Screening, production and characterization of extracellular microbial surfactants

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DISSERTATION

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Abstract

Amphiphilic molecules are widespread phenomena in natural systems. They feature surface active characteristics and thus are also termed surfactants. Surfactants represent key building blocks of cell membranes acting as natural barriers and they serve to solubilize hydrophobic compounds in hydrophilic environments. Furthermore surfactants are applied in a wide range of products of daily use. They can be build up of a variety of hydrophobic substances, either naturally derived or based on petrochemicals. Structurally versatile and an interesting group of surfactants are biosurfactants, amphiphilic molecules of natural origin that can either be directly produced by all domains of live, be catalyzed by enzymes from renewable resources or be synthesized from ingredients restricted to natural origin. Microbial communities produce a wide range of secondary metabolites, and the diversity of amphiphilic molecules elucidated within is immense, so are their physiologic properties. The growing interest in biosurfactants is reflected by a incresing amount of published patents (Sekhon and Rahman 2015). The driving force for the discovery and application of novel biosurfactants can be precised in three main reasons:

1 The huge and growing market of surfactants and the resulting economic potential.
2 The great diversity of amphiphilic microbial products with variable structural characteristics and unique properties that is difficult to be achieved by conventional chemically synthesizing.
3 Growing ecological awareness and the drive towards the establishment of a bio-based economy in times with consequently rising oil prices.

This thesis, conducted within the framework of the 'European Research Area – Industrial Biotechnology (ERA-IB) project BioSurf - Novel Production Strategies for Biosurfactants', focuses on the screening for, and the isolation of biosurfactant producing strains as well as the production, purification and structural elucidation of compounds produced with the aim to find yet unrevealed microbial surfactants.

Chapter 1 introduces surfactants and in particular biosurfactants. Physiological and structural characteristics, applications as well as concepts for screening (Müller et al. 2012) and structural elucidation are explained from a molecular, a human and a microbial point of view. The motivation for this work as well as the research proposal is presented.

Chapter 2 elaborately examines surfactants that are derived from an extraordinary group of prokaryotes and the number one producers of secondary metabolites: the class *Actinobacteria*. In the first section, a comprehensive overview of the knowledge available on actinobacterial surfactants, their types, structures, applications and original habitats of their producers is given aiming to deliver insight into the extensive nature of the biosurfactants (Kügler et al. 2015c). In the following sections, two case studies are dedicated to the finding of novel microbial surfactants from *Tsukamurella* spp. member of the class *Actinobacteria*. The first study addresses the production, the structural determination and the examination of trehalose lipids (Kügler et al. 2014). In the second study, a rare class of biosurfactants containing aromatic moieties is detected and partially elucidated (Kügler et al. 2015b).

Chapter 3, in its first two sections addresses the isolation of, and screening for novel surfactant producing prokaryotes from populations of circumspectly chosen ecological niches. Unknown biosurfactant structures seem to be more likely to be found within strains not yet described as surfactant producing organism. Consequently these are expected to occur in habitats not yet examined for their presence. One far distanced and extreme example of these habitats is a Dry Valley of Antarctica, where surfactant producing species were isolated from soil sampled underneath seal carcass skin. Another example, in much closer proximity are surfactant producing species isolated from peat, rich in humic substances and derived from a raised bog in the northern

Black Forest. The third section of this chapter is dedicated to *Rouxiella* sp. DSM 100043 a peat land bog isolated strain that is used for the recovery of glycolipids via foam-fractionation. These glycolipids were elucidated to contain glucose amd the rare sugar talose as hydrophilic moieties (Kügler et al. 2015a), the latter not yet described within microbial glycolipids.

Zusammenfassung

Amphiphile Moleküle sind in der Natur weit verbreitet, wirken grenzflächenaktiv und werden daher auch als Tenside bezeichnet. Tenside fördern die Löslichkeit hydrophober Moleküle in hydrophilem Millieu und als elementarer Bestandteil unserer Zellmembranen agieren sie als natürliche Grenze. Sie werden alltäglich in einer Vielzahl von Produkten angewandt und können aus verschiedenen Inhaltstoffen aus natürlichem oder petrochemischem Ursprung hergestellt werden. Eine strukturell vielseitige und dadurch interessante Gruppe der Tenside sind amphiphile Moleküle natürlichen Ursprungs, sogenannte Biotenside. Diese werden von allen Domänen des Lebens gebildet und können durch Enzyme oder chemisch aus nachwachsenden Rohstoffen katalysiert werden. Mikroorganismen produzieren etliche unterschiedliche Sekundärmetabolite. Die amphiphilen unter ihnen weisen vielseitige Strukturen und physiologische Eigenschaften auf. Die immense Zahl von Patenten auf dem Gebiet der Tensidforschung spiegelt das wachsende Interesse an der Suche nach neuen Biotensiden sowie deren potentiellen Anwendungen wider (Sekhon and Rahman 2015). Drei Hauptargumente treiben derzeit Forschung und Investitionen an:

1 Der große Wachstumsmarkt von Tensiden und das daraus resultierende ökonomische Potential.
2 Die große Vielfalt und die verschiedenen Eigenschaften von strukturell unterschiedlichen mikrobiellen Biotensiden, welche nur schwer durch herkömmliche chemische Synthesen hergestellt werden können.
3 Ein steigendes ökologisches Bewusstsein und die damit zusammenhängende Entwicklung hin zu einer Bio-basierten Wirtschaft in Zeiten stetig steigender Ölpreise.

Die folgende Dissertation, angefertigt im Rahmen des Forschungsrojektes ‚European Research Area – Industrial Biotechnology (ERA-IB) BioSurf - Novel Production Strategies for Biosurfactants' befasst sich mit dem Screening nach und der Isolierung von Biotensid-produzierenden Mikroorganismen, sowie mit der Produktion, der Aufreinigung und der Strukturaufklärung der gefundenen Produkte mit dem Ziel, bisher unbekannte mikrobielle Tenside zu beschreiben.

Kapitel 1 erörtert die Begriffe Tenside und Biotenside und geht auf deren physiologische und strukturelle Eigenschaften, sowie daraus resultierende Anwendungen ein. Verschiedene Screeningkonzepte (Müller et al. 2012) und Methoden der Strukturaufklärung von Biotensiden werden vorgestellt. Hierbei wird das Thema aus der molekularen, der mikrobiellen und der menschlichen Perspektive betrachtet.

Kapitel 2 beschäftigt sich mit einer sehr außergewöhnlichen Gruppe von Prokaryoten, welche für Ihre umfangreiche Sekundärmetabolitproduktion bekannt ist: die Klasse *Actinobacteria*. Der erste Teil des Kapitels gibt einen ausführlichen Überblick über die Vielfalt bekannter aktinobakterieller Tenside. Die verschiedenen Typen und Strukturen sowie deren Anwendung und die ursprünglichen Habitate der Produzenten werden in einer umfangreichen Zusammenstellung wiedergegeben (Kügler et al. 2015c). Die beiden folgenden Teile widmen sich zwei Fallstudien, welche neuartige mikrobielle Tenside aus *Tsukamurella* spp., Genera innerhalb der Klasse *Actinobacteria,* beschreiben. Während sich die erste Studie mit der Produktion und Strukturaufklärung von Trehaloselipiden und der physiologischen Untersuchung der gebildeten Produkte (Kügler et al. 2014) befasst, liegt der Fokus der zweiten Studie auf der partiellen Strukturaufklärung einer seltenen Klasse von Tensiden, welche aromatische Molekülbestandteile aufweist (Kügler et al. 2015b).

Kapitel 3 befasst sich in den ersten beiden Teilen mit der Isolierung von und dem Screening nach tensidbildenden Prokaryoten, welche aus sorgfältig ausgesuchten ökologischen Nischen stammen. Eine größere Wahrscheinlichkeit hierbei ein bisher unbeschriebenes Tensid zu finden besteht bei Stämmen aus denen noch keine Biotenside isoliert wurden. Diese wiederum finden sich am ehesten in bisher noch nicht

untersuchten Habitaten. Ein extremes Beispiel dieser Habitate ist ein Trockental der Antarktis, aus dem Bodenproben unterhalb von Seelöwenkadavern entnommen und auf Tensidbildner untersucht wurden. Auch im näheren Umfeld lassen sich unbekannte Strukturen finden, wie ein Beispiel von tensidbildenden Bakterien, die aus dem Torf eines Hochmoores im Nordschwarzwald isoliert wurden, zeigt. Die Verwendung einer dieser Torfisolate, *Rouxiella* sp. DSM 100043 zur Produktion von Glycolipiden, welche durch Schaumfraktionierung abgetrennt wurden, wird im dritten und letzten Teil des Kapitels dargestellt. Die hydrophilen Teile dieser Tenside konnten als Glukose und als Talose identifiziert werden (Kügler et al. 2015a) wobei Talose ein seltener und bisher noch nicht in mikrobiellen Glycolipiden beschriebener Zucker ist.

Publications

This thesis is partly based on research articles and reviews published accompanying in peer-reviewed scientific journals. Extracts and full articles are embedded with adjusted formatting. Extent, contribution and reference are stated at the beginning of each study.

Papers published during the thesis timeperiod that are integrated in this thesis:

Müller MM, Kügler JH, Henkel M, Gerlitzki M, Hörmann B, Pöhnlein M, Sydatk C Hausmann R (2012). Rhamnolipids–next generation surfactants? *Journal of Biotechnology*, *162*:366-380

in chapter 1a **A molecular perspective**

III

Kügler JH, Le Roes-Hill M, Syldatk C, Hausmann R (2015). Surfactants tailored by the class *Actinobacteria*. *Frontiers in Microbiology*, 6:212

as chapter 2a **Surfactants tailored by the class *Actinobacteria***

Kügler JH, Muhle-Goll C, Kühl B, Kraft A, Heinzler R, Kirschhöfer F, Henkel M, Wray V, Luy B, Brenner-Weiss G, Lang S, Syldatk C, Hausmann R (2014). Trehalose lipid biosurfactants produced by the actinomycetes *Tsukamurella spumae* and *T. pseudospumae*. *Applied Microbiology and Biotechnology*, 98:8905-8915.

as chapter 2b **Trehalose lipid biosurfactants produced by the actinomycetes *Tsukamurella spumae* and *T. pseudospumae***

Kügler JH, Kraft A, Heißler S, Muhle-Goll C, Luy B, Schwack W, Syldatk C, Hausmann R (2015). Extracellular aromatic biosurfactant produced by *Tsukamurella pseudospumae* and *T. spumae* during growth on n-hexadecane. *Journal of Biotechnology*, 211:107-114

as chapter 2c **Extracellular aromatic biosurfactant produced by *T. pseudospumae* and *T. spumae* during growth on n-hexadecane**

Kügler JH, Muhle-Goll C, Hansen SH, Völp AR, Kirschhöfer F, Kühl B, Brenner-Weiss G, Luy B, Syldatk C, Hausmann R. Glycolipids produced by *Rouxiella* sp. DSM 100043 and isolation of the biosurfactants via foam-fractionation. *AMB Express*, in press. Accepted December 5th 2015

in chapter 3c **Glycolipids produced by *Rouxiella* sp. DSM 100043 and isolation of the biosurfactants via foam-fractionation**

Papers published during the thesis timeperiod that are not integrated in this thesis:

Henkel M, Müller MM, Kügler JH, Lovaglio RB, Contiero J, Syldatk C and Hausmann R (2012). Rhamnolipids as biosurfactants from renewable resources: Concepts for next-generation rhamnolipid production. *Process Biochemistry*, 47:1207-1219

Oey M, Ross IL, Stephens E, Steinbeck J, Wolf J, Radzun KA, Kügler J, Ringsmuth AK, Kruse O, Hankamer B (2013). RNAi knock-down of LHCBM1, 2 and 3 increases photosynthetic H2 production efficiency of the green alga *Chlamydomonas reinhardtii*. *PloS one*, 8:e61375.

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Kügler JH, Hansen SH, Völp, AR, Syldatk C, Hausmann R (2013). From isolated producer strains to heterologous producing clones: Biosurfactants from a peat-bog sample. *DECHEMA international conference 'Biosurfactants – Challenges and Perspectives' 16.-17.05.2013, Frankfurt am Main, Germany*

Conference paper:

Floris R, Scanu G, Fois N, Kuegler J, Malavenda R (2014). Bioactive compounds production by bacteria isolated from the intestinal gilthead sea bream (*Spaurus aurata* linnaeus, 1758) from coastal marine environments of Sardinia Island (Italy). *45° Congresso della Societá Italiana di Biologia Marina, 19.-23.05.2014,Venice, Italy*

Poster presentations:

Henkel M, Kügler JH, Syldatk C and Hausmann R (2012). Biosurfactants from renewable resources: concepts for next-generation rhamnolipid production. *DECHEMA international conference 'Industrial use of renewable raw-materials', 14.-15.02.2012, Frankfurt am Main, German*

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Thies S*, Kügler JH*, Zwick M, Syldatk C, Rosenau F, Jäger KE, Hausmann R (2013). Heterologous production of serrawettin, a promising biosurfactant. *DECHEMA international conference 'Biosurfactants – Challenges and Perspectives' 16.-17.05.2013, Frankfurt am Main, Germany* *contributed euqally to this presentation

Kügler JH, Völp AR, Hansen SH, Syldatk C, Hausmann R (2013). Accessing novel biosurfactant compounds from cultivation based and environmental approaches. *Association for general and applied microbiology VAAM 'annual meeting', 10.-13.03.2013 Bremen, Germany*

Floris R, Scanu G, Fois N, Kügler JH, Malavenda R (2014). Bioactive compounds production by bacteria isolated from the intestinal gilthead sea bream (*Spaurus aurata* linnaeus, 1758) from coastal marine environments of Sardinia Island (Italy). *45° Congresso della Societá Italiana di Biologia Marina, 19.-23.05.2014, Venice, Italy*

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Contents

Microbial surfactants

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JK has concepted and written text passages of section 3.2.1 'Screening concepts' and participated in compilation of table 4 'Comparison of various screening methods for biosurfactants production modified according to Walter et al. (2010).

1a A molecular perspective

Surfactants

The expression surfactant is a compressed version of the words 'surface active agent'. No matter if naturally produced or chemically synthesized, it describes compounds that are surface active, act wetting, emulsifying and/or dispersing. The molecular character of these molecules is amphiphilic and their structure is classically partitioned into hydrophilic and lipophilic moieties (Figure 1.1). Due to their lipophilic moiety, surfactants tend to accumulate at interphases of aqueous liquids or in the transition of air to liquid or liquid to solid. The surface between the phases is reduced due to interfacial adsorption of the surfactants. This, in turn changes wetting and dispersing abilities of the solution formed. With increasing concentration, surfactants expand at interphases up to a certain concentration whereat a saturation of the surface is reached. To avoid contact of the lipophilic moiety with its aqueous surrounding, surfactants self-aggregate into lamellas, micelles or vesicles. This reversible rearrangement is performed once exceeding the critical micelle concentration (cmc), the point marking a saturation of the surface and the lowest possible surface tension that can be reached (Figure 1.1). The cmc directly depends on temperature of, and ion concentration in

the liquid it is solved and further on type and structure of the surfactant present thus displaying an individual characteristic for each surfactant molecule.
The lipophilic tail of surfactants is typically composed of alkyl chains that vary in length but in average consist of 8 to 20 carbon atoms. According to their charge, surfactants are classified into the four major groups anionic, cationic, non-ionic and amphoteric as displayed in Table 1.1. Depending on their molecular weight, they can be classified as low molecular surfactants typically not exceeding 1000 Da and high molecular weight surfactants composed of oligomeric or polymeric compounds.

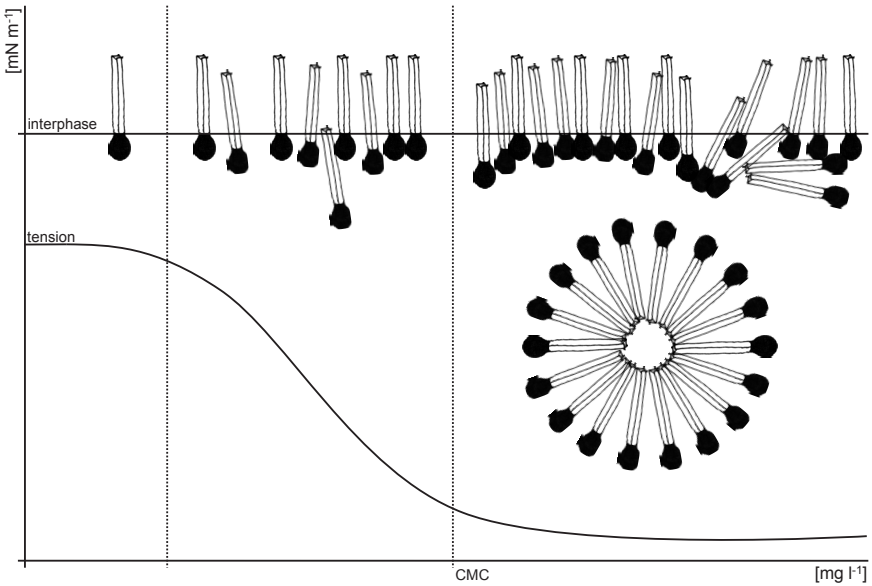

Figure 1.1: Scheme of concentration dependent accumulation of surfactant molecules at interphases and alteration in its tension caused. Black dots: hydrophilic moiety, white rods: lipophilic moiety; aqueous phase is plotted beneath the interphase. CMC: critical micelle concentration; the interphase is saturated and surfactant molecules favor the formation of micelles, the point exhibiting the lowest surface tension

Biosurfactants

Surface active metabolites that are naturally produced, mainly by plants or various microorganisms are termed biosurfactants. They have been identified to occur in all domains of live: *Bacteria*, *Archea* (Chooklin et al. 2014) and *Eucaryota*. Microbial derived, they either keep adhered to the membrane or are excreted by the cells. Varying in hydrophilic and lipophilic moiety, nature holds the capacity to host an uncountable amount of different structures of biosurfactants, mainly of anionic or non-ionic nature. Cationic surfactants are very rarely found as microbial metabolites, one example is polymyxin B, an antibiotic cationic lipopetide. Biosurfactants can, according to their hydrophilic moiety be divided into the low molecular weight structural classes of glycolipids and lipopeptides, as well as into the high molecular weight polymeric biosurfactants (Lang and Trowitzsch-Kienast 2002; Ron and Rosenberg 2001). Best known biosurfactants are glycolipids with sophorose, rhamnose, trehalose or mannosyl erithritol as sugar moiety. Lipopeptides often contain cyclic amino acid sequences of variable lengths that are assembled by non-ribosomal peptide synthetases, but also linear lipopeptdies occur. In both cases the lipophilic moiety, named aglycon remains highly versatile. Polymeric biosurfactants often are polysaccharide-protein complexes with strong bioemulsifying properties (Uzoigwe et al. 2015). Chapter 2a gives an overview and discusses the vast variety of biosurfactant structures that are produced within species of the class *Actinobacteria*.

Table 1.1: Classification of surfactants according to charge

Type	Property	Functional group	Example
Anionic surfactant	negative charge at hydrophilic group	carboxylates (-COO^-), solfonates (-SO_3^-) and sulfates (-SO_4^{2-})	Sodium dodecyl sulfate (SDS)
Cationic surfactant	positive charge at hydrophilic group	quaternary ammonia groups (^+N-R_4)	dodecyl trimethylammonium bromide (CTAB)
Nonionic surfactant	no charge	alcohols (-OH) and ether (-O-)	alkyl ethylene glycol
Amphoteric or zwitterionic surfactant	both positive and negative charge at hydrophilic group	combination of anionic and cationic groups	phosphatidylcholine

Detection of microbial surfactants

The main characteristics of biosurfactants are lowering of surface tension, the capacity to emulsify hydrocarbons and the ability to dissolve oil or lipids. These can be detected by various methods applied in screening experiments. Requirements for the revealing of biosurfactant producing species are methodologies that are far-reaching to detect various kinds of different surfactants types in small quantities and short time. For the pre-selection of interesting microorganisms, high throughput applicable tests are favoured as fast screening methods allow the screening of entire bacterial libraries or large numbers of isolated microorganisms in a relatively short time. Walter et al. (2010) and Satpute et al. (2008) compare different screening approaches. Several methods have been adapted for high throughput screening. Although these methods

Table 1.2: Screening methods for the detection of biosurfactants refined according to Walter et al. (Walter et al. 2010) and Müller et al. (Müller et al. 2012).

