 is a marine *n*-alkane-utilizing bacterium. 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 was detected. The exact position of succinate is confirmed at C 2 atom of trehalose. This is the first report of succinate on trehalose of a glycolipid (Passeri et al. 1991).

Rhodococcus wratislaviensis BN38 (Tuleva et al. 2008)

Glycolipids were produced by *R. wratislaviensis* BN38 when grown on 2% *n*-hexadecane. The glycolipids were isolated by chromatography on silica gel columns, and the main product was characterized as 2,3,4,2′-trehalose tetraester with molecular mass of 876, esterified with two decanoic, one octanoic, and one succinic acid (Tuleva et al. 2008). Trace amounts of another 2,3,4,2′-trehalose tetraester of molecular mass 849 were also detected.

Micrococcus luteus BN56 (Tuleva et al. 2009)

The soil strain *M. luteus* BN56 can produce a mixture of two trehalose tetraester biosurfactants when grown aerobically on *n*-hexadecane. The biosurfactants were extracted and purified from whole cultures and characterized by NMR and mass spectrometry (Fig. 8). The two major components consisted trehalose tetraesters with molecular mass of 876 and 848. In both cases, α,α-trehalose was linked at C-2 or C-4 with a succinic and at C-2′ with a decanoic acid. The trehalose lipid with molecular mass of 876 was esterified at C-2, C-3, or C-4 with an octanoic and a decanoic acid. The other one with MW 848, of lower molecular mass, was esterified at the same positions with two octanoic acids (Tuleva et al. 2009).

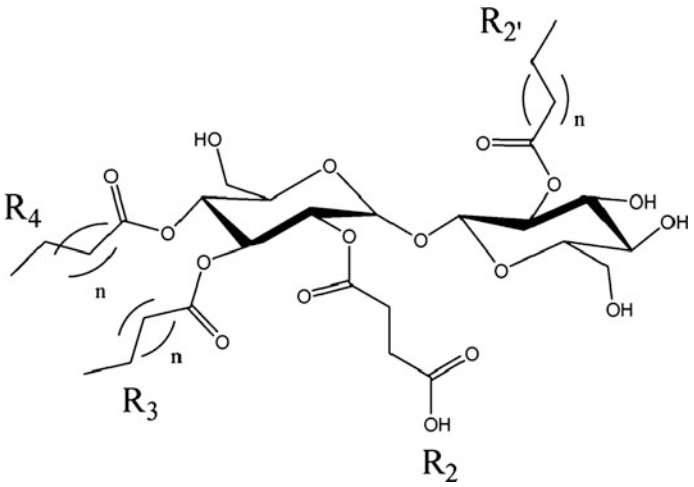


Fig. 8 Chemical structure of trehalose tetraesters produced by *M. luteus* BN56 (n = 6–11; R2 = succinic acid) (Tuleva et al. 2009)

Rhodococcus erythropolis 51 T7 (Marques et al. 2009)

The chemical structure of trehalose lipids produced by strain 51 T7 was further studied by LC-MS, with tetradecane as the carbon source (Marques et al. 2009). This analysis revealed that this surfactant is a mixture of at least six components, with the pseudomolecular ions being between *m*/*z* 905 and 821 (using negative ion mode). The most abundant component is the pseudomolecular ion 876, occupying 43.9% of the total lipids after 72 h in culture. This molecular weight may correspond to either trehalose–succinic acid–C9–C9–C10 or trehalose–succinic acid–C11–C10–C7. The remaining components had a lower and more constant concentration during growth. Worth to note, the trehalose tetraesters of *R. erythropolis* 51 T7 differed with the results of a previous report in the main component (Espuny et al. 1996). The authors postulated that this may have resulted from variations in the carbon source used.

3.2.3 Octaacyltrehalose from *Rhodococcus* sp. H13-A

Growing with hexadecane, *Rhodococcus* sp. H13-A produced a mixture of trehalose lipids with one major and ten minor components(Singer et al. 1990). The components of hydrophobic tail are relatively complex, constituting of normal C10–C22 saturated and unsaturated fatty acids, C35–C40 mycolic acids, hexanedioic and dodecanedioic acids, and 10-methyl hexadecanoic and 10-methyl octadecanoic acids. The major glycolipid was identified as 2,3,4,6,2′,3′,4′,6′-octaacyltrehalose, plus minor components of di-, tetra- and hexa-acyltrehalose derivatives.