Screening method	Detection property	High throughput	References
surface/interfacial tension measurement	surface activity	no	Cooper and Goldenberg 1987; Syldatk et al. 1985
atomized oil assay	surface activity	yes	Burch et al. 2010
drop collapse assay	surface activity	yes	Jain et al. 1991
microplate assay	surface activity	yes	Chen et al. 2007; Cottingham et al. 2004; Vaux and Cottingham 2007
penetration assay	solubilization	yes	Maczek et al. 2007
oil spreading assay	solubilization	no	Morikawa et al. 2000
emulsification capacity assay (E24)	emulsification	no	Cooper and Goldenberg 1987
solubilization of crystalline anthracene	solubilization	potentially	Willumsen and Karlson 1997
bacterial adhesion of hydrocarbons assay	several	no	Rosenberg 2006; Rosenberg et al. 1980
hydrophobic interaction chromatography	amphiphilic nature	no	Smyth et al. 1978
replica plate assay	several	no	Rosenberg 1981
salt aggregation assay	several	potentially	Lindahl et al. 1981
CTAB agar assay	anionic surfactants	yes	Pinzon and Ju 2009b; Siegmund and Wagner 1991
methylen blue colorimetric assay	anionic surfactants	yes	Pinzon and Ju 2009a
hemolysis assay	hemolysis	yes	Mulligan et al. 1984
orcinol assay	reducing sugars	potentially	Chandrasekaran and Bemiller 1980
anthrone assay	reducing sugars	potentially	Chayabutra et al. 2001; Hodge and Hofreiter 1962
thin-layer chromatography	amphiphilic nature	potentially	Satpute et al. 2010

have a rather qualitative than quantitative character, a fast pre selection can be made, followed by extensive measurements of the positive hits. These procedures significantly speed up the screening process towards the detection of biosurfactant producer strains. Different screening approaches and their capability to be used in high throughput screening are listed in table 1.2.

Cottingham et al. (2004) used micro well plates to determine the surface tension by using a modified plate reader that detects the curvature of the surface caused by low surface tension (Cottingham and Vaux 2007). Chen et al. (2007) further developed this method by identifying single biosurfactant-producing strains from a mixture of several microorganisms using serial dilutions and by holding the microtiter plate upon a rectangular grid and observing changes in the surface tension from a bird's eye view. Advancement of the drop collapse technique to detect extracellular biosurfactants is given by a method described by Burch et al. (2010) that benefits from a repelling effect of atomized paraffin droplets of surfactant producing colonies. Interfusion caused by the bleeding of stained culture supernatant into a defined drop of oil has been demonstrated as a detection method suitable for automated screening (Maczek et al. 2007).

Slightly greater volumes of supernatant are necessary to examine the emulsification capacity of hydrophobic substances after mixing of supernatant with hydrocarbons. Biosurfactants can be extracted from the supernatant using various kinds of solvents. The hydrophobic part of the molecule is carried within the hydrophilic part into the solvent. Using thin layer chromatography, these substances can be distinguished according to their hydrophobicity, and be visualized and characterized by various staining methods.

The results of the screening methods applied are strongly influenced by salt concentrations, in particular for anionic surfactants as well as the temperature, particularly playing an effect for non-ionic surfactants (Butt et al. 2004).

1b A human perspective

8 *Applications*

Amphiphilic molecules are applied throughout our everyday life and are essential in a wide range of industrial processes. Biosurfactants hold a great potential to replace chemically synthesized surfactants as major constituents or additives in products ranging from various cleansers to stabilizers and emulsifiers in creams used in cosmetics- and pharmaceutical applications as well as in food industry and as biocides. Further applications are found in bioremediation, the production of paper and varnish and in enhanced oil recovery processes. Many biosurfactants are described to exhibit bioactive properties thus are interesting for biomedical applications (Gudiña et al. 2013). Since it is difficult to conclude to physical performances of a biosurfactant from its chemical structure, several gram amounts of the different compounds are necessary for extensive testing of potential applications (Hausmann and Syldatk 2014).

Within well known low molecular weight biosurfactants, some particular structures have already been used in product formulations or have been extensively tested for. A large amount of patents for the use of biosurfactant in products and processes and their production and purification methodologies have been published. Good overviews are

given by Hames et al. (2014) and Sekhon and Rahman (2015). Well examined is the class of glycolipids. Sophorose-lipids produced by the yeast *Starmerella* (*Candida*) *bombicola* are currently used in detergents; rhamnose-lipids produced by *Pseudomonas* spp. have been tested for various applications including detergents and insecticides. The lipopeptide daptomycin is used as an antibiotic agent, more extensively discussed in chapter 2a.
Main advantages for the utilization of biosurfactants over chemically synthesized surfactants are their high activity at low cmc and their versatile structural properties. The revealing of novel biosurfactant structures holds the potential to find molecules that have superior properties to be made use of in very particular products that demand particular physical performances. An example is given by defoaming or low-foaming surfactants necessary for its use in laundry machines and the desire for high-foaming dish-washing liquid preferred by consumers.

Economic potential

Surfactants share a huge market. According to the European committee of organic surfactants (CESIO), 2.98 million tons of surfactants have been produced in the EU in 2013 (CESIO 2013), the overall global surfactant market generated a revenue of 27 billion dollars in 2012 (Geys et al. 2014). Despite the huge theoretic potential of biosurfactants in various applications, its actual implementation relies on only few product formulations. This can be traced back to high costs as the main reason. The low economic feasibility can be cut down to high costs in production processes and product recovery as well as low biosurfactant yields of the producing organism. Further, the classification of many surfactant producer strains as facultative pathogens (risk group 2 organisms according to the German Technical Rules for Biological Materials (TRBA)) (DSMZ webpage) are connected to expensive precautions necessary during the production process. Recent aims of research and development focuses on the utilization of cheap substrates gained from waste material (Henkel et al. 2012), the optimization of fermentation conditions and product recovery. More efficient production can as well be tackled by the screening for novel wild type producer strains with higher yields (Geys et al. 2014) and by metabolic engineering to increase the yield of various producer strains. Practical usage of biosurfactants is promising and industrial interest

arises although their actual presence on the market is negligible (Fleurackers 2014).

Advantages of biosurfactants

Surfactants form a considerable destination for crude oil and petrochemical derived products (Pérez-Carrera et al. 2010). Its widespread utilization has, for a long time resulted in a thread to the environment. The presence of residual surfactants in waste water effluents has let to pollution in the different environmental compartments soil, water and sediment (Ivankovic and Hrenovic 2010). To overcome pollution, most petrochemical based surfactants that are used today are based on even numbered straight hydrophobic moieties thus readily-biodegradable and only show low toxicity to the environment compared to branched surfactants commonly used in the past. Examples for easily biodegradable surfactants are alkyl polyglycosides (APG), C12 alkyl based sulfates, ethoxy sulfates, benzen sulfonates or alcohol ethoxylates (Pérez-Carrera et al. 2010). However, in many applications these structures are not always optimal from a performance point of view. Still they partly rely on fossil-based materials and in the search for alternatives the impact of renewable materials as feedstock for the production of surfactants and other valuable products arises. The superiority of biosurfactants over conventional chemically synthesized is most importantly the great diversity of the different structures that are produced. About 2000 different structures of amphoteric microbial metabolites are reported to occur (Hausmann and Syldatk, 2014). Further, a direct utilization of biosurfactant producing organisms can be used for the bioremediation of hydrocarbon contaminated soils and the removal of heavy metals in bioremediation processes. This displays a pressing issue for the utilization of both, biosurfactants and/or biosurfactant producing organisms for an environmental justifiable removal of contaminations aroused from recent oil spills by tankers and large sea contaminations by drilling rigs (Jackson et al. 2015).

Biotechnological production

Exploratory urge for microbial metabolites and their potential applications in various fields has resulted in different areas of exploitation of microbial surfactants. Microorganisms still remain the main promising source for the discovery and production of novel metabolites (Berdy 2005). Biotechnological production processes are optimized for maximum yields by feeding microorganisms with extensively chosen nutrients, by using sophisticated devices with parameters adjusted to perfect physical state. Bubbles of air, perfectly shaped and distributed to be consumed turn microorganisms into fast-growing populations and producers of valuable products. That accounts to approximately 0.1 % of them. 1 % will be in frail health, feel stressed and grow poorly. 99 % will just not grow at all. One could conclude human's efforts to imitate microbial niches as a failure. However, examples of successful productions of microbial derived products are plentiful and these numbers rather reveal the unexplored potential hosted by nature. In vitro cultivation will never perfectly imitate a natural niche; missing competition (and symbiosis) to other bacterial species can be named as only one reason for non-cultivability.

One attempt to overcome constraints in cultivation and within limits in the production of valuable secondary metabolites is given by a shortcut that directly takes advantage of the coding regions necessary to produce a biosurfactant. Different habitats are known to inherit a great amount of biosurfactant producing species, including hydrocarbon contaminated soils, seawater sediments, tube worms and spinges (Jackson et al. 2015). A direct isolation of environmental DNA from these habitats and the expression of biosurfactant synthesis cluster in metagenomic libraries can not only lead to a production of secondary metabolites from non cultivable organisms but also reveal potential cryptic information that lead to valuable products.

Purification and structural elucidation

Downstream processes for the recovery of biosurfactants are as versatile as the surfactant structures themselves. Foam-separation, two phase solvent extraction, precipitation, filtration, crystallization, adsorption or chromatography and combinations thereof are

mostly applied (Desai and Banat 1997; Mukherjee et al. 2006). Besides side products or other co-purified components, biosurfactants usually are not produced as single molecules, but in a consortium of structural similar compounds. A crucial step for structural elucidation of unknown compounds is the isolation of single compounds in a pure form. This is mainly achieved by application of various chromatographic procedures after fractionation and extraction or precipitation of the components. Gel filtration or separation according to size is limited for low molecular range surfactants like glycolipids or lipopeptides and mainly used to eliminate contaminants of superior size. Method of choice are chromatographic separations based on hydrophobic interactions, most commonly used are silica 60 and reverse phase C18 columns.

Staining of thin layer chromatography separated compounds hints to information about functional groups present. Further information about other structural groups can be revealed by spectroscopic measurements in ultraviolet or infrared light. Isolated compounds can be analyzed as whole or in a hydrolyzed form to determine structural characteristics of both, hydrophilic and lipophilic moieties. Mass spectrometric measurements give information about size and fragmentation profile of the substances detected. However, full structural elucidation is highly dependent on one and two dimensional nuclear magnetic resonance spectroscopic measurements (NMR) and interpretation of the data acquired.

1c A microbial perspective

Habitats of producer strains

Microorganisms reside in every yet little corner and settle at places that are restricted to all other known living. They are survivalists, their populations rather dorm than die and a wide diversity of the environments they settle in inherit biosurfactant producing microorganisms. They have been isolated from different soils, often contaminated with hydrocarbons, but also sea water, sediments or extreme environments. The overall fraction of biosurfactant producing culturable species among different samples is estimated to be rather small. Bodour et al. (2003) compared twenty different sampling sites and described an average of 3.4% of isolates from undisturbed soils to produce biosurfactants, whereas in hydrocarbon or metal contaminated soils the percentage is 8.4%, slightly higher and thus suggesting a selective pressure of contaminated soils for the production of biosurfactants. Differences in chemical structure and surface properties of the wide range of biosurfactants described suggest advantages for populations in particular ecological niches (Ron and Rosenberg 2001). Even within a producing genus, the range of different types of biosurfactants produced differs. An example are *Pseudomonas* spp. that produce rhamnose containing glycolipids found

within *P. aeruginosa* but also cyclic lipopeptides described to be produced by several species including *P. fluorescens* (Raaijmakers et al. 2006).

Functions for the cell

Microorganisms are making use of surfactants in very much the same way humans do: to overcome solubility problems. They are produced as both, intra- or extracellular compounds and have the role to modify surfaces by wetting, dispersing or emulsifying of compounds in the natural habitat (Holmberg 2001) although they do not seem necessary for growth and survival of the individual species (Hausmann and Syldatk 2014). The production of a biosurfactant requires energy and nutrient resources. The reason for microorganisms to conduct the effort of production must display a distinct advantage for competition and survival. The complex mechanisms of surfactant synthesis are thus linked to ecological circumstances the organisms live in. They might be the reason microorganisms survive in their habitat and are the result of adaptation. Since chemical structures and physiological properties of biosurfactants are divers and since they are produced by such a wide variety of organisms, it is difficult to interpret into a general conclusion about the natural role of surfactants. Hypotheses that suggest the physiological roles of microbial surfactants are described mainly based on the study of pure cultures and will be shortly introduced in the following sections.

Biosurfactants as boundaries and its use in mobility

Surfactants are not only produced as secondary metabolites, they are ubiquitous in natural systems and display the main barrier between an organism and it's surrounding: biological membranes. Besides constituting as boundaries, they are also active at boundaries. An alteration in the hydrophobicity of a cell can be caused by the attachment of surfactants to the cell wall. This not only results in a greater surface area but also alters the living conditions present. A phenomena microorganisms use within the attachment and detachment to surfaces as demonstrated by *Acinteobacter calcoaceticus* (Ron and Rosenberg 2001). Another role of microbial surfactants is

described for the flagella mediated movement over solid surfaces, a property termed swarming. Different non ribosomal produced cyclic lipopeptides mediate swarming in *Serratia* spp. and *Bacillus* spp.. *Pseudomonas* spp. excrete glycolipids to promote swarm extension (Kearns 2010).

Biosurfactants for the acquisition of nutrients

Carbon sources, nitrogen sources and salts are the main nutritional prerequisite for microorganisms. Some need light, some are obliged or obstructed to air or can handle both. All need water to live, maintain and reproduce. Some habitats seem to particularly show a great potential to host surfactant producing microorganisms. Mostly these have a common element: nutrient sources that are difficult to access. The bioavailability of the carbon source can be enhanced by the excretion of surfactants that disperse or emulsify and thus benefit to the cell in two ways: first, it alters the cell hydrophobicity facilitating access and subsequent uptake of the hydrophobic substrates. Second, it feazes the substrate into smaller proportions in proximity to the cell. It has been demonstrated that the solubility and degradation of polyaromatic hydrocarbons has been increased manifold by the presence of the polymeric biosurfactant alasan produced by *Acetinobacter radioresistens* (Rosenberg and Ron 1999). The dependence on a production of rhamnolipid surfactants for the uptake and utilization of hydrophobic carbon sources has been detected in examinations of *P. aeruginosa* mutants unable to grow in n-hexadecane (Koch et al. 1991).

Biosurfactants as an advantage over competitors

Several biosurfactants are antibiotics or inhibit antimicrobial, antifungal and antiviral activities (Rodrigues et al. 2006). Some of these compounds are discussed in more detail in chapter 2a the section on actinobacterial surfactants. Advantages due to the antibiotic properties of extracellular surfactant molecules are exhibited in the natural competition against other species. Bharali et al. (2013) examined the interaction of rhamnolipids produced by *P. aeruginosa* with the cell surfaces of other microorganisms

and described cell lysis of competing *Staphylococcus* sp. and *Klebsialla* sp. if the cmc of rhamnolipids in the cultivation media is reached. This disrupting effect of rhamnolipids is caused by an alteration of the cell hydrophobicity leading to enhanced cell permeability that causes cell damage. Other studies report on changes in membrane functions due to modifications in protein conformations (Gudiña et al. 2013; Van Hamme et al. 2006).

Biosurfactants in signaling and communication

The production of many biosurfactants is described to correlate to a high bacterial cell density, e.g. late exponential growth phases. It is noteworthy that the production of a diverse range of biosurfactants is regulated by quorum sensing, e.g. rhamnolipids produced by *P. aeruginosa* or the lipopeptides surfactin and serrawettin produced by *Bacillus* spp. respectively *Serratia* spp.. Sullivan (1998) concludes that a certain cell density is necessary to initiate the release of surfactants as virulence factors thus being able to accumulate a high enough concentration for a localized attack on the host. High cell densities are also present in biofilms, a protected form of life that allows cells to survive in hostile environments. Biofilms are described as efficient tool for the remediation of hydrophobic compounds (Tribelli et al. 2012). Cell attachment in biofilm formation is reported to be caused by stressfull environments (Costerton et al. 2003) so is the production of certain biosurfactants (Pacheco et al. 2012). A cell communication mediated production of both, polyhydroxy alkanoates and surfactants for the storage of carbons and a facilitated accession of nutrients caused thereof is reported by Tribelli et al. (2012), several bioemulsifying compounds bind surfaces in order to initiate the formation of biofilms. An enhanced production of alasan was reported by co-cultivation of different *Acinetobacter* spp. suggesting a horizontal transfer of bioemulsifying molecules (Ron and Rosenberg 2001).

1d Research proposal

A great diversity of amphiphilic microbial products have been described and revealed, often exhibiting variable structural characteristics. Interest for an application of microbial derived surfactants has extraordinary increased in the past depicted by more than 250 patents published (Sekhon and Rahman 2015). The upcoming interest is based on very unique properties that are difficult to be achieved by conventional chemically synthesizing of surfactants. Further, as natural products based on renewable resources, biosurfactants hold the potential to replace their chemical counterparts in a society with growing ecologically awareness and consequently rising oil prices.

To my opinion, the ability to reveal unknown microbial surfactants is limited to five factors:

1 The development and verification of screening protocols to detect surfactant molecules and its application in high throughput.
2 The proper selection of habitats or strain collections to find strains that produce surfactants as well as the isolation, identification and maintenance of the strains.

3 Adequate systems and techniques to produce surfactants from a selected strain in an amount large enough for extensive purification procedures.
4 The combination of several spectrometric and spectroscopic measurements and the interpretation of the data acquired to elucidate the compounds analyzed.
5 Certain luck to reveal an unknown microbial surfactant within the amphiphilic compound examined.

This thesis aims to find yet unrevealed microbial surfactants and its producing strains, focusing on isolation of producing strains, the application of screening methods, the establishment of production and purification procedures as well as on the structural elucidation of the biosurfactants produced.

Surfactant producers of the class *Actinobacteria*

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Surfactants tailored by the class *Actinobacteria*

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Contribution to this publication:

JHK has designed, conceived, and written this review, it's figures and tables as well as acquired and interpreted the relevant data used. All authors have fruitfully discussed content and structure of the review. In particular, ML has given substantial contributions related to actinobacteria and CS and RH have given substantial contributions related to biosurfactants.

2a Surfactants tailored by the class *Actinobacteria*

Microbially derived compounds that share hydrophilic and hydrophobic moieties, and that are surface active, are commonly referred to as biosurfactants. Many have been detected and described, and the majority are molecules of low molecular weight. Within this group of low molecular weight microbial surfactants, the classes of lipopeptides or glycolipids, where fatty acid or hydroxy fatty acid chains are linked to either peptides or carbohydrates, have been extensively studied (Hausmann and Syldatk, 2014). The combinations of different types of hydrophilic and hydrophobic moieties within surfactants are innumerable and highly biodiverse.

Due to their amphiphillic structures, surfactants act as emulsifying agents, resulting in low surface tensions of interphases. Often, microorganisms produce them when growing on hydrophobic carbon sources or when exposed to growth limiting conditions. It is hypothesized, that biosurfactants play a role in the uptake of various hydrophobic carbon sources thus making nutrients bioavailable, as well as the protection of bacteria from harsh environmental conditions (Ristau and Wagner, 1983; Vollbrecht et al., 1998; Philp et al., 2002). Some biosurfactants show antimicrobial effects and the distinction

of secondary metabolites as antibiotics or biosurfactants is often not strict.

Biosurfactants, compared to chemically derived surfactants, are independent of mineral oil as a feedstock, they are readily biodegradable and can be produced at low temperatures. Furthermore they are described to be less toxic, effective at low concentrations and show effects in bioremediation. Industrial interest in biosurfactants is not solely based on the bio-acitivity of these molecules, but is also due to the broader ecological awareness linked to their application, which in turn is driven by sustainability initiatives and green agendas (Marchant and Banat, 2012). Biosurfactants can be applied in various areas such as the nutrient-, cosmetic-, textile-, varnish-, pharmaceutical-, mining- and oil recovery industries (Henkel et al., 2012; Marchant and Banat, 2012; Müller et al., 2012).

An example of an actinobacterial biosurfactant that has already entered the market and found industrial application, is the lipopeptide antibiotic daptomycin. This antibiotic is used in the treatment of diseases caused by gram positive pathogens and has been marketed as Cubicin® by Cubist Pharmaceuticals. Other promising studies for the potential application of actinobacterial biosurfactants are in environmental applications such as bioremediation: Oil spills were successfully dispersed by biosurfactants produced by a *Gordonia* sp. (Saeki et al. 2008), a *Dietzia* sp. (Wang 2014) and a *Rhodococcus* sp. (Kuyukina and Ivshina, 2010); and trehalose lipids were applied in microbial enhanced oil recovery and the cleaning of oil storage tanks (Franzetti et al., 2010). In medical applications, the production of biosurfactants are generally considered safer than synthetically produced compounds due to high enzymatic precision during synthesis. Antiproliferation activities of cancerogenic cells could be induced by application of various glycolipids (Isoda et al. 1997; Sudo et al. 2000). In cosmetic applications, the use of trehalose lipids is favoured above that of sodium dodecyl sulfate as it causes less irritation (Marques et al., 2009).

Different types of biosurfactants or bioemulsifiers have been described to be produced as secondary metabolites within the class *Actinobacteria*, and to the best of our knowledge, all of the producing species belong to the order *Actinomycetales* (Figure 2.1). The following section of the review will focus on the different types of actinobacterial biosurfactants reported in literature as well as their key structural features and bio-activities.

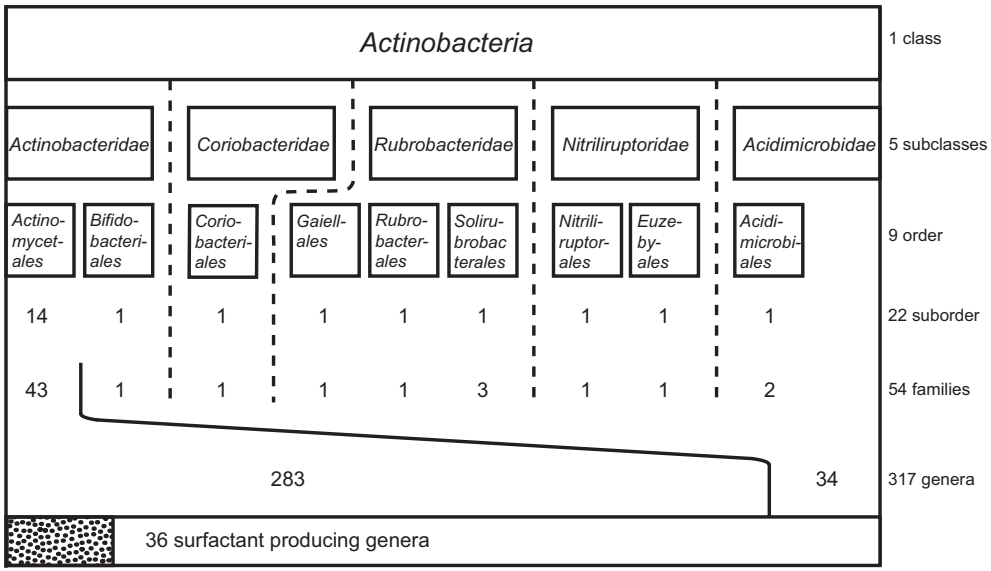


Figure 2.1: Systematic classification of the class *Actinobacteria* including subclasses and orders. Suborder, families and genera examined for the production of biosurfactants and bioemulsifying compounds are displayed in numbers. 36 surfactant-producing genera are reported, all belonging to the largest order within the *Actinobacteria*: *Actinomycetales.*

Metabolite production within the class Actinobacteria

Over the past few decades, there has been an increased interest in the discovery of bioactive metabolites with novel bioactive properties and their potential for application in medical- or industrial-based processes. Microbial products are still considered to be the most promising source for the discovery of novel chemicals or therapeutic agents (Berdy, 2005). In addition, vast microbial genetic resources remains untapped and can lead to the development of novel bioactive metabolites.

In contrast to primary metabolites, secondary metabolites often accumulate and have miscellaneous chemical compositions that are species-specific. These secondary metabolites often exhibit bioactivity and are therefore of great interest to various industries. The most dominant source of microbially derived bioactive compounds is a group of bacteria known to have relatively large genomes and constitutes one of the main phyla within the *Prokaryotes*: The class *Actinobacteria* (Ludwig and Klenk, 2001).

The class *Actinobacteria* play important roles in the environment, e.g. nutrient cycling, but also include major plant, animal and human pathogens (Embley and Stackebrandt, 1994), well known examples are the causative agents of leprosy and tuberculosis. Baltz (2008) assumed 5-10 % of their genome coding capacity to be used for the production of secondary metabolites and indeed more than 35 % of all known bioactive microbial metabolites and more than 63 % of all known prokaryotic bioactive metabolites arise from actinobacteria (Bérdy, 2012). Most secondary metabolite producers described belong to families of the *Actinomycetales,* but it is estimated that only ~ 1% of them are culturable (Bérdy, 2012). Many of these actinobacterial secondary metabolites exhibit antibacterial, antifungal, antitumor, anticancer and/or cytotoxic properties (Manivasagan et al., 2013). Antibiotics, with around 10,000 compounds described (Bérdy, 2012) is by far the largest group of metabolites isolated from actinobacteria. Depending on their chemical nature, the huge number of antibiotic compounds can roughly be classified into *peptides*, *aminoglycosides*, *polyketides*, *alkaloids*, fatty acids and *terpenes* (Manivasagan et al., 2013; Abdelmohsen et al., 2014). Besides antibiotics, other actinobacterial compounds described are bioactive compounds with pharmacological activity (pheromones, toxins, enzyme inhibitors, receptors and immunological modulators), with agricultural activity (pesticides, herbicides and insecticides) and other industrially relevant properties (pigments and surfactants). Most compounds are derived from members of the genus *Streptomyces,* however, other so-called "rare" actinomycetes are increasingly playing a more important role in the production of biocompounds (Bérdy, 2005; Kurtboke, 2010).

To fully understand the taxonomic distribution of the actinobacterial strains identified to produce biosurfactants and bioemulsifying compounds, taxonomic data of the class *Actinobacteria* was evaluated. Information were retrieved from the taxonomy browser of the National Center for Biotechnology Information (NCBI) considering 16S rRNA gene sequence based reclassifications according to Zhi *et al.* (2009) and Goodfellow and Fiedler (Goodfellow and Fiedler, 2010). The order *Thermoleophilales* that has been reclassified into a new class (Euzéby, 2013) has been excluded and the recently identified order *Gaiellales* has been included (Euzéby, 2012). Overall, the class *Actinobacteria* contains five subclasses and nine orders with a total of 54 families (Figure 2.1). The largest order, *Actinomycetales*, is divided into 14 suborders and contains

by far the highest diversity within the class *Actinobacteria*. It is therefore not surprising that biosurfactants reported in literature focuses on members of this order. The next few paragraphs will go into more detail around the different types of biosurfactants that have been identified to be produced by actinobacterial strains, their production, purification and structural elucidation, as well as the clear influence of the environment the producer organism is found in and their ability to produce biosurfactants.

Trehalose-comprising glycolipids

The best described biosurfactants amongst the actinobacteria are glucose-based glycolipids, most of which have a hydrophilic backbone consisting of two α,α-1,1 glycosidic linked glucose units forming a trehalose moiety. Different types of trehalose-containing glycolipids and their producers have been extensively reviewed (Asselineau and Asselineau, 1978; Asselineau and Lanéelle, 1998; Franzetti et al., 2010; Kuyukina and Ivshina, 2010; Shao, 2011; Khan et al., 2012). Those of the class *Actinobacteria* are mainly found within the genera *Rhodococcus, Mycobacterium*, *Nocardia, Arthrobacter* and *Corynebacterium*, and less frequently within the genera *Tsukamurella, Brevibacterium* and *Micrococcus* (Table 2.1 and Table 2.2). Different structures of trehalose lipid comprising amphiphilic molecules have been reported: Acyl chains with glycosidic linkages to glucose or trehalose units have been reported to vary in number of occurrence, length and type, as well as the position (and number) of their linkage to the sugar rings and exhibit different cellular functions.

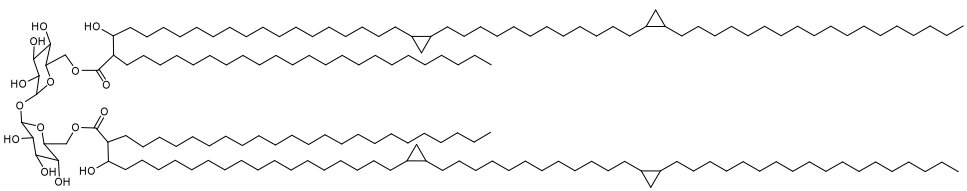
For the hydrophobic moiety of trehalose-comprising glycolipids, the structures of two main types of trehalose lipids have been elucidated: those carrying a mycolic fatty acid ester and those carrying a fatty acid ester.

The smallest hydrophilic backbone in glycolipids constitutes glucose, the building block of the sugar dimer trehalose. Complete structures of acylglucoses carrying mycolic acid esters have been elucidated and reported to be produced by isolates belonging to the genera *Corynebacterium* and *Mycobacterium* (Brennan et al., 1970) (Table 2.1), whereas acylglucoses carrying fatty acid esters have been described for *Brevibacterium* spp. (Okazaki et al., 1969) (Table 2.2).

Table 2.1: Mycolic and corynemycolic containing trehalose lipids that are of actinobacterial origin

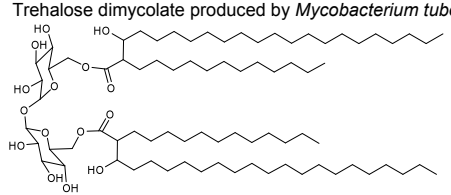
Species	Strain	TL mycolic acid ester	Reference
Arthrobacter paraffineus	KY 4303	TL mycolic (C32-C36)	Suzuki et al., 1969
Brevibacterium sp.	KY 4304/4305	TL mycolic (C32-36)	Suzuki et al., 1969
Brevibacterium vitarumen	12143	TL dimycolic (C28-C38)	Lanéelle and Asselineau, 1977
Corynebacterium diphtheriae	n.a.	Glucose mycolic (C32)	Brennan et al., 1970
Corynebacterium spp. *(fasciens, pseudodiphtheriae)*	KY 3543 KY 3541	TL mycolic (C32-36)	Suzuki et al., 1969
Corynebacterium matruchotii	ATCC 14266	TL dimycolic (C28-C38)	Datta and Takayama, 1993
Mycobacterium spp. *(smegmatis, tuberculosis)*	BCG, n.a.	Glucose mycolic (C32)	Brennan et al., 1970
Mycobacterium spp.* (*bovis, fortuitum, kansaii, malmoense, phlei, tuberculosis, smegmatis, szulgai,* etc.)	various	TL mycolic, dimycolic,	**Reviewed in:** Asselineau and Asselineau, 1978; Gautier et al., 1992; Asselineau and Lanéelle, 1998; Vergne and Daffé, 1998; Dembitsky, 2004; Ishikawa et al., 2009; Shao, 2011
Nocardia spp.	n.a.	TL mycolic (C32-36)	Suzuki et al., 1969
Rhodococcus spp.* (*erythropolis, opacus, ruber,* etc.)	various	TL mycolic,dimycolic,	**Reviewed in:** Asselineau and Asselineau, 1978; Lang and Philp, 1998; Kuyukina and Ivshina, 2010; Shao, 2011; Khan et al., 2012

Examples of mycolic acid containing trehalose lipids



1

Trehalose dimycolate produced by *Mycobacterium tuberculosis*



2

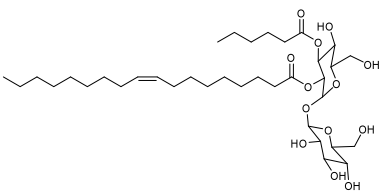
Trehalose dicorynemycolate produced by *Rhodococcus erythropolis*

*several producing species are reported; TL=trehalose lipid; n.a.=information not available

Table 2.2: Trehalose lipid ester of actinobacterial origin

Species	Strain	TL ester	Reference
Arthrobacter sp.	EK 1	TL tetraester (C12-C18)	Passeri et al., 1990
Brevibacterium thiogenitalis	No. 653	Glucose diester (C18)	Okazaki et al., 1969
Micrococcus luteus	BN56	TL tetraester (C9-C14)	Tuleva et al., 2009
Mycobacterium spp.* (*africanum, bovis, fortuitum, tuberculosis,* etc.)	various	TL ester	**Reviewed in:** Vergne and Daffé, 1998; Dembitsky, 2004; Shao, 2011
Mycobacterium tuberculosis	H37Rv	TL sulfolipid	Goren, 1970; Gilleron et al., 2004)
Nocardia farcinica	BN26	TL succinic tetraester (C7-12)	Christova et al., 2014
Rhodococcus spp.* (*erythropolis, longus, wratislavensis*, etc.)	various	TL ester, TL succinic ester	**Reviewed in:** Asselineau and Asselineau, 1978; Lang and Philp, 1998; Kuyukina and Ivshina, 2010; Shao, 2011; Khan et al., 2012
Tsukamurella pulmonis	PCM 2578T	TL diester (C18-20/C4-5)	Pasciak et al., 2010a
Tsukamurella spumae	DSM 44113, DSM 44114	TL diester (C16-18/C4-6)	Kügler et al., 2014
Tsukamurella pseudospumae	DSM 44117		
Tsukamurella tyrosinosolvens	DSM 44370	TL diester (C16-18/C2-6)	Vollbrecht et al., 1998

Examples of trehalose lipid esters



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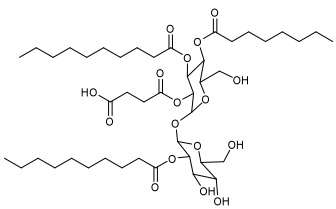
Trehalose diester produced by *Tsukamurella spumae*



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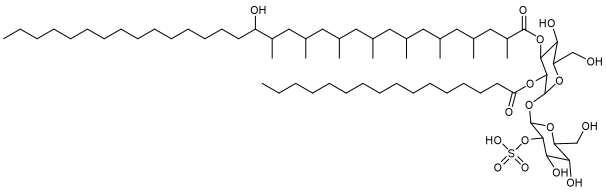
Succinic trehalose tetraester produced by *Nocardia farcinia*



5

Diacetylated trehalose sulfolipid produced by *Mycobacterium tuberculosis*

*several producing species are reported; TL=trehalose lipid

Trehalose lipid mycolic acid esters

Mycolic acids are long-chain fatty acids and a major component of the cell wall in various actinobacteria. Species-dependent, its lengths varies from 22 to 92 carbon atoms; they possess long β-hydroxy-α-branched acyl chains, including cyclopropane patterns and oxygenic groups. The synthesis of mycolic acids includes condensation reactions, and they are also referred to as eumycolic acid, corynemycolic acid and nocardio-mycolic acid, depending on their presence in *Mycobacterium* spp., *Corynebacterium* spp. and *Nocardia* spp., respectively (Asselineau and Lanéelle, 1998).

Mycolic acid comprising trehalose lipids (Table 2.1) can be distinguished into two different types, the trehalose mycolic lipids and the trehalose corynemycolic lipids. These mycobacterial trehalose mycolates or dimycolates are by far the most hydrophobic glycolipids. Linked to C6 (and C6') of the sugar rings, they vary among species in length and branching. They are shaped to form bilayers, implemented in the outer cell wall and usually not found on the bacterial cell surface (Vergne and Daffé, 1998). Trehalose dimycolates (1, Table 2.1), also referred to as "cord factor", serve a particular function for the cell. They act as virulence factors and have immuno-modulating activity (Shao, 2011). They may further be important to maintain a hydrophobic cell wall of the organism hence facilitating the uptake of hydrophobic carbon sources. The other type, trehalose lipids containing corynemycolic acid also carry β-hydroxy-α-branched fatty acid moieties and have been described to occur within the genus *Rhodococcus* (2, Table 2.1), carrying 30-56 carbon atoms and within the genus *Corynebacterium*, carrying 22-36 carbon atoms. They are also described to occur in mycobacteria (Brennan et al., 1970) and found in trehalose lipids of *Brevibacterium vitarumen* (Lanéelle and Asselineau, 1977), *Arthrobacter paraffineus* and a *Nocardia* sp. (Suzuki et al., 1969). Corynemycolic acids are much shorter than their mycobacterial counterparts: they lack functional groups and are often unsaturated. Within virulent strains of mycobacteria, five different sulfonated forms of trehalose esters have been found, varying in their acylation pattern (Khan et al., 2012).

Trehalose lipid esters

Actinobacterial trehalose lipid esters are mainly acylated at C6/C6' or at C2/C3 and are summarized in Table 2.2. The amount of hydrophobic chains linked to the trehalose unit varies from one to four, forming trehalose mono, di, tri- and tetraesters, but

also octaesters (Singer et al., 1990) (3, Table 2.2). The acyl chains varies in lengths from C8 to C20, show an unsaturated pattern or form short succinoyl acids, giving the trehalose lipid an anionic character (Lang and Philp, 1998; Tokumoto et al., 2009) (4, Table 2.2). They are reported to be linked to the chain length present in hydrophobic carbon source fed to the producing strain. These glycolipid-linked medium chain length fatty acids are found within the following actinobacterial genera: *Arthrobacter*, *Brevibacterium, Caseobacter, Micrococcus, Mycobacterium, Nocardia, Rhodococcus* and *Tsukamurella* (Table 2.2).

An exception among the trehalose lipid esters described, is sulfolipid 1 (Goren, 1970) (5, Table 2.2), a sulfonated and acylated trehalose lipid carrying phtio- and hydroxyphtioceranic compartments. They are known to contribute to the pathogenesis and virulence of *Mycobacterium tuberculosis*, the causative agent of tuberculosis. Diacyltrehalose sulphate, the biosynthetic precursor for sulfolipid 1, has recently been isolated from *M. tuberculosis* (Domenech et al., 2004) and has been used as a target for Tcell mediated recognisation and elimination of *M. tuberculosis* infected cells (Gilleron et al., 2004).

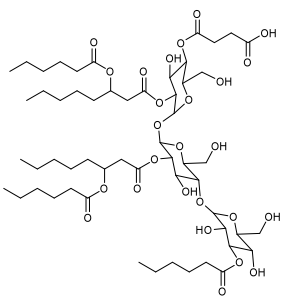
Oligosaccharide lipids

A glycosylated backbone of trehalose is found in oligosaccharide lipids (Table 2.3) carrying two to five sugar units. Trisaccharide lipids that have been reported for the class *Actinobacteria* all differ with respect to the acylation pattern of the third glucose unit. One sugar of the 1-1' linked di-glucose backbone is further linked to a third sugar unit at C2 in the hydrophilic moeity of oligosaccharides produced by *Mycobacterium leprae* (Brennan, 1989) and *Tsukamurella tyrosinosolvens* (Vollbrecht et al., 1998). The third sugar unit is linked at C3 in a terrestrial actinomycete reported by Esch *et al.* (1999) and at C4 in a *Rhodococcus* sp. (Konishi et al., 2014) (6, Table 2.3). They also differ with respect to their hydrophobic nature. The latter two are acylated at all three sugar units, both carrying a C6 fatty acid moiety at the third sugar unit and succinic acid at the first sugar unit. Something that is rather exceptional is the acylation pattern at the trehalose backbone that, in its hydrophobic moieties, carries at each unit an acyloxyacyl structure in the *O*-ester linkage to the carbohydrate where the 3-hydroxy C8 or C10 fatty acid moiety is further acylated with a C6 fatty acid (6, Table 2.3). The

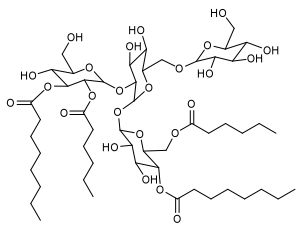
Table 2.3: Actinobacterial oligosaccharide lipids

Species	Strain	Oligosaccharid lipids	Reference
Mycobacterium spp.* (avium, *kansaii, leprae, linda, malmoense, smegmatis, szulgai, tuberculosis*)	various	oligosaccharide ester, phenolic glycolipids	**Reviewed in:** Saadat and Ballou, 1983;Brennan, 1989;Dembitsky, 2005b
Nocardia corynebacteroides	SM1	Pentasaccharide succinic octaester (C2-C8)	Powalla et al., 1989
Rhodococcus sp.	NBRC 1097287	Trisaccharid succinic tetraester (C8-*O*-C6/C6)	Konishi et al., 2014
Rhodococcus fascians	NBRC 12155		
Tsukamurella tyrosinosolvens	DSM 44370	Tri/tetrasaccharide ester (C8-10)	Vollbrecht et al., 1998

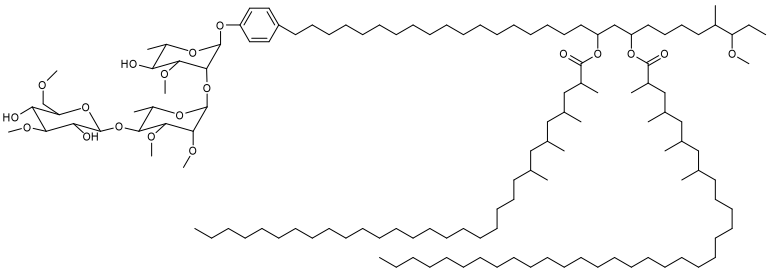
Examples of oligosaccharide lipids



6
Succinic trisaccharide lipid produced by *Rhodococcus fascians*



7
Tetrasaccharide lipid produced by *Tsukamurella tyrosinosolvens*



8
Methylated dirhamnose/glucose phenol phtiocerol named phenolic glycolipid I of *Mycobacterium leprae* (Brennan, 1989)

*several producing strains are reported

Tsukamurella sp. trisaccharide lipids are acylated at two sugar units, each carrying two ordinary C8-C10 fatty acid units. Furthermore, a tetrasaccharide lipid form of this glycolipid has also been found to occur (Vollbrecht et al., 1998) (7, Table 2.3).
Non-trehalose based oligosaccharide lipids are found within phenol-phtiocerol glycosides in various mycobacteria. These oligosaccharide lipids, also termed phenolic glycolipids, contain tri- and tetraglycosyl units composed of various methylated sugars that are mainly based on rhamnose and partly on fucose, glucose and arabinose (Brennan, 1989). The rarely described phenolic acylation pattern is bound to dimycocerosyl phtiocerol acyl groups. The phenolic glycolipid I of *M. leprae* carries three mycocerosyl acyl groups each in length of C30-C34 (Brennan, 1989) (8, Table 2.3).