4 Physicochemical Property

Physicochemical properties of trehalose lipids have been mainly examined in the genus *Rhodococcus,* while the glycolipids from mycobacteria are seldom investigated in this aspect due to their long hydrophobic moiety tightly attached on cell wall and limitations as a practical biosurfactant.

Recently, some trehalose lipids come to purification (Tuleva et al. 2008, 2009; Marques et al. 2009; Tokumoto et al. 2009; Peng et al. 2007; Niescher et al. 2006; Kretschmer et al. 1982; Kretschmer and Wagner 1983; Kim et al. 1990). Most of them showed strong surface activity by lowering water surface tension below 30 mN m^{-1} (ranging from 19 to 43 mN m^{-1}), lowering the interfacial tension against hexadecane (decane or kerosene) to 5 mN m^{-1}, even <1 mN m^{-1} (Table 1), while the CMC values can reach 0.7 mg l^{-1} in case of dicorynomycolates (Kretschmer and Wagner 1983), and the CMC values of most trehalose lipids varied from 0.7 to 37 mg l^{-1}. Examples are as follows.

The trehalose corynomycolates generated by *R. erythropolis* DSM43215 showed extremely low CMC in high-salinity solutions, and the interfacial properties were stabile in solutions with a wide range of pH and ionic strength(Kretschmer et al. 1982). The dicorynomycolates reduced interfacial tension from 44 to 18 mN m^{-1} and are less sensitive to salt concentrations than synthesized surfactants, therefore of potential application in enhanced oil recovery.

Succinoyl trehalose lipids (STL) from *R. erythropolis* SD-74 are quite efficient to lower water surface tension. The estimated CMC and γ_{CMC} values for STL-1 were 5.6×10^{-6} M (equivalent to 5 mg l^{-1}) and 19.0 mN m^{-1}, and those for sodium salt (Na-STL-1) were 7.7×10^{-6} M and 23.7 mN m^{-1}, respectively. Thus, STL-1 holds an excellent surface activity at remarkably low concentrations (Tokumoto et al. 2009).

The glycolipid synthesized by *Rhodococcus* species H13-A (Singer et al. 1990) exhibited a CMC of 1.5 mg l^{-1} and minimum interfacial tension value of 0.02 mN m^{-1} against decane, even much lower in interfacial tension to 6×10^{-5} mN m^{-1} in the presence of pentanol. In the case of the glycolipids purified from *M. luteus* BN56, they own CMC value of 25 mg l^{-1} and can reduce the surface tension of water to 24.1 mN m^{-1} and lower the interfacial tension against hexadecane to 1.7 mN m^{-1}. In addition, they have emulsion-stabilizing activity.

5 Biological Activity

Biosurfactants play an essential role in bacterial swarming motility and participate in cell signaling and differentiation as well as in biofilm formation. In addition, they can increase the bioavailability of hydrophobic substrates, trap heavy metals, and function as an antibiotic (Singh and Cameotra 2004; Rodrigues et al. 2006). Trehalose lipids accept the general merits being a biosurfactant. Moreover, some particular functions also exist, and worthy to be noted.

5.1 *Trehalose Lipids in Mycobacteria*

Trehalose lipids in mycobacteria draw attentions due to their role in pathogenicity and molecular immunology (Imasato et al. 1990; Ryll et al. 2001; Hunter et al. 2006; Ortiz et al. 2008, 2009; Zaragoza et al. 2009). TDM (cord factor) is the most abundant, granulomagenic, and toxic lipid in the cell surface of virulent *M. tuberculosis*(Hunter et al. 2006). TDM has been identified as the factor responsible for cord formation. It has been also detected in nontuberculous mycobacteria, including *M. avium* complex species (Fujita et al. 2005). In the mycobacterial cell envelope, together with arabinogalactan mycolate and trehalose monomycolate, TDM forms an integral part of the cell wall skeleton, resulting in highly hydrophobic cell surface properties and acid-fastness (Barry et al. 1998). In addition, it is believed that TDM is responsible for the low permeability of the membranes conferring appreciable drug resistance to the organisms.