In industrial and environmental processes the potential of trehalose lipids could become valuable as they have shown interesting properties in several studies that focus on the remediation of hydrocarbon contaminated soils, the removal of suspended solids from wastewater (Franzetti et al., 2010) and in enhanced oil recovery (Christofi and Ivshina, 2002). However, most research are centered around the bio-activity of trehalose lipid molecules that exhibit biomedical properties such as antimicrobial, antiviral (Azuma et al., 1987; Watanabe et al., 1999; Shao, 2011) and anti-tumor activities (Sudo et al., 2000; Franzetti et al., 2010; Gudiña et al., 2013). Due to their functions in cell membrane interactions they can act as therapeutic agents (Zaragoza et al., 2009; Shao, 2011) or have an impact on the pathogenesis of causative agents of infections, such as those caused by pathogenic *M. tuberculosis, Corynebacterium diphteriae*, and the opportunistic pathogens, *Mycobacterium avium, Mycobacterium intracellulare, Nocardia asteroides, Corynebacterium matruchotii* and *Corynebacterium xerosis* (Kuyukina and Ivshina, 2010). Trehalose lipids can be excreted into the cultivation supernatant or can be produced as non-covalently linked lipids bound to the cell wall or they can be cell wall integrated thus posing limits to quantities produced by the organisms, a disadvantage for its potential exploitation in large scale production processes.

Non-trehalose glyolipids

Hexose-comprising glycolipids

Besides the trehalose-containing biosurfactants and its congeners, several glycolipids have been elucidated that are produced by actinobacteria and share other hydrophilic moieties. By simply varying the carbon source in the growth media from nalkanes to either sucrose or fructose, the hydrophilic part of the surfactant produced was reported to be switched from trehalose to fructose by members of the genus *Arthrobacter, Corynebacterium*, *Nocardia, Brevibacterium* and *Mycobacterium* (Itoh and Suzuki, 1974) and sucrose in the case of the same genera except *Mycobacterium* (Suzuki et al., 1974). Compounds for which structures have been elucidated are listed in Table 2.4.

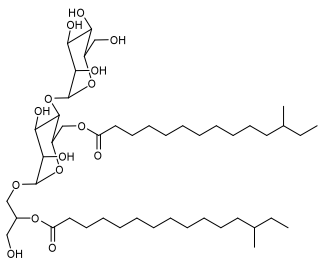
Besides the rhamnose-containing phenolic glycolipids mentioned in the oligosaccharide lipid section, the occurrence of other rhamnose-based lipids have recently been detected in a deep sea isolate identified as *Dietzia maris* (Wang et al., 2014) and has been identified as a C10:C10 di-rhamnolipid. This represents a unique occurrence within the class *Actinobacteria.* Other rhamnolipid producing actinobacteria are admittedly declared as producing strains in literature, however the surface active compounds produced have either not been elucidated or identified as rhamnolipids with debatable structural characterizations (*Rhodoccocus fascians* (Gesheva et al., 2010), *Renibacterium salonarium* (Christova et al., 2004), and a *Nocardioides* sp. (Vasileva-Tonkova and Gesheva, 2005)) (Table 2.11).

A different group of glycolipids are lipidic structures based on dimannose. Typically they are linked via a glycerol unit to different numbers of fatty acid chains. They have been reviewed in Shaw (1970) and structures have been identified for compounds produced by species belonging to the actinobacterial genera *Micrococcus* (Lennarz and Talamo, 1966), *Curtobacterium* (Mordarska et al., 1992), *Saccharopolyspora* (Gamian et al., 1996), *Rothia* (Pasciak et al., 2002; Pasciak et al., 2004), *Nocardiopsis* (Pasciak et al., 2004), *Arthrobacter* (Pasciak et al., 2010b) as well as the strain *Sinomonas atrocyaneus* (Niepel et al., 1997), formerly classified as *Arthrobacter atrocyaneus*. These di-mannose based glycolipids are composed of hydrophilic αDmannopyranose dimers linked with two C14 to C16 *iso* or ante*iso* fatty acid chains. One chain is directly esterified to the C6 hydroxyl group of one sugar unit, while the second fatty acid chain is linked via a glycerol moiety to the C3 of the same sugar unit. The glycerol moiety is monoacylated

Table 2.4: Non-trehalose comprising glycolipids produced by actinobacteria

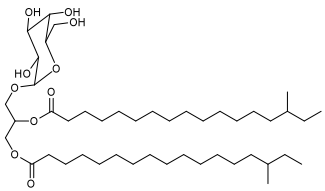
Species	Strain	Hexose lipids	Reference
Arthrobacter paraffineus	KY 4303	Sucrose mycolic (C32-C36)*	Suzuki et al., 1974
Arthrobacter paraffineus	KY 4303	Fructose coryne- and dicorynemycolic*	Itoh and Suzuki, 1974
Arthrobacter spp. *(globiformis, scleromae)*	ATCC 8010^T YH 2001^T	Dimannosylacyl (C15-C17) monoglyceride (C15-C17) Galactosyl diglyceride(C15-C17)	Pasciak et al., 2010b
Brevibacterium butanicum	KY 4332	Fructose coryne- and dicorynemycolic*	Itoh and Suzuki, 1974
Brevibacterium spp.	n.a.	Sucrose mycolic (C32-C36)*	Suzuki et al., 1974
Corynebacterium spp.	n.a.	Sucrose mycolic (C32-C36)*	Suzuki et al., 1974
Corynebacterium spp.	n.a.	Fructose coryne- and dicorynemycolic*	Itoh and Suzuki, 1974
Curtobacterium flaccumfaciens	ATCC 13437	Di- and trimannosylglyceride (C18-C19 cyclopropane)	Mordarska et al., 1992
Dietzia maris	MCCC 1A00160	Rhamnolipid (C10/C10)	Wang et al., 2014
Micrococcus lysodeikticus	ATCC 4698	Dimannosylglyceride (C14)	Lennarz and Talamo, 1966
Mycobacterium avium *Mycobacterium koda*	KY 3844 KY 3852	Fructose coryne- and dicorynemycolic*	Itoh and Suzuki, 1974
Nocardia butanica *Nocardia convulutus*	KY 4333 KY 3907	Sucrose mycolic (C32-C36)*	Suzuki et al., 1974
Nocardia rubra *Nocardia butanica* *Nocardia convulutus*	KY 3844 KY 4333 KY 3907	Fructose coryne- and dicorynemycolic*	Itoh and Suzuki, 1974
Nocardiopsis dassonvillei	PCM 2492T (ATCC 23218)	Dimannosylacyl (C15) monoglyceride (C16)	Pasciak et al., 2004
Rothia dentocariosa	PCM 2249^T (ATCC 17931)	Dimannosylacyl monoglyceride (C16-C19)	Mordarska et al., 1992; Pasciak et al., 2002
Rothia mucilaginosa	PCM 2415^T (ATCC 25296^T)	Dimannosylacyl (C15) monoglyceride (C16)	Pasciak et al., 2004
Saccharopolyspora spp. *(erythraea, hirsuta, rectivirgula,* sp.*)*	ATCC 27875^T ATCC 11635^T IMRU1258 LL-100-46)	Dimannosylacyl (C15-C16) monoglyceride (C16)	Gamian et al., 1996; Pasciak et al., 2002; Pasciak et al., 2004
Sinomonas artrocyaneus	LMG 3814^T	Dimannoseylacyl (C14) monoglyceride (C16)	Niepel et al., 1997

Examples of non trehalose comprising hexoselipids



9

Dimannosylacyl monoglyceride produced by *Rothia mucilaginosa*



10

Galactosyl diglyceride produced by *Arthorbacter globiformis* and *Arthrobacter scleromae*

n.a.= no information available; *= sucrose and fructose based surfactants are variants of trehalose lipids.

at either the primary or secondary methylene position (9, Table 2.4) and its acylation site can be used to distinguish taxonomic properties of the different producer strains. These compounds have been isolated intracellularly and they act as precursors and cell membrane anchors for the synthesis of lipoarabinomannan, a polymeric surfactant and actinobacterial cell wall component (Pakkiri and Waechter, 2005) (see section on polymeric biosurfactants).

The coexistence of galactosyl diglycerides (10, Table 2.4) in *Arthrobacter scleromae* and *Arthrobacter globiformis* (Pasciak et al., 2010b) have been described and can be used as a glycomarker to distinguish these strains from the opportunistic pathogens, *Rothia mucilaginosa* and *Rothia dentocariosa* (Pasciak et al., 2002 and 2004).

Macrocyclic glycosides

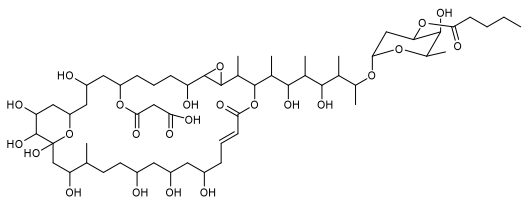
Among the biosurfactants produced by actinobacteria, macrocyclic glycosides (Table 2.5) and macrocylcic dilactones (Table 2.6) can be distinguished and are often known to exhibit bio-activity against a range of organisms. The aliphatic macrolide antibiotic, brasilinolide, is produced by *Nocardia brasiliensis* and exhibits both antifungal and antibacterial activity. Three different variants have been described by Tanaka *et al.* (1997), Mikami *et al.* (2000) and Komatsu *et al.* (2004). All consist of a C32-membered macrolide with a sugar moiety but differ with regards to the acylation site of a malonic acid ester side chain (11, Table 2.5). The C16-membered dimeric macrolide elaiophylin and its variants have been isolated from various *Streptomyces* spp. including high producer strains. It exhibits bio-active properties against intestinal worms as well as antimicrobial, antitumor and immunosuppressant activities. A putative 95 kbp biosynthetic gene cluster of elaiophylin has been proposed (Haydock et al., 2004). Dembitsky (2005a;c) reviewed the different types of C14-membered lactam rings that are attached to an aminosugar (12, Table 2.5). Fluvirucin has been isolated from various *Actinomadura* spp., *Streptomyces* spp., *Microtetraspora* spp. and *Saccharotrix mutabilis*. The different fluvirucins share a common lactam ring unit but differ in terms of glycosylation. All of them act as potent antifungal agents against *Candida* spp. and show antiviral properties against influenza A virus (Dembitsky, 2005c).

Among the macrocyclic dilactones, glucolypsin, an acylglucose dimer has been isolated from *Streptomyces purpurogeniscleroticus* and *Nocardia vaccinii* by Qian-Cutrone *et al.* (1999). This extraordinary glycolipid is formed out of two glucose units linked

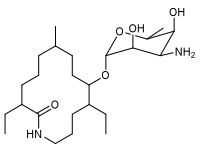
Table 2.5: Macrocyclic glycosides produced by actinobacteria

Species	Strain	Macrocyclic glycoside	Reference
Actinomadura spp.* *(roseorufa roseorura, vulgaris, yumaensis)*	various	Fluvirucin (14C macrolide)	**Reviewed in:** Dembitsky, 2005a;c
Microtetraspora pusilla	R359-5	Fluvirucin B1 (14C macrolide)	Dembitsky, 2005a
Microtetraspora tyrrheni	Q464-31	Fluvirucins (14C macrolide)	Dembitsky, 2005a;c
Nocardia brasiliensis	IFM 0406	Brasilinolide A, B, C (32C macrolide)	Tanaka et al., 1997; Mikami et al., 2000; Komatsu et al., 2004
Saccharothrix mutabilis	R869-9	Fluvirucin A2 (14C macrolide)	Dembitsky, 2005a
Streptomyces spp.* (*antibioticus, erythreus, felleus, hygroscopicus, melanosporus, narbonensis, spinichromogenes, violaceoniger*)	various	Elaiophylin and derivates (16C macrolide) Fluvirucin (14C macrolide)	**Reviewed in:** Dembitsky, 2005a

Examples of macrocyclic glycosides



11
Brasilinoide A produced by *Nocardia brasiliensis*



12
Fluvirucin B1 produced by *Actinomadura vulgaris* subsp. *lanata*

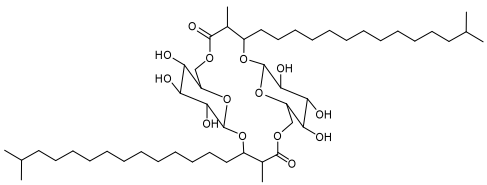
*several producing strains are reported

to identical iso-branched C18 acyl chains that each carry a methyl group at C2 and a hydroxyl group at C3 of the acyl chain. By connecting the C6' of the glucose molecule to the carboxy-C1 of the fatty acid chain, a rotationally symmetric dimer is formed (13, Table 2.6). Glucolypsin variants with C18 and C17 fatty acid chains of the same type also occur. Glycolypsin is reported to increase the activity of glucokinases by relieving its inhibition via long chain fatty acyl CoA esters (Qian-Cutrone et al., 1999). Derivates of glucolypsin that share a common backbone, have been shown to exhibit antiviral and antibiotic properties. In contrast to glucolypsin, the acylglucose dimer of fattiviracins (C24/C26) and cycloviracins (C24/C33) are built up out of trihydroxy fatty acids, each of them glycosidic linked to a further glucose unit at the third hydroxyl group. Cycloviracins are characterized by a fifth glucose unit bound to the C26 fatty acid chain, the three non-cyclic sugar units are methoxylated at C2,

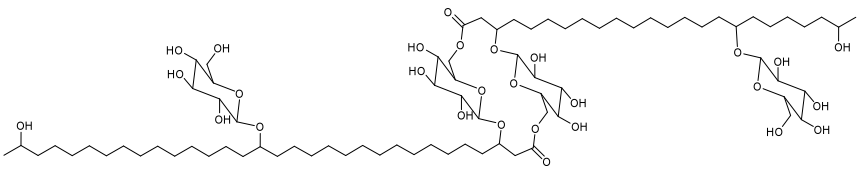
Table 2.6: Macrocyclic dilactones produced by actinobacteria

Species	Strain	Macrocyclic dilactones	Reference
Kibdelosporangium albatum	*ATCC 55061*	Cycloviracin B1 and B2 (C23/C26)	Tsunakawa et al., 1992a; Tsunakawa et al., 1992b
Nocardia vaccinii	WC65712	Glucolypsin A and B (C19/C19)	Qian-Cutrone et al., 1999
Streptomyces microflavus	No.2445	Fattiviracin a1 (C22-28/C22-24)	Uyeda et al., 1998; Yokomizo et al., 1998
Streptomyces purpurogeniscleroticus	WC71634	Glucolypsin A and B (C19/C19)	Qian-Cutrone et al., 1999

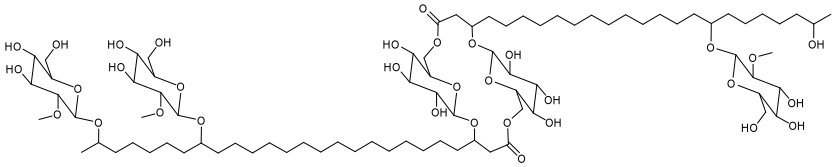
Examples of macrocyclic dilactones



13
Glucolypsin A produced by *Nocardia vaccinii* and *Streptomyces purpurogeniscleroticus*



14
Fattiviracin produced by *Streptomyces microflavus*



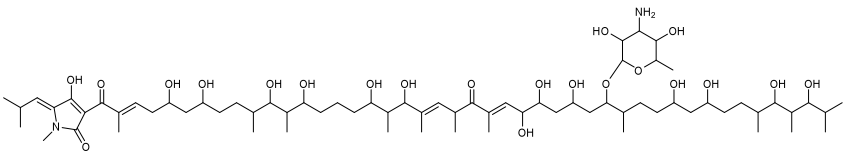
15
Cycloviracin B_1 produced by *Kibdelosporangium albatum*

and the methyl branches at C2 of the fatty acid moieties are missing. Congeners of fattiviracin are divided into five families according to the length of their fatty acid moiety with each family showing similar antiviral activity against herpes, influenza and human immunodeficiency viruses (Uyeda, 2003). No alterations in the fatty acid chain length of cycloviracins have been reported. Fattiviracins (14, Table 2.6) have been shown to be produced by *Streptomyces microflavus* (Uyeda et al., 1998) and cycloviracins (15, Table 2.6) by *Kibdelosporangium albatum* (Tsunakawa et al., 1992b).

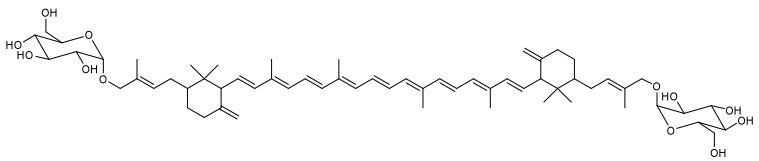
Table 2.7: Terpenoid and terpene-containing biosurfactants produced by actinobacteria

Species	Strain	Terpenoids and terpenes	Reference
Amycolatopsis sp.	DSM 12216	Vancoresmycin (65C terpenoid)	Hopmann et al., 2002
Arthrobacter sp.	M3	Corynexanthin mono- and diglycosides (C50 terpene)	Arpin et al., 1972; Dembitsky, 2005
Corynebacterium sp.	CMB 8	Corynexanthin (C50 terpene)	Weeks and Andrewes, 1970
Micrococcus yunnanensis	AOY-1	Sarcinaxanthin, sarcinaxanthin mono- and diglucosides (C50 terpene)	Osawa et al., 2010
Rhodococcus rhodochrous	RNMS1	Carotenoid (C40 terpene) glycoside (C36-C50 mycolic)	Takaichi et al., 1997

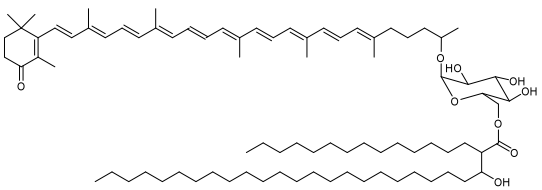
Examples of terpene glycosides



16
The terpenoidic glycoside vancoresmycin produced by *Amycolatopsis* sp.



17
Sarcinaxanthin diglycoside produced by *Micrococcus yunnanensis*



18
Carotenoid glycoside containing esterified with a rhodococcus type mycolic acid produced by *Rhodococcus rhodochrous*

Terpenoids and terpene glycosides

Actinobacterial terpenoid and terpene glycosides are summarized in Table 2.7. Vancoresmycin is a C65 highly oxygenated terpenoid glycoside produced by an *Amycolatopsis* sp. It contains a tetramic acid unit and is glycosidic linked to a methylated carbohydrate moiety containing one amino group (16, Table 2.7). Antimicrobial effect against various bacteria was reported by Hopemann *et al.* (2002), most notably against species resistant to the antibiotic vancomycin (often considered to be the antibiotic of last resort for the treatment of resistant bacteria). Besides the terpenoid glycoside, several different types of terpene glycosides are produced by actinobacterial strains. They are surfactants that mostly carry terminal hydrophilic groups linked by a hydrophobic carotenoid moiety.

Terpene glycosides have been elucidated as products obtained from members of the following genera: *Corynebacterium* (Weeks and Andrewes, 1970), *Arthrobacter* (Arpin et al., 1972), *Rhodococcus* (Takaichi et al., 1997) and *Micrococcus* (Osawa et al., 2010) (Table 2.7). Most of them share a backbone of a C50 atom carotenoid. They can either be linked to one or two hydroxyl groups at the terminal ends (decaprenoxanthin and sarcinaxanthin) or one hydroxyl group and one glycosidic moiety (corynexanthin, decaprenoxanthin monoglycoside and sarcinaxanthin monoglycoside). Di-glycosylated forms are found within *Arthrobacter* and *Micrococcus* (decaprenoxanthin diglucoside and sarcinaxanthin diglucoside) (17, Table 2.7) and further exist as an acetylated form at all hydroxyl groups. The terpene glycosides produced by *Rhodococcus rhodochrous,* differ from the one mentioned above, as they contain a monocyclic carotenoid backbone linked to a glucopyranosyl residue at the non-cyclic end (18, Table 2.7). The glucose unit is further acylated at C6 to a C36-C50 mycolic acid moiety leading to carotenoid glucoside mycolic acid esters. These terpene glycosides are mainly found in pigmented bacteria and it is hypothesized that they act as antioxidants to protect organisms from injuries caused by free radicals (Osawa et al., 2010).

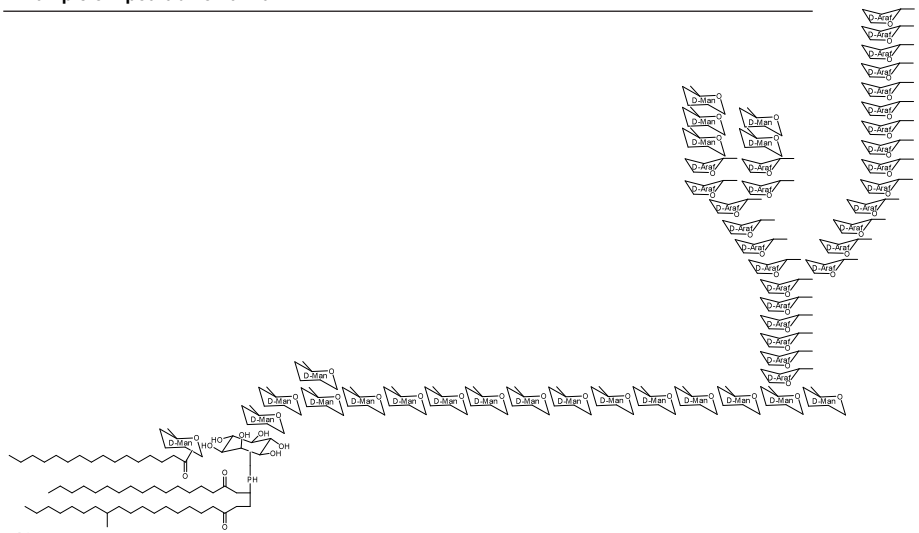
Polymeric biosurfactants

The most common polymeric surfactants produced by actinobacteria are macro-amphiphilic lipoglycans such as lipoarabinomannan and its precursors, lipomannan and phosphatidylinositol mannosides. In contrast to the core of the actinobacterial cell wall, arabinogalactan and peptidoglycan, these polymeric lipoglycans are non-covalently attached to the cell membrane although phosphatidylinositol mannides are structurally related to lipomannan and lipoarabinomannan anchor units. These polymeric glycolipids have been isolated from *Mycobacterium* spp., *Gordonia* spp., *Rhodococcus* spp., *Dietzia maris, Tsukamurella paurometabolus, Turicella otitidis* and *Amycolatopsis sulphurea* (Table 2.8). Except for *A. sulphurea*, all of these strains belong to the suborder *Corynebacteridae* that are known to contain mycolic acids in their cell wall. It comprises the presence of mycolic acids and contain lipid rich cell envelope structures (Sutcliffe, 1997) forming an extremely robust and impermeable cell envelope (Berg et al., 2007). Lipoarabinomannans are well known to cause immunorepressive functions in diseases such as tuberculosis and leprosy that are caused by the pathogenic mycobacterial strains *M. tuberculosis* and *M. leprae*. However, non-pathogenic species have also been shown to produce lipoarabinomannans and are reported to have an opposite effect thus stimulating pro-inflammatory responses (Briken et al., 2004). The mannan core of lipoarabinomannan and the number of branching units is species dependent. Further differences in its structure is traced back to capping motifs present at the non-reducing termini of the arabinosyl side chains. Mannan caps are mainly present in pathogenic strains, whereas inositol phosphate caps are present in non-pathogenic mycobacteria (Briken et al., 2004). Lipoarabinomannans show structural similarity to its precursors lipomannan and phosphatidylinositol mannoside and consist of an α-1,6 linked mannan core with frequent α-1,2 mannose branches leading to a mannan backbone of approximately 20-25 mannose residues substituted with arabinofuran residues that carry terminal extension motifs, which vary among the producer species (Berg et al., 2007). The lipophilic part consists mainly of C16 glycerides that are linked to the mannan core by a phosphate group (19, Table 2.8).

Table 2.8: Polymeric glycolipids of actinobacterial origin

Species	strain	Polymeric glycolipid	Reference
Corynebacterium matruchotii	NCTC 10207	Lipoarabinomannan	Sutcliffe, 1995
Turicella otitidis	DSM 8821	Lipoarabinomannan	Gilleron et al., 2005
Dietzia maris	N1015	Lipoarabinomannan	Sutcliffe, 2000
Mycobacterium spp.* *(avium, bovis, chelonae, fortuitum, kansaii, leprae, smegmatis, tuberculosis, etc.)*	various	Lipoarabinomannan and lipomannan	**Reviewed in:** Chatterjee and Khoo, 1998 Briken et al., 2004 Nigou et al., 2003 Brennan, 2003
Gordonia bronchialis	N654^T	Lipoarabinomannan, phosphatidylinositol mannoside	Garton and Sutcliffe, 2006
Gordonia rubripertincta	ATCC 25689	Lipoarabinomannan, phosphatidylinositol mannoside	Flaherty and Sutcliffe, 1999
Rhodococcus spp.* (*equi, rhodnii, ruber,* etc)	various	Lipoarabinomannan	**Reviewed in:** Sutcliffe, 1997
Tsukamurella paurometabola	DSM 20162	Lipoarabinomannan	Gibson et al., 2004
Amycolatopsis sulphurea	DSM 46092	Lipoarabinomannan	Gibson et al., 2003

Example of lipoarabinomannan



19

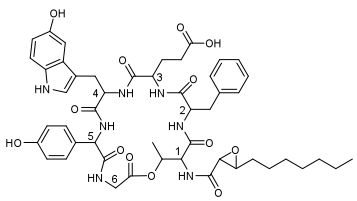
Simplified structure of lipoarabinomannan produced by *Mycobacterium tuberculosis* with only one arabinofuran branch shown. Modified from Berg et al. (2007)

*several producing strains are reported

Table 2.9: Lipopeptides produced by actinobacterial strains

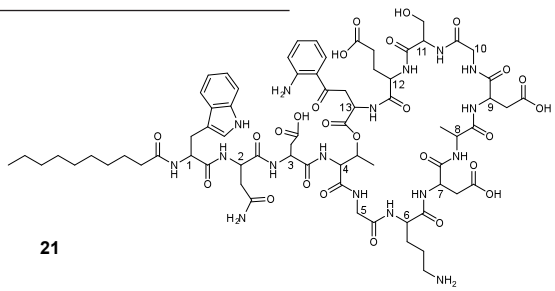
Species	Strain	Lipopeptide	Reference
Actinoplanes sp.	ATCC 33076	Ramoplanin (glycosylated 17aa, C8-C10)	Ciabatti et al., 1989; Gastaldo et al., 1992
Kitasatospora cystarginea	NRRL-B16505	Cystargamide (6aa, 2'-3'epoxy-C10)	Gill et al., 2014
Rhodococcus sp.	MCCC 1A00197	rhodocfactin	Peng et al., 2008
Streptomyces roseosporus	NRRL 11379	A21978C (daptomycin) (13aa, C10-12)	Debono et al., 1987
Streptomyces tendae	Tü 901/8c	Streptofactin	Richter et al., 1998
Streptosporangium amethystogenes subsp. *fukuiense*	AL-23456	TAN-1511 A, B, C	Takizawa et al., 1995

Examples of lipopeptides



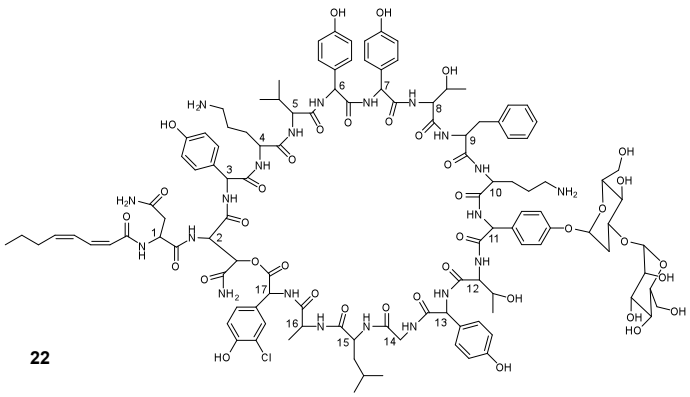
20

Cystargamide produced by *Kitasatospora cystarginea*



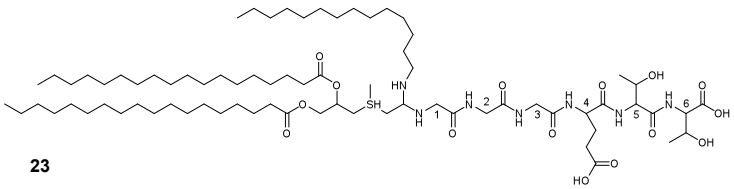
21

Daptomycin reacylated with decanoic acid from the core complex A21978C, produced by *Streptomyces roseasporus*



22

Dimannosylated ramoplanin produced by *Actinoplanes* sp.



23

Linear TAN-1511 A produced by *Streptosporangium amethystogenes* subsp. *fukuiense*

aa=amino acid

Lipopeptides

Cyclic and linear lipopeptides are produced by various actinobacterial strains and are summarized in Table 2.9.

Cyclic lipopeptides

Cyclic lipopeptides are the most common type of lipopeptides and consist of a peptide chain of various types and numbers of amino acids circularized and linked to mainly one fatty acid chain. A surfactant often falsely cited to be produced by an actinobacterium but not of actinobacterial nature, is the eleven amino acid cyclic lipopeptide arthrofactin. It was initially postulated to be produced by an *Arthrobacter* sp. (Morikawa et al., 1993) but later corrected to originate from a *Pseudomonas* strain (Roongsawang et al., 2003).

Cyclic lipopeptides that have been reported within the class *Actinobacteria* are the six amino acid containing cystargamide produced by *Kitasatospora cystarginea* (Gill et al., 2014) (20, Table 2.9), the thirteen amino acid containing daptomycin produced by *Streptomyces roseosporus* (Debono et al., 1987) (21, Table 2.9) and the depsipeptide ramoplanin, containing sixteen amino acids, and which is produced by an *Actinoplanes* sp. (Ciabatti et al., 1989) (22, Table 2.9). All of them are cyclic due to an ester linkage between the carboxyl terminus and a hydroxyl group of either a threonine or hydroxyl-asparagine.

In cystargamide, the smallest cyclic lipopeptide, an uncommon 2,3 epoxy fatty acid chain (C10) is linked to the threonine amine. Besides proteinogenic amino acids, cystargamide further contains rare 5'-hydroxy-trypthophan and 4'-hydroxyphenylglycine (20, Table 2.9). No antimicrobial activity of cystargamide could be demonstrated (Gill et al., 2014).

An outstanding example of successful screening for a surfactant with bioactive properties are A21978C complexes, known as precursors of daptomycin. They were structurally elucidated in 1987 (Debono et al., 1987) and A21978C comprises thirteen different amino acids, ten of them in the cyclic part of the structure and three in the extension of the hydrophobic tail (21, Table 2.9). Three different lipophilic tails are known, C10 ante*iso*, C11 *iso* branched and C12 ante*iso*. The most bioactive form of A21978C is daptomycin and has been generated by enzymatic deacylation of the

mixture of lipophilic tails and chemical reacylation with a decanoyl fatty acid moiety. It was approved by the U.S. Food and Drug Association (FDA) in 2003 as the first antibiotic of its kind, and commercialized as cubicin®. It is active against various gram positive bacteria including the methicilin-resistant pathogen *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae* and vancomycin resistant enterococci (Miao et al., 2005). Its ability to act as an antimicrobial requires the presence of calcium. The cyclic lipopeptide oligomerizes and uses its C10 hydrophobic tail to interact with the bacterial membrane creating a membrane perforation and cell death. This displays a novel mode of action among antimicrobial agents. Daptomycin shows high activity and a resistance to its mechanism is more difficult to generate compared to conventional antibiotics (Vilhena and Bettencourt, 2012). It is produced by a non-ribosomal peptide synthetase (NRPS) in *S. roseosporus*. The NRPS contains three subunits whose main genes have recently been identified in a 128kb cluster as *dpt*A, *dpt*BC and *dpt*D (Miao et al., 2005) with several other genes necessary to synthesize an active form of daptomycin. Its production yield of approximately 0.5 g l^1, is relatively low compared to industrial production of other microbial products. Current attempts for a heterologous production not only target novel congeners of daptomycin but also the search for high producing strains. Similar production yields compared to the wild type strain have been reported for heterologous production which was developed using a combination of metabolic flux analysis and genetic modifications (Huang et al., 2012).

Antimicrobial activity against gram positive bacteria has also been detected for ramoplanin produced by an *Actinoplanes* sp. It contains 17 amino acids, 16 of which are part of the cyclic section of the compound. It is further glycosylated at a hydroxyphenylglycine with either di-mannose (Ciabatti et al., 1989) or mannose (Gastaldo et al., 1992), thus its classification as a glycolipopeptide. Besides its glycosylation pattern, members of ramoplanin can be differentiated by their acyl amides that consist of different di-unsaturated fatty acids linked to the distal hydroxyl-asparagine. The fatty acid chain varies in length between C8 and terminal branched C9 and C10 (22, Table 2.9).

A peptide-based surfactant produced by *Streptomyces tendae,* streptofactin, was found to contain hydrophobic amino acids, but lacked fatty acid chains (Richter et al., 1998).

Linear lipopeptides

Linear lipopeptides have been found in *Streptosporangium amethystogenes* (Takizawa et al., 1995). They are reported to protect against infections in patients with leucopenia caused by cancer therapies by stimulating bone marrow cells. Different structures of these compounds are described, all share a 4'-thio C7 fatty acid chain with two ester linked C16-C19 fatty acid chains and one amide linked C13-C15 fatty acid chain. Three glycine amino acids are linked at the amide bond of the thio fatty acid with three to four proceeding amino acids varying in type (23, Table 2.9).

Other actinobacterial biosurfactants

Phenazine ester

Phenazines are a rare class of alkaloid esters. A marine *Streptomyces* sp. has been described to produce a phenazine ester that contain the desoxy pyranose quinovose esterified at either C3 or C4 to the carboxyl end of the phenazine. This phenazine-quinovose ester has been shown to exhibit antimicrobial activity. Several different types of the compound have been characterized also varying in hydroxylation and acetylation pattern at the desoxyglucose unit (Pathirana et al., 1992) (24, Table 2.10).

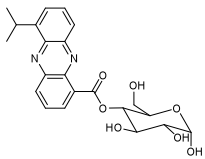
Amide glycosides

Various surfactants with nucleoside fatty amide glycoside structure are produced by actinobacteria. A group of amide glucosides is based on the uracil and disaccharide-containing tunicamycin, a glycoprotein with antibacterial properties (Dembitsky, 2005c). In this glycoprotein, two saturated or unsaturated partly branched fatty acid chains varying in length are linked via an amide to the galactosamine/glucosamine disaccharide. Besides tunicamycin, produced by *Streptomyces* spp., the tunicamycin-based surfactants streptovirudin (containing dihydrouracil) and corynetoxin (25, Table 2.10) have been reported. The latter is produced by *Corynebacterium rathayi*, a pathogen of rye grass. The organism multiplies within the galls of sheep spreading the toxic metabolite (Frahn et al., 1984). In addition, the inhibitors of bacterial peptidoglycan synthesis, liposidomycin A, B and C, have been reported to be produced by *Streptomyces griseosporus.* Liposidomycin A contains the so far uniquely described

Table 2.10: Other biosurfactants produced by actinobacteria

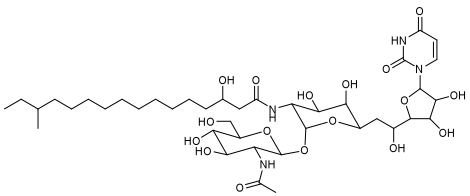
Species	Strain	Compound	Reference
Streptomyces sp.	CNB-253	Phenazine-quinovose	Pathirana et al., 1992
Streptomyces spp.* (*griseoflavus, griseosporus, halstedii, lysosuperficus, nursei, vinausdrappus*)	various	Fatty acid amide glycoside (Tunicamycin, Streptovirudin, Liposidomycins)	**Reviewed in:** Dembitsky, 2005c
Corynebacterium rathayi	n.a.	Corynetoxin	Frahn et al., 1984

Examples



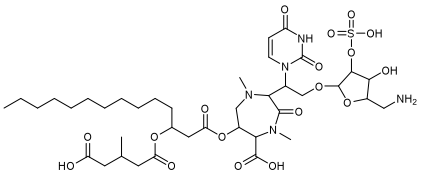
24

Phenanzine-quinovose ester produced by *Streptomyces* sp.



25

Corynetoxin produced by *Corynebacterium rathayi*



26

Liposidomycin A produced by *Streptomyces* sp.

*several producing strains are reported

fatty acid composition of 3'-hydroxy-7,10-hexadecanoic acid (Dembitsky, 2005c) (26, Table 2.10).

Not yet elucidated surfactants and their producing strains

Surface or emulsifying activity has been observed to occur from secondary metabolites of other members of the class *Actinobacteria*. Table 2.11 gives an overview of strains that are described to produce surface active compounds. Only some of the structures of these compounds have been partially elucidated.

Partly characterized surface active flocculants consisting of lipids, fatty acids and

corynemycolic fatty acids of *Corynebacterium lepus* have been described by Cooper *et al.* (1979b). In addition, eleven different glycolipids that consist of hexoses and pentoses linked to diverse fatty acid moieties that vary in length of C10 to C18 have also been described.

Besides *D. maris* (see glycolipid section), three other putative rhamnolipid-producing actinobacteria have been described. Vasileva-Tonkova *et al.* (2005) and Gesheva *et al.* (2010) detected thin layer retention values equal to L-rhamnose after acid hydrolysis of a biosurfactant produced by a *Nocardioides* sp. and *Rhodococcus fascians*. The putative rhamnolipid was not further examined in terms of the hydrophilic moiety or fatty acid compositions. Christova *et al.* (2004) reported the production of rhamnolipid by *Renibacterium solmonarium* in comparison to commercial rhamnolipids in thin layer chromatography and infrared spectroscopy. The infrared spectra showed homologies to ester and carboxylic groups; thin layer chromatographic data were not shown in the study. In all cases the detection of rhamnolipids were putative and further structural analyses remains necessary for confirmation.

Other surface active compounds were only putatively classified based on the component analysis of the crude extract towards lipid, peptide and carbohydrate compositions. Based on this limited information, it was concluded that the production of either glycolipids or lipopeptides took place (Table 2.11).

Mass spectroscopic analysis greatly assisted to partly characterize the putative wax esters produced by *D. maris* (Nakano et al., 2011). In addition, Kiran *et al.* (2010a,b and 2014) described the production of furan-containing glycolipids in *Brachybacterium* spp., *Brevibacterium* spp. and *Nocardiopsis* spp.. By analyzing hydrophilic and hydrophobic moieties after acid hydrolization, database comparison of gas chromatography-mass spectroscopic plots were used. ^{1}H NMR evaluation of compounds from the two latter strains were described to approve the resulting structure, however relative data were not shown.

Similar results have been observed for surface active extracts with a majority of peptidic compounds in the hydrophilic part in *Brevibacterium aurum* (Kiran et al., 2010c) where fractions of the biosurfactant showed molecular weights of C9-C29 methyl esters and a mass that putatively confers to a proline-leucine-glycine-glycine amino acid chain. However, mass spectroscopic database comparisons remains putative. *Leucobacter komagate* is described to produce surfactin or a surfactin-like lipopeptide. This was

Table 2.11: Actinobacterial strains identified to produce surface active compounds for which no structures have been elucidated

Species	Strain	Compound	Reference
Actinopolyspora sp.	A18	n.d. GLP	Doshi et al., 2010
Amycolatopsis tucumanensis	DSM 45259	n.d. (bioemulsifier)	Colin et al., 2013
Brachybacterium paraconglomeratum	MSA21	n.d. GL (putative furan lipid/C12)	Kiran et al., 2014
Brevibacterium aureum	MSA13	n.d. LP (putative brevifactin/C18)	Kiran et al., 2010c
Brevibacterium casei	MSA19	n.d. GL (putative furan lipid/C18)	Kiran et al., 2010a
Corynebacterium hydrocarboclastus	n.a.	n.d. polymer	Zajic JE, 1977
Corynebacterium lepus	n.a.	n.d. LP	Cooper et al., 1979a
Corynebacterium lepus	n.a.	n.d. GL	Cooper et al., 1979a
Corynebacterium lepus	n.a.	p.d. (lipid, fatty acid, mycolic acid)	Cooper et al., 1979b
Corynebacterium xerosis	n.a.	n.d. LP	Margaritis et al., 1979
Dietzia maris	WR-3	p.d. (putative wax-ester)	Nakano et al., 2011
Dietzia sp.	S-JS-1	n.d. LP	Liu et al., 2009
Frankia sp.	CpI1	n.d. GL	Tunlid et al., 1989
Gordonia amarae	SC1	n.d. (extracellular with high molecular weight)	Iwahori et al., 2001
Gordonia rubripertincta	DSM 46038	n.d.	Pizzul et al., 2006
Gordonia sp.	ADP	n.d.	Pizzul et al., 2006
Gordonia sp.	BS29	n.d. GL	Franzetti et al., 2010
Gordonia sp.	JE-1058	n.d. (extracellular)	Saeki et al., 2008
Kocuria marina	BS-15	n.d. LP	Sarafin et al., 2014
Leucobacter komagatae	183	p.d. LP	Saimmai et al., 2012b
Microlunatus sp.	NA2	n.d.	Saimmai et al., 2012a
Nocardia erythropolis	ATCC 4277	n.d. GL, PL	Macdonald et al., 1981
Nocardioides sp.	A-8	n.d. GL (putative Rhamnolipid)	Vasileva-Tonkova and Gesheva, 2005
Nocardiopsis alba	MSA10	n.d. LP	Gandhimathi et al., 2009
Nocardiopsis lucentensis	MSA04	n.d. GL (putative furan lipid/C9)	Kiran et al., 2010b
Oerskovia xanthineolytica	CIP 104849	p.d. GL (hexose, pentose C10-C18)	Arino et al., 1998
Pseudonocardia sp.	BSNC30C	n.d.	Ruggeri et al., 2009
Renibacterium salmoninarum	27BN	n.d. GL (putative Rhamnolipid)	Christova et al., 2004
Rhodococcus fascians	A-3	n.d. GL (putative Rhamnolipid)	Gesheva et al., 2010
Streptomyces sp.	n.a.	n.d. GL	Khopade et al., 2011

GL=Glycolipid; GLP=Glycolipiopeptide; LP=Lipopeptide; PL=Phospholipid;
n.a.= information not available; n.d.=not determined; p.d.=partly determined

concluded from mass spectroscopy, ^{1}H NMR and infrared spectral data by Saimmai *et al.* (2012b), but the full elucidation of the structures could not be achieved.