On the other hand, TDM and its stereoisometric derivatives (TDCMs) were evidenced to inhibit tumor metastasis, and TDCMs are more potential to suppress tumor growth and inhibit tumor metastasis than TDM (Watanabe et al. 1999).

5.2 *Trehalose Lipids from* Rhodococcus

Beside their known industrial applications, trehalose lipids from *Rhodococcus* recently attracted attention due to their functions in cell membrane interaction and the prospects as a therapeutic agent (Isoda et al. 1995; Zaragoza et al. 2009, 2010; Aranda et al. 2007; Harland et al. 2009; Ortiz et al. 2008, 2009; Imasato et al. 1990).

- The succinoyl trehalose lipid (STL-1) of *R. erythropolis* SD-74 shows effects on cell differentiation. Isoda et al. (1995) reported that STL-1 markedly inhibited the growth of a human monocytoid leukemic cell line and induced its morphological alteration along a monocyte-macrophage lineage. STL-1 markedly increased differentiation-associated characteristics in macrophage, such as phagocytic activities in U937. Furthermore, the U937 cells activated with STL-1 exhibited cytotoxic activity against human carcinoma cell line, while it has low cytotoxicity against normal human cells (Isoda et al. 1995).
- Hemolytic activity of a succinoyl-containing trehalose lipid: the trehalose lipid produced by *Rhodococcus* sp. was observed to cause the swelling of human erythrocytes followed by hemolysis at concentrations well below its CMC value. It works by a colloid-osmotic mechanism, most likely by formation of enhanced permeability domains, or "pores" enriched in the biosurfactant, within the erythrocyte membrane (Zaragoza et al. 2010).
- Interactions with phosphatidylethanolamine membrane: Ortiz et al. (2008) purified a trehalose lipid from *Rhodococcus* sp. and examined its effect on the thermotropic and structural properties of phosphatidylethanolamine membranes of different chain length and saturation. They find that the trehalose lipid

presents good miscibility both in the gel and the liquid crystalline phases of the membrane and affects the gel to liquid crystalline phase transition. The trehalose lipid was suggested to incorporate into the membrane bilayers and produce structural perturbations, which might affect the function of the membrane. Similar conclusions were drawn based on the results with phosphatidylserine membranes (Ortiz et al. 2009):

- Mechanism of membrane permeabilization by the STL (Zaragoza et al. 2009): The partition constant of the trehalose lipid to palmitoyloleoylphosphatidylcholine membranes indicates that trehalose lipid behaves as a weak detergent, which prefers membrane incorporation over micellization. Addition of the trehalose lipid to large unilamellar vesicles results in a size-selective leakage of entrapped solutes to the external medium. Further studies support that the trehalose lipid incorporates into phosphatidylcholine membranes and segregates within lateral domains, which may constitute membrane defects or "pores."
- Synthetic trehalose glycolipids confer desiccation resistance to supported lipid monolayers (Harland et al. 2009). Harland et al. (2009) presented the first synthetic trehalose glycolipids capable of providing desiccation protection to membranes of which they are constituents. They deduced that interactions between the trehalose headgroup and surrounding molecules are the determining factor in dehydration protection.

6 Biosynthesis

The biosynthesis pathways of biosurfactant have been extensively studied in rhamnolipids and lipopeptides, and related genes have been determined in some cases (Nakano et al. 1991, 1992; Ochsner et al. 1994; Campos-Garcia et al. 1998). However, the corresponding researches on trehalose lipids are hard to be retrieved from literatures.

With an aim to clarify the synthesis pathway of trehalose dicorynomycolates, Kretschmer and Wagner (1983) characterized biosynthetic intermediates of the trehalose lipids in *R. erythropolis* DSM 43215. Free corynomycolic acid intermediates were observed. The corynomycolic acid moiety and the trehalose moiety therefore are synthesized independently and are subsequently esterified. Trehalose is first esterified to form the monomycolate and then the dimycolate. Based on the intermediates, a biosynthetic pathway was suggested (Fig. 9) (Kretschmer and Wagner 1983).

7 Production

The production of most trehalose lipids is growth-associated, whereas production is also observed under growth-limiting conditions or by resting cells. Compared to rhamnolipids, the yield of trehalose lipids reported in the literature is lower, usually below 3 g l^{-1}, considering the production of purified products.