The long list of non-elucidated actinobacterial surface active compounds underlines the extraordinary potential of finding novel biosurfactants in actinobacteria and displays the great need for structure elucidation to allow for a better understanding of the novelty and biodiversity of the compounds produced.

Structural elucidations of actinobacterial surfactants

Various factors have been shown to influence the production, extraction, purification and structure elucidation of novel biosurfactants produced by actinobacterial strains. Due to their phenotypic growth characteristics, distinct membrane compositions and their function within the utilization of hydrocarbons, the surfactants produced are often membrane integrated, membrane associated, extracellular or a mixture of the above, and is always dependent on their particular function within the producing strains. Commonly the compounds produced exhibit antimicrobial properties, on the one hand proposing wide ranging applications, on the other resulting in opposing challenges during the production process. Special considerations are necessary when aiming for the extraction of the compound in an adequate amount and purity for structural elucidation as well as surfactant characterization. This section gives an overview of the most common techniques used to achieve successful structural elucidations.

Detection

Novel surfactant producing strains can be detected through the use of screening assays that determine a surfactant's activity either from liquid culture (cell-free supernatant or culture broth) or from solid agar plates. Various detection methods have been described, but they mostly focus on changes observed in surface tension or the solubilization and emulsification of hydrocarbons. High throughput compatible assays can be distinct from more precise assays that need several milliliters of the compound to be tested. The latter often are also applied to characterize the activity of a purified biosurfactant. Good reviews on screening techniques have been summarized by Walter *et al.* (2010) and Satpute *et al.* (2010).

Production

The manufacturing capacity of biosurfactants by a bacterial culture is limited. Wild type producing strains of the best described microbial surfactants, cultured with optimized process methods in suitable media and culture vessels reach production quantities of up to 422 g l^{-1} for sophorose lipids (Daniel et al., 1998), 112 g l^{-1} for rhamnose lipids (Giani et al., 1996), 110 g l^{-1} for spiculisporic acids (Tabuchi et al., 1977), 106 g l^{-1} for mannosylerythritol lipids (Morita et al., 2008) and 3,6 g l^{-1} for surfactin

(Yeh et al., 2005). These are rare exceptions within the typical amounts produced by microorganisms, which usually do not exceed milligram amounts. The production level is strongly influenced by non-favorable growth and production conditions due to a lack of knowledge about the organism used and compound produced when initially screening for novel surfactants or novel producer strains.

With a few exceptions (Qian-Cutrone et al., 1999; Kügler et al., 2014), the average minimum volume for successful structure elucidation of an actinobacterial biosurfactant, is typically 20 liters. Harvesting of the surfactants is type dependent and either whole cell broth (intracellular or membrane associated surfactants) or cell free supernatant is used as a starting point.

Glycolipids

A typical method for the extraction of surfactants from culture broth or supernatant is the use of two phase extractions. In a first step, if appropriate, non-polar solvents (e.g. n-hexane) are used to remove residual hydrocarbons from the cultivation broth. If extraction is carried out from whole cell broth or wet cell mass, glycolipids are either captured by direct cell extraction or by cell treatment (e.g. sonication) prior to the extraction.

In a second step, the surfactant is removed by repeated agitation with a medium polar solvent or solvent mixture. Most commonly, combinations of chloroform and methanol or polar aprotic solvents such as ethyl acetate or methyl-*tert*-butyl ether are used. A frequency solvent distribution for the extraction of glycolipids from "rare" actinobacteria is shown in Figure 2.2, comprising data of 47 two-phase extraction methods used to enrich surfactants produced from either cell-free supernatant or the culture broth. Depending on the chemical characteristics of the glycolipid, an acidification step (pH2 – pH3) with subsequent incubation (4 °C) prior to the extraction process could result in enhanced product recoveries (Passeri et al., 1990; Konishi et al., 2014). Often, after dehumification, further washing steps are applied, either of a hydrophilic (e.g. ultrapure water) or a hydrophobic (e.g. n-hexane) nature. For the polymeric glycolipid lipoarabinomannan and related structures, a hot-phenol water method is almost exclusively used (Sutcliffe, 2000).

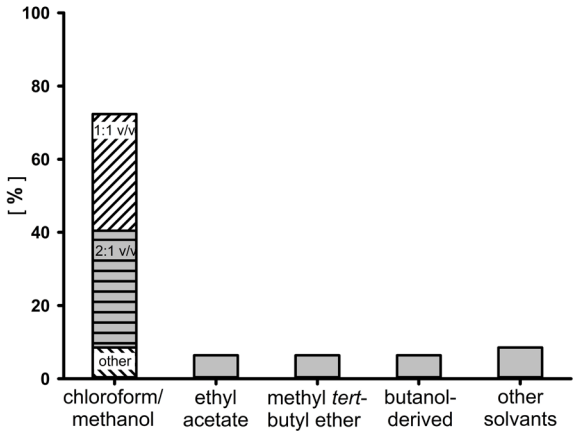


Figure 2.2: Frequency distribution of solvents used for the enrichment of surfactants by two-phase extraction from the culture broth or cell free supernatant of 47 "rare" actinobacteria

The glycolipids produced, mainly present in mixtures of different forms, need to be separated for structural analysis. This procedure is usually performed by combinations of chromatographic steps using either gradient columns or preparative medium- and high pressure chromatography. In addition, preparative planar chromatographies are reported as an additional purification step for the isolation of pure compounds (Powalla et al., 1989; Pasciak et al., 2002; Pasciak et al., 2004). Rarely applied is the use of absorbers within the cultivation process. The number one choice for chromatography is the use of hydrophobicity affiliated separations with silicic acids as an absorbing material. In approximately 80 % of structure reports from "rare" actinobacteria, silicic acid is used with various elution gradients of non-polar and polar solvents. Separated compounds are often further purified by repetitive silica chromatography using different gradients or by subsequent (or preceding) steps with different column material. Therefore, either reverse-phase C18 chromatography or cellulose-based ionic interaction chromatography are widely used.

Lipopeptides

The diversity of different peptide-based surface active compounds produced by actinobacterial strains is much smaller than that of reported glycolipids. Depending on the lipopeptide produced, two different approaches for the concentration of the surfactants are used. Either the lipopeptide can be precipitated from the liquid culture/supernatant by either using cold acetone, methanol, salt concentrations, acidic environments, or a direct extraction by medium polar solvents similar to those

used for glycolipids have been reported. Besides the chromatographic purification steps used for glycolipids, gel filtration has been successfully used as an additional step (Takizawa et al., 1995).

Structural elucidation

Once a compound is purified to a sufficient extent, component analysis, specific staining methods and mass spectroscopic examinations are widely used to get a first hint about the type of surfactant produced. A more detailed schematic of the surfactant can be deduced from mass spectroscopy fragmentation studies, often revealing mass abundances of separated hydrophilic and hydrophobic parts of the glycolipid. However, complete structure examinations (of complete compounds or hydrolyzed components) rely on multi-dimensional nuclear magnetic resonance spectroscopy.

Natural habitats of biosurfactant-producing actinobacteria

With the exception of a few strains, the great majority of surfactant-producing actinobacteria have been isolated from three different environments. These are: (1) Hydrocarbon contaminated soils, (2) infections caused by the actinobacterium itself, and (3) marine-derived samples. Obviously, this must not reflect the distribution of surfactant-producing actinobacteria in nature, but it is clear that there is a link between the type of environment and the ability of actinobacteria to produce biosurfactants and can be considered to be environmentally-driven.

Hydrocarbon contaminated soil

The formation of various actinobacterial surfactants is mainly observed during growth in a range of different hydrophobic carbon sources such as nparaffin, nhexadecane or vegetable oils. Occurrences of surfactant-producing microorganisms seems to correlate to environments in which hydrophobic carbon sources are present, no matter if these are oil contaminated or oil enriched (Powalla et al., 1989; Arino et al., 1998; Christova et al., 2004; Pizzul et al., 2006; Liu et al., 2009; Ruggeri et al., 2009; Christova et al., 2014). Evoked by their hydrophobic cell wall due to incorporation and association of various lipoglycosides, actinobacteria preferably grow in hydrophobic droplets that are

dispersed in the aqueous phase when cultured in cultivation devices. The surfactants produced facilitate the uptake of these difficult–to-access carbon sources by dispersing it into small droplets that can easily be pre-digested by extracellular enzymes.

Infections

A second feature of surfactants is the antimicrobial property exhibited by most of these compounds. Endowed with nutritional and growth advantages towards surrounding organisms, surfactant producers can become rampant, and are often less affected by substances present during its growth, e.g. antimicrobial drugs. They have been found in patients that suffer from infections/diseases caused by human deficiency viruses (Guérardel et al., 2003), patients with lung infections and infections of the oral cavity (Datta and Takayama, 1993; Sutcliffe, 1995; Tanaka et al., 1997). In addition, biosurfactant-producing actinobacterial strains have also been isolated from infected plant tissue (Frahn et al., 1984).

Marine habitat

Many actinobacteria are specialists in survival and native to a wide range of extreme environments. Surfactant-producing genera have been isolated from various marine-associated habitats (Passeri et al., 1990; Khopade et al., 2011; Nakano et al., 2011). Several of these environments exhibit rather extreme conditions, amongst which are deep sea sediments or hydrothermal fields (Peng et al., 2008; Konishi et al., 2014; Wang et al., 2014), ornithogenic exposed soil (Vasileva-Tonkova and Gesheva, 2005) as well as actinobacteria isolated from sponges (Gandhimathi et al., 2009; Kiran et al., 2010a,b,c, 2014) and hard corals (Osawa et al., 2010). An antimicrobial effect of surfactants produced in a highly procaryotic populated sponge tissue is apparent. However, the reason for the frequent occurrence of surfactant producers within the other marine habitats, still remains to be understood.

Summary and Conclusion

A wide range of unique and diverse surfactants produced by actinobacteria have been reported. Various glycolipids, lipopeptides and other surfactant types are produced by numerous species, all belonging to the order *Actinomycetales.* Taking into account the fact that only a minority of actinobacteria is culturable and the given list of surfactant producing strains without structurally elucidated compounds (Table 2.11), the sheer magnitude of actinobacterial surfactants that still remain undetermined is evident. The ability of actinobacteria to produce biosurfactants seems to be influenced by their natural habitat. From the three main sources of surfactant producing actinobacteria it can be concluded that the compounds produced mainly serve for either gaining access to hydrophobic carbon sources or as a bioactive agent against competing strains.

In order to pave the way towards biotechnological applications of actinobacterial surfactants, emphasis should be placed on (1) structural elucidation of described, but not identified biosurfactants, (2) the identification of novel actinobacterial surfactants by the implementation of next generation screening methods; (3) the production of sufficient amounts of surfactants for application based studies; and (4) production processes that result in high yields and that would cut down on the production costs.

(1) Actinobacterial strains with a surface active culture broth or supernatant often are declared as "novel" biosurfactant producing strains, without elucidation of the surface active compound(s) produced and a list of producing strains is given in this article whose surfactant structures remain to be identified (Table 2.11). For a successful structural identification of the compound, sufficient quantities of the isolated surface active compound at an adequate purity is necessary in order to apply the various analytical methods necessary. This aspect was reviewed in the structural elucidation of actinobacterial compounds section. Quite a few of the studies cited lacked sufficient strain information and further research can only be ensured if the strains reported have designated strain numbers and thus are available for other researchers to pursue the production of these potentially novel biosurfactants.

(2) Approaches for the identification of novel biosurfactants mainly remain traditional by the detection of interesting producing strains and subsequent isolation and characterization of the compound produced. To further expand the variety of actinobaterial surfactants, alternative screening methodologies that are already known to be used for the detection of novel lead molecules in the pharmaceutical industry could be applied. Genome-based information technology to reveal pathways that can be implemented into artificial surfactant synthesis cascades are currently being investigated. These attempts would allow for access to both undetected and cryptic pathways present in actinobacteria. By direct sequencing of metagenomic derived DNA, enzyme information acquired could be expanded to information gained from non-culturable and slow growing species.

(3) Many of the surface active compounds produced by actinobacteria potentially show interesting properties as biotechnological products or additives. Often, as is the case for many of the compounds summarized in this article, an application based study is lacking. This is most probably due to low availability of the product and can be traced back to the use of low quantity producing strains. Focus on a novel actinobacterial surfactant, along with progress in the development towards novel biotechnology-based products, will only be made possible if enough substance for initial studies on bioactivity or other interesting applications can be acquired. If an adequate amount of substance is not achievable by standard bioprocess engineering attempts, metabolomic approaches and flux analysis could lead the way. Furthermore, the identification of enzymes involved in the synthesis and their genetic regulation can give an important input into the improvement of fermentation processes. An implementation of the surfactant's synthesis through adequate heterologous production strains could lead to higher quantities of the different surfactants produced. Potential applications of a novel compound is a guarantee of success in white biotechnology and negates the efforts made with regards to its production, purification and elucidation.

(4) Currently, comparatively high production costs combined with low production yields restrict the development of compounds as valuable products, and are mainly limited to high purity applications, e.g. the drug industry. Several examples in the past have shown that once a potential application for a specific compound is foreseen,

intensive research is set in motion to facilitate production and purification processes, cutting costs, enhancing yields and, although research often lasts for decades, compounds might end in industrial scale production and application.

One example of an actinobacterial surfactant that successfully underwent the process from detection to application is the antimicrobial agent daptomycin. It was initially produced semi-synthetically in a three step procedure, but later a direct synthesis of daptomycin was achieved by feeding toxic decanoic acid to a carbon-limited production culture (Huber et al., 1988). Production rates were further increased by 10 – 30 % by using a mixture of less toxic decanal and a solvent to solubilize the hydrophobic carbon feed (Bertetti et al., 2012). Mutagenesis approaches (Yu et al., 2011; Li et al., 2013), genome shuffling (Yu et al., 2014) and directed overexpression (Huang et al., 2012), have recently led to further increases in production yields. Other examples of success stories, are non-actinobacterial surfactants that have been pushed to application: sophorolipids, mannosyl erythritol lipids and the lipopeptide surfactin have found application in cosmetic industries (Fracchia et al., 2014). Sophorolipids are even applied in low cost cleaning products.

Actinobacteria clearly represents a unique and vast untapped resource for the discovery of novel and potentially useful biosurfactants. The surfactants produced by members of the class *Actinobacteria* are a highly interesting group of products that could be of great importance in the future in both the area of basic research and application-oriented industrial research.

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Trehalose lipid biosurfactants produced by the actinomycetes *Tsukamurella spumae* and *T. pseudospumae*

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Contribution to this publication:

JK has concepted and written this study, performed experiments, collected and interpretated the relevant data used. RH has contributed to production, purification and discussion within his Bachelor Thesis (Heinzler 2012). CMG, WV, BL and SL contributed within aquiring and interpreation of NMR data, BK and GBW contributed within aquiring and interpreation of MS data. All authors have fruitfully discussed content and structure of this study.

2b Trehalose lipid biosurfactants produced by the actinomycetes *Tsukmurella spumae* and *T. pseudospum*ae

Introduction

Actinomyceteales is a group of mycolic acids containing gram positive microorganisms with characteristic filamentous growth behaviour. Members of this group are known to produce a large variety of biological substances. With 45 % of all known bioactive microbial metabolites those of actinomycete origin represent the largest group (Berdy 2005). Compared to other bacteria they have relatively large genomes and Baltz *et al*. (2008) assumed that 5-10 % of their genome coding capacity is used for the production of (mainly cryptic) secondary metabolites. Often, these secondary metabolites have antimicrobial properties.

When grown on hydrophobic carbon sources some microorganisms produce secondary metabolites in form of biosurfactants, a structurally versatile group of surface-active agents. Best studied low molecular microbial surfactants belong to the classes of lipopeptides or glycolipids whereat either peptides or carbohydrates carry fatty acid or hydroxy fatty acid chains. Due to their amphiphilic structure, they lower the tension of interphases, act as emulsifying agents and may facilitate the uptake of hydrophobic carbon sources. Some biosurfactants show antimicrobial effects and their production is often

connected to growth limiting conditions (Philp et al. 2002; Ristau and Wagner 1983; Vollbrecht et al. 1998). Biosurfactants are considered to be more sustainable compared to chemical surfactants as they have the advantage of being readily biodegradeable. They show effects in bioremediation, are effective at low concentrations, are less toxic and independent of mineral oil as a substrate. These facts, sustainability regulations and a broader ecological awareness of end-users have led to an increased interest of biosurfactants in nutrient-, cosmetic-, textile-, varnish-, pharmaceutical-, mining- and oilrecovery industry (Cameotra and Makkar 2004; Henkel et al. 2012; Marchant and Banat 2012; Müller et al. 2012).

Different types of low molecular biosurfactants are reported to be produced as secondary metabolites by the actinomycetes *Rhodococcus sp.* (Lang and Philp 1998; Philp et al. 2002; Ristau and Wagner 1983), *Streptomyces sp.* (Khopade et al. 2011; Richter et al. 1998), *Tsukamurella sp.* (Choi et al. 1999; Vollbrecht et al. 1998), *Nocardiopsis sp.* (Kiran et al. 2010), *Nocardia sp.* (Kim et al. 2000; Powalla et al. 1989), *Gordonia sp.* (Iwahori et al. 2001; Jackisch-Matsuura et al. 2014), *Arthrobacter sp.* (Morikawa et al. 1993) and myco- and corynebacteria (Desai and Banat 1997). The surfactants produced are glycolipids or lipopeptides either excreted into the cell culture or produced as cell-associated glycolipids. A reason for the attachment to cell membranes often is due to a characteristic hydrophobic chemotype IV cell wall (Embley and Stackebrandt 1994).

Mainly produced by *Rhodococcus sp*. and best described within actinomycetal biosurfactants are glycolipids that share a hydrophilic backbone of two glucose units α,α-1,1 glycosidic linked to form trehalose. Bound to trehalose are hydrophobic acyl chains that vary in number of occurrence, length and type, as well as the position of their glycosidic bond at the sugar rings. They are classified into non-ionic trehalose mono-, di- and trimycolates and anionic trehalose tetraesters and succinoyl trehalose lipids (Kuyukina and Ivshina 2010). In several studies, trehalose lipids have shown interesting properties in the remediation of mainly hydrobcarbon-contaminated soils, the removal of suspended solids from wastewater (Franzetti et al. 2010) as well as in enhanced oil recovery (Christofi and Ivshina 2002). Trehalose lipids have been reported to be less toxic than their synthetic counterparts (Franzetti et al. 2010) and feature several biomedical properties like antimicrobial, antiviral (Azuma et al. 1987; Shao 2011; Watanabe et al. 1999) and anti-tumor activity (Franzetti et al. 2010; Gudiña et

al. 2013; Sudo et al. 2000). Moreover they can act as therapeutic agents due to their functions in cell membrane interactions (Shao 2011; Zaragoza et al. 2009).
The current article reports on the production, purification and structure elucidation of two trehalose lipid biosurfactants by the actinomycetes *Tsukamurella spumae* and *T. pseudospumae* grown on hydrophobic carbon sources under nitrogen limiting conditions. *Tsukamurella spumae* and *T. pseudospumae* hold an advantage for production of biosurfactants, as they are regarded as risk group one organism by classification according to the German Technical Rules for Biological Materials (TRBA) (DSMZ webpage)

Materials and Methods

Unless stated otherwise, all experiments were conducted as triplicates.

Microorganisms

Six non-pathogenic strains of the actinomycetes *Tsumakurella sp.*, (*Tsukamurella spumae* DSM44113, DSM44114, DSM44115, DSM44116 and *Tsukamurella pseudospumae* DSM44117 and DSM44118), originally isolated from activated sludge foam (Nam et al. 2003; Nam et al. 2004) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Strains were stored in cryo-stocks at -80 °C in 15 % glycerol and GYM media containing per liter 4 g glucose, 4 g yeast extract and 10 g malt extract.

Microbial production

For cultivation, a loop of a single colony from a GYM agar plate was inoculated in GYM and grown at 30 °C and 130 rpm for three days (100 ml baffled conical flasks, 20 ml broth volume) using an incubation shaker (Multitron II, Infors, Bottmingen, Switzerland). 1 ml was transferred into a mineral media modified from Vollbrecht et al. (1998) containing per liter: 1.24 g $(NH_4)_2SO_4$, 20 g sunflower oil, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 0.01 g $CaCl_2 \cdot 2\ H_2O$, 100 ml 1M K_2HPO_4/KH_2PO_4 pH 7.0, 0.444 mg $ZnSO_4 \cdot 7\ H_2O$, 0.075 mg $CuSO_4 \cdot 2\ H_2O$, 6.3 mg $FeSO_4 \cdot 7\ H_2O$, 0.255 mg $MnSO_4 \cdot 1\ H_2O$, 0.672 mg $CoSO_4 \cdot 7\ H_2O$, 0.081 mg $NiSO_4 \cdot 6\ H_2O$, 0.078 mg

$(NH_4)Mo_7O_{24}$ • 7 H_2O, 0.186 mg H_3BO_3, 0.03 mg KI, 250 mg Na_2EDTA • 2 H_2O. Actinomycetes were grown for three days, 30 °C and 110 rpm (500 ml baffled conical flasks, 100 ml broth volume) prior to inoculation into a bioreactor. Alternatively 20 g glyceryltrioleate were used instead of sunflower oil as carbon source.

For batch fermentation 500 ml parallel bench-scale bioreactors (Sixfors, Infors, Bottmingen, Switzerland) filled with equal media to the precultures were inoculated with 10 % (v/v) to a total broth volume of 440 ml and the process was run for 96 h. Physiological activity was monitored by an internal pO_2 electrode and pH 7.0 was controlled and adjusted by an internal pH-electrode using 4M H_3PO_4 and 4M NaOH. 30 °C temperature, airflow of 0.25 vvm and stirring speed of 800 rpm were kept constant throughout the process.

During fermentation, 5 ml culture samples were taken at different time points for the analysis of growth characteristics:

Samples were mixed 1:1 with n-hexane and centrifuged for 20 min at 4643 x g and 4 °C. After removal of the hydrophobic hexane phase and the aqueous supernatant, dry cell mass was determined gravimetrically by washing the cell pellet with 1.5 ml 0.9 % (w/v) NaCl, centrifuging, decanting and drying to constant weight.

The content of sunflower oil in the culture was determined gravimetrically after evaporation of the n-hexane phase of the sample at 40 °C, 50 mbar and 2000 rpm using a vacuum concentrator (ScanSpeed MiniVac Evaporator, Saur, Reutlingen, Germany).

The ammonium ion concentration in the supernatant was determined by an ammonia essay using photometric quantification (Spectroquant 114752, Merck, Darmstadt, Germany) according to the supplier's manual.

Ammonia ion data points were fitted using a sigmoidal model with three parameters (SigmaPlot, version 9.01, Systat Software, Inc., Washington, USA) using equation (Eq. 1):

$$y = \frac{a}{1 + e^{\left(\frac{x - x_e}{b}\right)}} \qquad \text{Eq. 1}$$

Sunflower oil data points were fitted using an exponential decay with three parameters using equation (Eq. 2):

$$y = a\, e^{-b\,x} \qquad \text{Eq. 2}$$

pO_2 were plotted exemplarily for one bioreactor (Figure 2.4).

Critical micelle concentration of the TL A and TL B mixture of fraction 38 (Figure 2.5, lane 4) and alteration in the surface tension of the culture supernatant were monitored against air at room temperature using the Du Noüy ring method (1919) on a Tensiometer (Lauda TD1, Lauda-Königshofen, Germany) according to the supplier's manual.

Surface tension data points were fitted using a logistic model with four parameters using equation (Eq. 3):

$$y = y_0 + \frac{a}{1 + \left(\frac{x}{x_0}\right)^b} \qquad \text{Eq. 3}$$

Emulsification index (E_{24}) was measured by a method modified from Bicca et al. (1999). 2 ml of the TL A and TL B mixture of fraction 38 (Figure 2.5, lane 4) and 2 ml kerosene (purum, Sigma-Aldrich; Germany) was mixed with a vortex for 1 min in a 10 ml screw cap glas vial at room temperature. After 24 hours, the E_{24} index was determined as percentage of the height of emulsified layer by the total height of the liquid column.

Purification and detection of trehalose lipids

For each sample, 4 ml of the supernatant was extracted twice with 5 ml ethyl acetate by mixing vigorously and centrifugation for 20 min at 4643 x g and 4 °C. The organic phases were combined and evaporated at 40 °C, 50 mbar and 2000 rpm in a vacuum concentrator (ScanSpeed MiniVac Evaporator, Saur, Reutlingen, Germany).

The bioreactors were harvested after the fermentation process. Residual sunflower oil was removed by extraction with 300 ml hexane of the fermentation broth of each bioreactor. The aqueous phase was extracted twice with ethyl acetate in a ratio (1:1.25 v/v). The combined organic phases from three bioreactors were concentrated to dryness using a rotary evaporator (Heidolph Laborota 4000, Schwabach, Germany) at 40 °C and 240 mbar followed by vacuum concentration at 40 °C, 2000 rpm and 50 mbar in order to gain a crude trehalose lipid extract.

Qualitative measurment of the trehalose lipids was performed by thin-layer-chromatography (TLC) using the stationary phase 60 Å silica (Alugram Xtra SIL G, Macharey-Nagel, Düren, Germany) and the solvent system

chloroform / methanol / acetic acid (65:15:2 v/v/v). Trehalose lipids were detected using a freshly prepared solution of acetic acid / anis aldehyde / sulphuric acid (100:1:2 v/v/v) and development under 150 °C air stream for 2-4 min (Daniel et al. 1998), trehalose lipids resulted in blue to purple spots.

Trehalose lipids were dissolved in chloroform / methanol (9:1 v/v) and further purified for structural analysis by medium-pressure-liquid-chromatography (SepacoreX50, Büchi, Flawil, Switzerland) using 40-63 µm particle size silica stationary phase with 60 Å pore size (150 mm column length, 12 mm column diameter and 17 ml bed volume) and a chloroform / methanol solvent system at a flow rate of 5 ml min^{-1} (gradient: 20 min 90 %-90 % $CHCl_3$; 25 min 90 %-10 % $CHCl_3$; 10 min 10 %-10 % $CHCl_3$). The eluate was collected in 5 ml fractions. Fractions 34 and 38 were used for structural analysis (Figure 2.5).

Structural analysis

MALDI-ToF / MS experiments of the purified fractions were carried out with a 4800 Plus MALDI-ToF-ToF mass spectrometer (Applied Biosystems / MDS SCIEX, Foster City, CA) equipped with a Nd:YAG pulsed laser (355 nm wavelength of <500 ps pulse and 200 Hz repetition rate). The software used for analysis were the 4000 Series Explorer software (V 3.5.3) and the Data Explorer software (V 4.9). Data acquisition was performed in the reflector positive ion mode. For each mass spectrum an average of 500 laser shots over the whole spot were accumulated.

For analysis with MALDI-ToF / MS, 0.5 µl of each sample was mixed with 0.5 µl of the matrix solution (10 mg ml^{-1} α-cyano-4-hydroxy cinnamic acid (CHCA) in 50 % acetonitrile with 0.1 % trifluoroacetic acid).

For MALDI-ToF / MS / MS analysis a relative precursor mass window range around the set precursor masses at $[M + Na^+]^+$ 727, 725, 699 and 697 was specified with ± 50.00 full-width at half maximum.

For high mass accuracy 0.5 µl of a manufacturer's calibration kit (des-Arg-Bradykinin m/z = 904.47 Da, Angiotensin I m/z = 1296.69 Da, Glu-Fibrinopeptide B m/z = 1570.68 Da, ACTH clip 1-17 m/z = 2093.09 Da, ACTH clip 18-39 m/z = 2465.20 Da) dissolved in matrix solution was used for calibration. The mixture was spotted in two 0.5 µl steps onto the MALDI target and air dried.

For NMR spectroscopy 20 mg of purified fraction 34 was dissolved in 0.8 ml

$CDCl_3$ / CD_3OD (70:30 v/v) (Sigma-Aldrich; Germany). One dimensional ^1H NMR spectroscopy and two dimensional ^1H-^1H correlation spectroscopy (COSY), total coherence transfer spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY) and ^1H-^{13}C heteronuclear single quantum coherence spectroscopy (HSQC) and heteronuclear multiple-bond correlation spectroscopy (HMBC) were recorded on a Bruker AVANCE II+ 600 MHz spectrometer (Bruker AG, Rheinstetten, Germany) equipped with a BBI probe head. Spectra were analyzed with Topspin 3.2 (Bruker AG) and Spinworks 3.1.8 software (Marat). Intensities were measured from a 1D ^1H spectrum acquired with a single scan. Chemical shifts are referenced to the ^1H and ^{13}C resonance of the residual $CHCl_3$ signal.

Results

The production of trehalose lipids (TL) has been observed during growth of *Tsukamurella spumae* DSM44113, *Tsukamurella spumae* DSM44114 and *Tsukamurella pseudospumae* DSM44117 on sunflower oil and its main component glyceryltrioleate as sole carbon source in a mineral salt media previously described by Vollbrecht *et al.* (1998). No production occurred when grown on glyceryltridecanoate

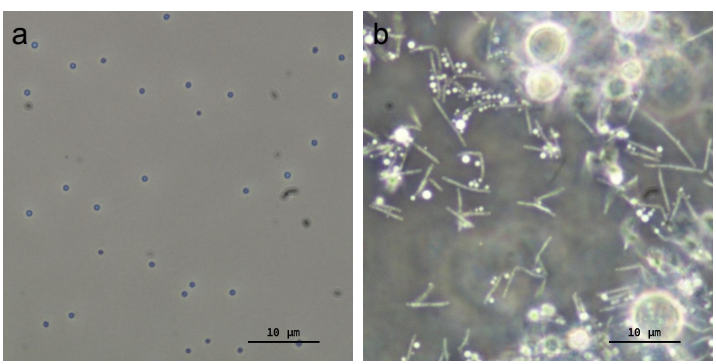


Figure 2.3: Light microscopic image with 1000x magnification and phase contrast of a 48 hour shake flask culture of *Tsukamurella spumae* in a medium containing sunflower oil as sole carbon source. Same amount of culture broth is spotted (a) taken from the aqueous phase of the culture and (b) taken from the oily phase of the same culture. Whereas only small droplets of oil are visible in the aqueous phase, an accumulation of cells (rod shaped) in the oily phase can be observed where the actinomycetes emulsify the oil (light spots) into smaller droplets

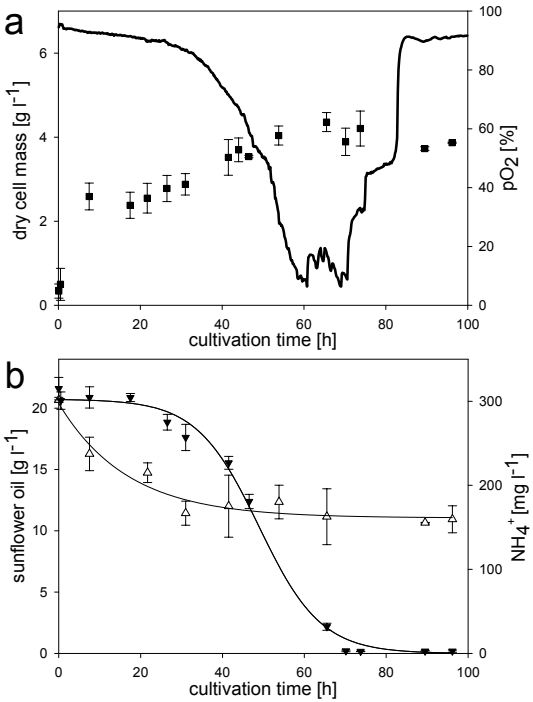


Figure 2.4: Time course of dry cellmass (black square) and pO_2 values (black line) (a) and depletion of sunflower oil (open triangle) and ammonia ions (filled inverted triangle) of *Tsukamurella spumae* DSM44113 during batch cultivation

or glycerol as sole carbon source. During growth of all *Tsukamurella* strains tested, cells accumulate in the hydrophobic phase and are present in the aqueous phase of the broth only later during cultivation (Figure 2.3). Throughout the process an actinomycetal characteristic agglomeration of the cells and its adherence to the glass surface of the cultivation device was observed.

Trehalose lipids from *Tsukamurella spumae* DSM44113 have been produced in 400 ml bench scale bioreactors as triplicates under nitrogen limiting conditions. A rapid decrease of sunflower oil in the beginning of the fermentation process combined with a rapid increase of cell mass (hours 1-10, Figure 2.4) is probably due to the adhesion of residual oil to the cells' surface that distort the gravimetric measurements of the parameters mentioned. Due to the strong appearance of cells at the glass surfaces the absolute dry cellmass is estimated to be superior to the broth's dry cellmass determined during fermentation. The production of trehalose lipids was observed already in the preculture and increased under nitrogen limiting conditions in the fermentation process visualized by an increasing density after staining equal amounts of extracts via

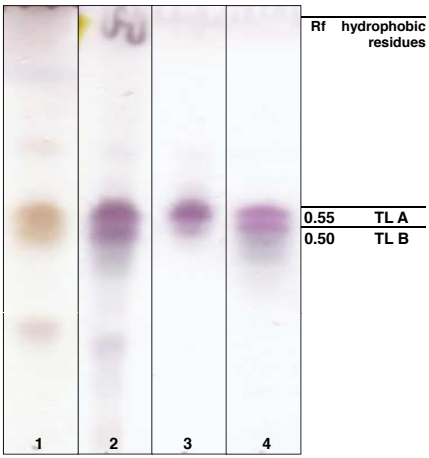


Figure 2.5: Thin layer chromatography of trehalose lipid (TL) crude ethyl acetate extracts produced by *Tsukamurella spumae* DSM44113 during growth on glyceryltrioleate (lane 1) and sunflower oil (lane 2) as sole carbon source and their retention factor values (Rf). Extracted TL from the sunflower oil cultivation are further purified into fraction 34, containing mainly TL A (lane 3) and fraction 38 containing both, TL A and TL B (lane 4)

TLC (appendices Figure A-2b-1). The surface tension of non-inoculated media was 51.77 (± 1.63) mN m^{-1} and immediately dropped after inoculation of the bioreactor to 40.73 (± 0.34) mN m^{-1}. It was then lowered gradually until it reached a value of 32.77 (± 0.60) mN m^{-1} at the end of the fermentation process (96 hours). The total yield of crude trehalose lipid extract was 1.28 g l^{-1}.

After extraction of the supernatant with ethyl acetate the mixture of trehalose lipids produced by *Tsukamurella spumae* in sunflower oil and glyceryltrioleate as carbon source are stained on a TLC as two double spots (TL A and TL B) with retention factors of

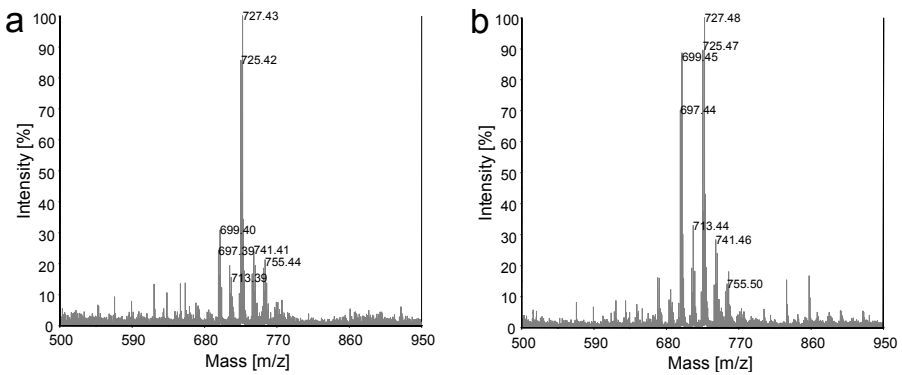


Figure 2.6 Mass spectra of the trehalose lipids produced. Shown are intensities of masses present in the fractions containing (a) mainly TL A and (b) both TL A and TL B

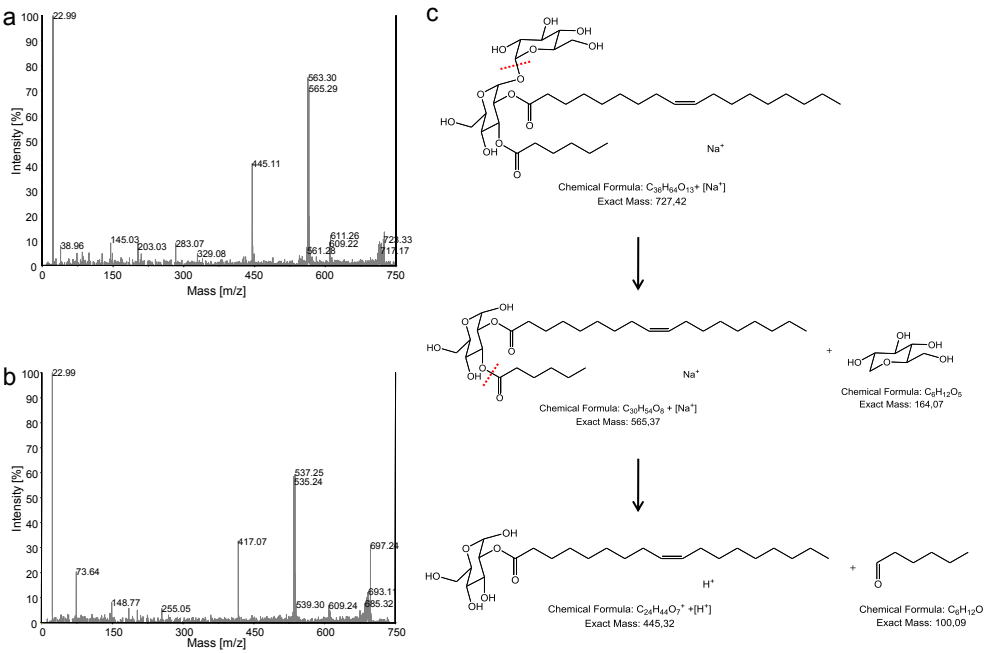


Figure 2.7: Mass spectra and MS/MS-fragmentation at m/z $[M + Na^+]^+$ 727.48 (a) and 699.45 (b). Fragmentation is shown exemplarily for monounsaturated TL A with $[M+Na^+]^+$ 727.42 (c)

0.55 and 0.50 (Figure 2.5). In order to extract potential cell bound biosurfactants, cell mass of *Tsukamurella spumae* was extracted with several solvents such as ethyl acetate, diethyl ether, chloroform, methanol and a mixture of chloroform and methanol (2:1 v/v), but none of these combination led to the isolation of the compound extracted from the supernatant as determined by TLC.

For structural analysis of the glycolipid, extracted trehalose lipid crude extract was separated by silica gel column middle pressure liquid chromatography and led to fraction 34 containing mainly TL A and fraction 38, a mixture of TL A and TL B (Figure 2.5, lane 3 and lane 4). MALDI-ToF / MS measurements of these fractions revealed sodium adducts with an m / z $[M + Na^+]^+$ of 727 for TL A and 699 for TL B that partly concur to some of the sodium adduct masses of GL1 of *Tsukamurella sp.* (Vollbrecht et al. 1998) later classified as *Tsukamurella tyrosinosolvens.* Further, 2 Da smaller derivates were observed for TL A ($[M + Na^+]^+$ 725) and TL B ($[M + Na^+]^+$ 697) indicating the occurrence of mono- and double-unsaturated fatty acids chains of the trehalose

lipid molecules (Figure 2.9). Fragments formed during MALDI-ToF-ToF / MS / MS measurements of the trehalose lipids at $[M + Na^+]^+$ 727, 725, 699 and 697 revealed a single glucose unit bound to C18 / C6 and C16 / C6 with one and two double bonds ($[M + Na^+]^+$ 565 / 563 and 537 / 535 respectively). Further fragmentation of the compound results in a glucose unit only carrying the C18:1 or the C16:1 chain ($[M + H^+]^+$ 445 and 417) (Figure 2.7).

To confirm the structure of the trehalose lipids, one and two-dimensional ^1H and ^{13}C-NMR of fraction 34 containing mainly TL A allowed to assign the hydrogen nuclei in the molecule to the trehalose and fatty acid moieties present (Figure 2.8, Figure 2.9 and Table 2.12). Full chemical shift assignment of the sugar moiety was achieved in ^1H COSY (Figure 2.8b and 2.8c), ^1H TOCSY and $^1H^{13}C$-HSQC spectra (Table 2.12 and appendices Figure A-2b-1 and Figure A-2b-2). This confirmed the 1 / 1' glycosidic bond of the two sugar molecules forming the trehalose unit (4.90 ppm, and 5.08 ppm). Hydrogens 2' and 3' of one of the glucoses are downfield shifted (4.67 ppm and 5.25 ppm opposed to 3.34 ppm and 3.60 ppm for the other sugar unit) (Figure 2.8 and Figure A-2b-3), a clear indication that the two fatty acids are glycosidically linked to the corresponding carbons.