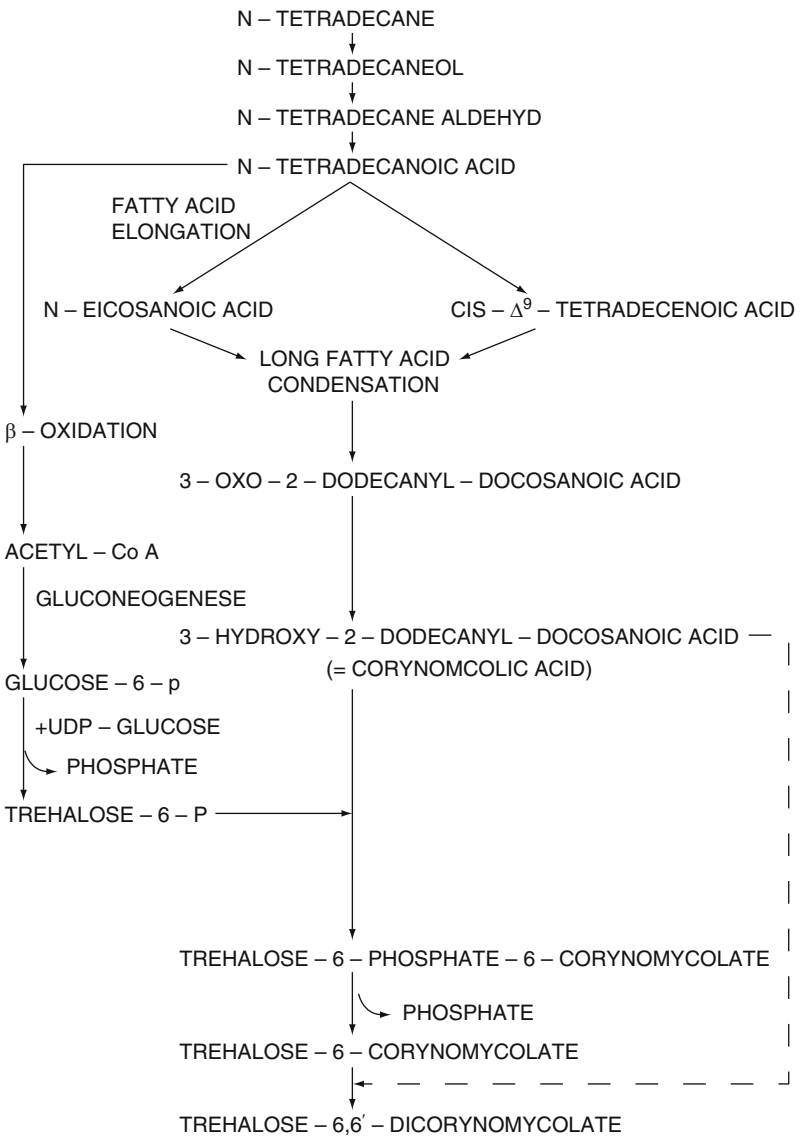


Fig. 9 Trehalose dicorynomycolate synthesis pathway from *n*-tetradecane by *Rhodococcus erythropolis* DSM 43215 (Kretschmer and Wagner 1983)

7.1 Substrates Used for Trehaloselipids Production

- *n*-alkanes: In the case of oil degrading bacteria of *Rhodococcus, n*-alkanes are usually the optimal substrates for glycolipids production. Although bacteria of *Rhodococcus* can utilize alkanes of a wide length range, C14 and C16 alkanes

seem to be the best for high yielding (Niescher et al. 2006), especially for the trehalose lipids with medium length chains(Tokumoto et al. 2009).

- Nonalkanes: Trehalose dicorynomycolates have also been produced in the absence of *n*-alkanes or other lipophilic carbon sources by *Brevibacterium vitarumen* 12143 (Laneelle and Asselineau 1976), *Corynebacterium diphtheriae* (Adam et al. 1967), and different pathogenic *Mycobacteriaceae* (Asselineau and Asselineau 1978).

7.2 Cell Wall Association

The usual pattern of production of nonionic trehalose glycolipids is generally cell wall-associated (Suzuki et al. 1969; Kretschmer et al. 1982). Usually, the lipids of long-chain fatty acids tend to be cell wall-associated. Especially at early stage of cultivation, the products mainly attach to the cell wall.

Growing on *n*-alkanes as a sole source of carbon and energy, *R. fascians* produced both an extracellular and cell-bound surface-active mixture of trehalose lipids that reduced the surface tension of water to 32 mN m (Yakimov et al. 1999). When *R. erythropolis* strain 51 T7 was cultivated with both alkanes and waste lubricant oil as a sole carbon substrate, it produced extracellular glycolipids with surface activity. *Rhodococcus* sp. strain 51 T7 produced trehalose lipids between 0.48 and 1.12 g l^{-1}(Espuny et al. 1996). However, in a previous report of this strain (named Coryneform bacterium 51 T7 at first), trehalose lipids are associated mainly with the cell wall on incubation up to 80 h. After 80 h, the trehalose lipids are mainly extracellular (Martin et al. 1991).

7.3 Growth-Associated Production

Growth-associated production of trehalose lipids is popular (Table 1), such as the production of a trehalose dicorynomycolate in *R. erythropolis* DSM43215 (Rapp et al. 1979). The production of a trehalose dicorynomycolate in *R. opacus* 1CP is also growth-associated (Niescher et al. 2006) (Fig. 10).

However, the formation of anionic trehalose ester by *R. erythropolis* DSM43215 seems to be uncoupled from growth and occurs in the stationary phase (Ristau and Wagner 1983; Kim et al. 1990). The growth-uncoupled production also was observed in a paraffin-oxidizing bacterium *R. erythropolis* cultivated in shake flasks on a mixture of C14 and C15 *n*-alkanes or kerosene. The growth-limiting conditions, such as nitrogen deficiency, caused the formation of α, α-trehalose-2,3,4,2′-tetraesters.

Contrarily, also being a trehalose 2,3,4,2′-tetraester, one of the *Rhodococcus* sp 51 T7 is produced in growth-associated mode. When grown on hydrocarbon, cells were highly segmented and accumulated lipid granules in the cytoplasm. Under

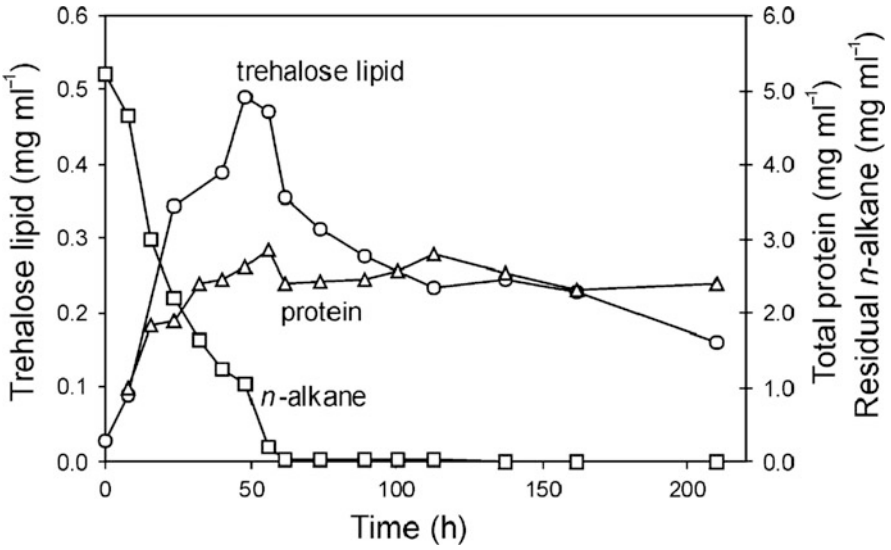


Fig. 10 Correlation of cell growth (protein), alkane consumption, and glycolipid formation of *R. opacus* 1CP (Niescher et al. 2006). Batch cultures (20 ml), each containing mineral medium and 1% (w/v) *n*-tetradecane, were inoculated with identical amounts of glucose-grown *R. opacus* 1CP and incubated at 30°C with constant shaking (130 rpm)

optimal concentrations of sodium nitrate, potassium phosphate and iron (2.5 g l^{-1}, 1.5 g l^{-1} and 0.01 g l^{-1}, respectively), production was increased from 0.5 to 3 g l^{-1} (Espuny et al. 1996).

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