Trehalose unit:	**C Shift [ppm]**	**H Shift [ppm]**	**Multiplicity**	**Coupling [Hz]**
–**C^{1}H**–O–	94.0	4.90	d	3.6
–**C^{2}H**–	71.5	3.34	dd	3.8; 9.9
–**C^{3}H**–	72.9	3.60	t	9.3
–**C^{4}H**–	69.8	3.22	t	9.2
–**C^{5}H**–	72.1	3.41	m	n.d.
–**C^{6a}H**–	60.9	3.53	d	5.2
–**C^{6b}H**–	60.9	3.50	t	3.5
–**C$^{1'}$H**–O–	91.1	5.08	d	3.6
–**C$^{2'}$H**–O–*R*	70.4	4.67	dd	3.6; 10.2
–**C$^{3'}$H**–O–*R*	71.8	5.25	t	9.5
–**C$^{4'}$H**–	68.6	3.37	t	9.7
–**C$^{5'}$H**–	72.1	3.76	m	n.d.
–**C$^{6a'}$H**–	61.0	3.65	dd	2.5;12.1
–**C$^{6b'}$H**–	60.9	3.52	d	3.0
Acyl moiety:	**C Shift [ppm]**	**H Shift [ppm]**	**Multiplicity**	**Coupling [Hz]**
–**CH3**^{a}	13.6/13.4	0,66-0,70	t	6.2
–**CH3**^{b}	13.1	0.75	t	7.4
–**CH2**–	22.0–22.2 28.5-29.4 30.9-31.6	1.06-1.18	m	n.d.
–$^{\beta}$**CH2**–C–CO–O–*R*	18.0/24.6	1.35-1.44	t	7.4
–**CH2**–C=C–**CH2**–	26.7	1.80-1.85	m	n.d.
–$^{\alpha}$**CH2**–CO–O–*R*	35.9/34.0/33.6	2.13–2.09	m	7.7
–C=C–**CH2**–C=C–	25.2	2.57	degenerate	6.6
–**CH=CH**–	127.5-129.9	5.11-5.17	m	n.d.

Table 2.12 Chemical shifts of carbon and hydrogen nuclei, multiplicity of the peak observed and its coupling constant of both, the hydrophilic moiety and the acyl chains. Carbon atoms of the two sugar units are numbered from 1-6 and 1'-6' respectively. d=doublet, t=triplet, m=multiplet

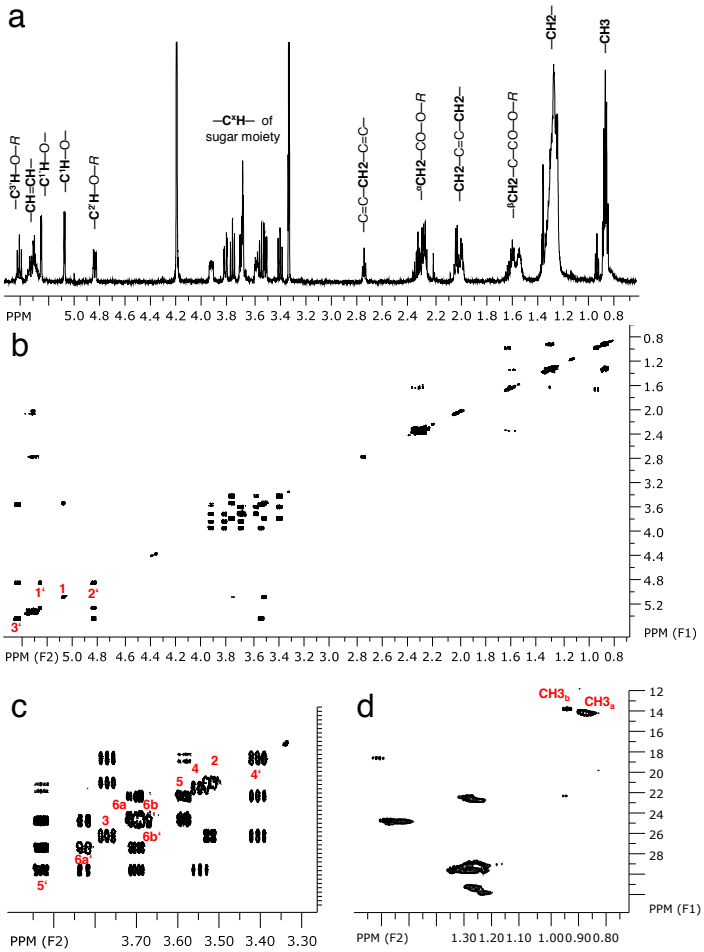


Figure 2.8: NMR of the purified fraction containing mainly trehalose lipid TL A. (a) ^{1}H NMR spectra, relevant carbon and hydrogen groups are assigned in bold text. (b) Two dimensional correlation spectroscopy of ^{1}H NMR (COSY). (c) Close-up of the sugar fraction and assignment of hydrogens at the relevant carbon atoms, the acylated groups are assigned in (a). (d) close-up of the CH_3 groups in ^{1}H / ^{13}C heteronuclear single quantum correlation spectroscopy (HSQC)

Characteristic peaks at 5.11 ppm with carbon chemical shift of ~129 ppm, respectively, confirm the presence of one or two double bonds. Furthermore, an allylic proton inbetween two double bonds was identified by its characteristic chemical shift of 2.57 ppm and further allylic protons were identified at 1.80 - 1.85 ppm. This corroborates the presence of mono- and double-unsaturated fatty acid chains and explains the 2 Da mass difference of each trehalose lipid. The intensity of this proton is less than expected (0.8 instead of 2) revealing that it is present in less than 50% of the compounds.

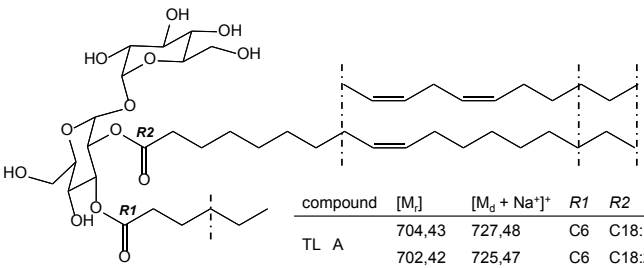
The exact position of the double bond within the fatty acid chain can only be estimated.

Neither TOCSY, nor NOESY nor HMBC spectra allowed a direct connection to either C1 – C3 of the fatty acid chain, the glycosidic moiety or to the methyl end group of the fatty acid. A connection was only observed to the -CH_2-groups resonating around 1.1 ppm (^1H) and between 22.0 and 31.6 ppm (^{13}C), therefore the double bond(s) must be located in the central part of the long acyl chain, but not the C6 unit.

Three different signals for -CH_3 ends of the acyl chains were observed (Figure 2.8d) with an intensity ratio of 0.7 / 6.6. Two signals would have been expected, one for each acyl chain. Thus, the fraction analyzed contains more than one substance, differing in - at least - one fatty acid chain corroborating the mass spectrometric analysis.

The slightly shifted methyl resonance at 0.94 ppm belongs to a short spin system that could be unambiguously identified in TOCSY and HMBC spectra. This methyl group is coupled only to -$^{\alpha}CH_2$- and -$^{\beta}CH_2$- groups, but neither to further -CH_2- groups nor hydrogen atoms close to oleofinic ones (Figure A-2b-3) and appears to be butyric acid. However this fatty acid is not part of the major substance in the fraction analyzed, because the intensity of its methyl group is lower than that of the other methyl groups (0.7 in contrast to 6.6 for the peak at 0.7 ppm comprising the other methyl groups). The remaining signals could not be further assigned to defined fatty acids.

The purified trehalose lipids from fraction 38 containing TL A and TL B (Figure 2.5, lane 4) were able to lower the surface tension of water to air to 32.51 (± 0.19) mN m^{-1} with a critical micelle concentration (CMC) of around 50 mg l^{-1} (Figure 2.10). A concentration dependent increase of the emulsification capacity of TL A and TL B with kerosene was observed reaching a maximum of 46.23 (± 4.93) % at a concentration of 250 mg l^{-1}. The capacity decreased with concentrations beyond the CMC (Figure 2.10).



compound	$[M_r]$	$[M_d + Na^+]^+$	*R1*	*R2*
TL A	704,43	727,48	C6	C18:1
	702,42	725,47	C6	C18:2
TL B_a	676,40	699,45	C6	C16:1
	674,39	697,44	C6	C16:2
TL B_b	676,40	699,45	C4	C18:1
	674,39	697,44	C4	C18:2

Figure 2.9: Structures of the different trehalose lipids produced by *Tsukamurella spumae* DMS44113, their relative masses (M_r) and the masses observed during MALDI-ToF mass spectrometry in positive mode $[M_d+Na^+]^+$. Different compositions of the acyl chains *R1* and *R2* linked to either $C^{2'}$ or $C^{3'}$ of the trehalose unit are indicated by dashed/dotted lines.

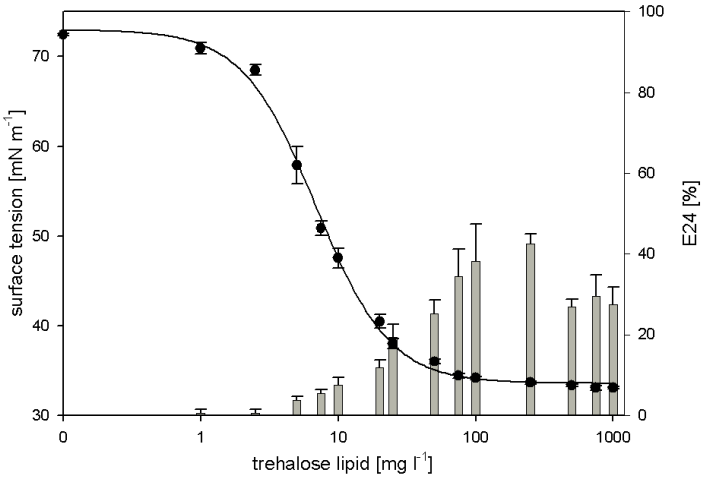


Figure 2.10: Concentration dependent surface tension values (filled circle) against water and emulsification indices (E_{24}, grey columns) of purified trehalose lipid from fraction 38 (TL A and TL B)

Discussion

Tsukamurella spumae DSM44113, *Tsukamurella spumae* DSM44114 and *Tsukamurella pseudospumae* DSM44117 are non pathogenic actinomycetes that produce a glycolipidic biosurfactant in a crude concentration of approximately 1.28 g l^{-1} within the cultivation parameters tested. Surfactants were also produced when using pure glyceryltrioleate, the main component of sunflower oil.

Structure elucidation of the two main compounds TL A and TL B produced showed that the glycolipids formed are trehalose lipids with two acyl chains linked to the 3' and 2' carbon atom of one ring system. The length of the bond acyl chains was determined to be C4 or C6 for the smaller chain and C16:1 / C16:2 or C18:1 / C18:2 for the longer chain by MS-MS and NMR experiments (Figure 2.9). The intensity ratio of the methyl group that belongs to the C4 acyl chain (Figure 2.8d) to those of the longer chains (C6 to C18) suggests that the C4 chain present in fraction 34 is due to the small amount of TL B present in the sample. This is supported by mass spectra analysis revealing a 30 % intensity of TL B in fraction 34 (Figure 2.6).

Mass fragmentation of TL A and TL B suggests that a saturated and mono-unsaturated fatty acid chain is present as smaller acyl chains in either of the two compounds. After fragmentation and loss of one glucose unit the remaining glucose unit with

both acyl chains showed two masses with the characteristic mass difference of 2 Da. A further fragment of a glucose unit only carrying the longer acyl chain and lacking the 2 Da mass difference suggested the presence of two forms (saturated and mono-unsaturated) to be present at the small acyl chain. However fragmentation of the longer chain from the glucose unit and the presence of a single sugar unit bound to the smaller acyl chain could not be ionized with the experimental procedure used during MALDI-ToF-ToF / MS / MS experiments (Figure 2.7). In NMR spectroscopy the presence of small acyl chains (C4 and C6) carrying a mono-unsaturated fatty acid was not observed suggesting that the 2 Da mass differences in both forms of the trehalose lipid (TL A and TL B) were due to alteration in the long acyl chain between mono- and double-unsaturated fatty acids chains. In contrast to the mono-unsaturated acyl chain, the double-unsaturated form was either fragmented more easily or was more difficult to be ionized.

Several trehalose lipids of microbial origin are reported in literature but they either differ in structure or nature of the trehalose lipid mixture produced. Most trehalose lipids are reported to originate from *Rhodococcus sp*. namely the non-ionic mono-, di- and trimycolates as well as anionic succinoyl trehalose lipids and trehalose tetraesters (Niescher et al. 2006, Tokumoto et al. 2008). Further trehalose lipids are known to be produced by mycobacteria, *Nocardia* and corynebacteria (Asselineau and Asselineau 1978). All of them differ from the Tsukamurella trehalose lipids described in this study by the amount of acyl chains or masses. Solely the production of trehalose lipids observed by the opportunistic pathogenic relative *Tsukamurella tyrosinosolvens* (Vollbrecht et al. 1998) contains TL A and TL B as part of a mixture of oligosaccharide surfactants. Choi et al. (1999) described in a different approach the production of a biosurfactant by a further *Tsukamurella sp.* strain proposing trehalose as the hydrophilic part, unfortunately the structure of this surfactant was not completely elucidated. Unlike in a fermentation of *Tsukamurella tyrosinosolvens* (Vollbrecht et al. 1998) a change in the composition of its glycolipids from trehalose lipids to oligosaccharide lipids under nitrogen limiting conditions could not be observed via thin layer chromatography in the fermentation of *Tsukamurella spumae* DSM44113 (Figure A-2b-1).

The trehalose lipids produced by *Tsukamurella spumae* and *Tsukamurella pseudospumae* differ from most rhodococci produced trehalose lipids as they only carry two relatively short acyl chains (C4 / C6 and C16 / C18) compared to up to C70 acyl chains present

in mycolic trehalose lipids described (Asselineau and Asselineau 1978, Vollbrecht et al. 1998). These relatively short acyl chains give them a more hydrophilic character that could be interesting for various water based surfactant applications. With a CMC of around 50 mg l^{-1} and a minimal surface tension to water of 32.51 (± 0.19) the Tsukamurella produced trehalose lipids TL A and TL B compete with CMCs and surface tensions of other trehalose lipids ranging from 24.4 to 39 mN m^{-1} with a CMC of 4 to 173 mg l^{-1} (Kuyukina and Ivshina 2010). Their capacity to emulsify kerosene is concentration dependent and shows increased intensity at values close to the CMC determined (between 25 mg l^{-1} and 250 mg l^{-1}) with a maximum of 46.23 (± 4.93) % at a concentration of 250 mg l^{-1}. These values are in the range of emulsification indices determined for rhodococci trehalose lipids ranging from 20 % to 69 % towards various hydrocarbons (Kuyukina and Ivshina 2010). Further *Tsukamurella spumae* and *Tsukamurella pseudospumae* are, in contrast to their pathogenic counterparts classified as risk group 1 organisms and therefore hold potential advantages for their use in industrial scale production and medical applications.

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Extracellular aromatic biosurfactant produced by *Tsukamurella pseudospumae* and *T. spumae* during growth on n-hexadecane

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Contribution to this publication:

JK has concepted and written this study, performed experiments, collected and interpretated the relevant data used. AK has contributed to production, purification and discussion within his Bachelor Thesis (Kraft 2012). SH contributed within aquiring and interpreation of FTIR data, CMG and BL contributed within aquiring and interpreation of NMR data. WS contributed within aquiring of mass spectrometry data. All authors have fruitfully discussed content and structure of this study.

2c Extracellular aromatic biosurfactants produced by *Tsukamurella pseudospumae* and *T. spumae* during growth on n-hexadecane

Introduction

Surfactants encounter human's daily routine in various applications. Besides cleaning and household, their surface-active and solubilising properties find use in nutrient-, cosmetic- and textile industries, in pharmaceuticals, bioremediation and in the processes of oil recovery (Fracchia et al. 2014; Gudiña et al. 2013; Müller et al. 2012). Mainly, they consist of chemically synthesized molecules that, at least partly originate from crude oil residues. More sustainable and readily biodegradable are surface active compounds produced by microorganisms termed biosurfactants. They mainly consist of glycolipids or lipopeptides, low molecular compounds whereat fatty acid residues are linked to either sugar units or peptides. Biosurfactants have become a focal point of interest not only due to their production based on renewable resources, but also due to the great variety of surface active molecules produced and speciality applications arising thereof (Henkel et al. 2012; Kügler et al. 2015; Marchant and Banat 2012).
A diverse variety of biosurfactants can be found amongst the order *Actinomycetales* that are gram positive, guanine and cytosine rich bacteria that often comprise complex morphologic occurrences. They are known as producers of secondary metabolites with

miscellaneous chemical compositions, amongst bioactive molecules, antibiotics and surfactants (Bérdy 2012). Besides well studied trehalose lipids from *Rhodoccocci* spp. and *Mycobacteria* spp., various other biosurfactants are reported to be produced from so called "rare" actinobacteria (Kügler et al. 2015).

Biosurfactants produced by members of the family *Tsukamurellaceae* were firstly detected within various oligosaccharide lipids produced by *Tsukamurella tyrosinosolvens* when cultivated in sunflower seed oil as carbon source (Vollbrecht et al. 1998). Since then, the production of trehalose lipids have been described within the genera *T. pulmonis* (Pasciak et al. 2010), *T. spumae* and *T. pseudospumae* (Kügler et al. 2014). Choi et al. (1999) purified a potential trehalose lipid from a *Tsukamurella* sp. during cultivation on n-hexadecane.

This study reports on the production, purification and characterization of biosurfactants synthesized by *T. pseudospumae* and *T. spumae* cultivated on n-hexadecane as carbon source. The composition of the surface active compounds isolated differs from biosurfactants so far described within the family *Tsukamurellaceae*. Aromatic moieties have been detected within the surface active molecules that are so far only described to occur within the family *Mycobacteriaceae* (Brennan 1989; Kügler et al. 2015).

Materials and Methods

Microorganisms:

Non-pathogenic *Tsukamurella* spp. (*Tsukamurella spumae* DSM44113, DSM44114, DSM44115, DSM44116 and *Tsukamurella pseudospumae* DSM44117 and DSM44118), originally isolated from activated sludge foam (Nam et al. 2003; Nam et al. 2004) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). So was the facultative pathogenic reference strain *Tsukamurella tyrosinosolvens* DSM44370. The actinobacterial strains were stored in cryo-stocks at -80 °C in 15 % glycerol and glucose yeast malt (GYM) media containing per litre 4 g glucose, 4 g yeast extract and 10 g malt extract.

Cultivation

Cultures in GYM media were inoculated with single colonies from GYM agar plates

(containing additional 2g l^{-1} $CaCO_3$) and grown (100 ml baffled conical flasks, 10 ml broth volume) at 30 °C and 130 rpm for three days using an incubation shaker (Multitron II, Infors, Bottmingen, Switzerland). 1 ml of the GYM culture was transferred into a shake flask (1 l baffled conical flasks, 100 ml broth volume) containing a mineral media described by Choi et al. (1999) (per litre: 2.0 g $NaNO_3$, 44.35 g hexadecane, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 0.2 g $CaCl_2 \cdot 2\ H_2O$, 0.2 g yeast extract, 0.2 g tryptone, 100 ml 1M K_2HPO_4/KH_2PO_4 pH 7.0) and grown for three days at 30 °C and 110 rpm.
Alternatively, hexadecane was replaced by 20 ml l^{-1} sunflower seed oil in shake flask experiments.
Batch fermentations were conducted as triplicates using *Tsukamurella pseudospumae* DSM44118 in a 2.5 l bench-scale bioreactor (Minifors, Infors, Bottmingen, Switzerland) and a 5 l bioreactor (Biostat B5, Satorius, Göttingen, Germany). Both bioreactors were filled with a mineral media equal to shake flask experiments, except of 12.5 mM K_2HPO_4/KH_2PO_4 at pH 7.0 being used. Bioreactor cultivations were run for approximately 100h at 30 °C. pO_2 and pH were recorded by internal electrodes, the pH was adjusted automatically using 4M H_3PO_4 and 4M NaOH.
The 2.5 l bioreactor was inoculated with 10 % (v/v) of a mineral media containing shake flask culture to a total broth volume of 1 l. Airflow of 0.2 vvm and a stirring speed of 500 rpm were kept constant throughout the process. The process was controlled and recorded using Iris software (Infors, Bottmingen, Switzerland).
The 5 l bioreactor was inoculated with 5 % (v/v) of a mineral media containing shake flask culture to a total broth volume of 3.315 l. Airflow of 0.3 vvm and a stirring speed of 400 rpm were kept constant throughout the process. The process was controlled and recorded using MFCSwin software (Satorius, Göttingen, Germany).
For the analysis of growth characteristics 5 ml culture samples were taken as duplicates at different time points from both reactor types. Samples were centrifuged for 20 min at 4643 x g and 4 °C. The liquid phase was transferred into a new tube and used for the determination of nitrate ions.
The hydrophobic cell mass and solid hexadecane pellet was extracted twice with 2 ml hexane by manual shaking. Samples were centrifuged for 10 min at 4643 x g and 4 °C and the organic phase containing hexane and hexadecane discarded. Dry cell mass was determined gravimetrically by washing the cell pellet with 2 ml 0.9 % (w/v) NaCl, centrifuging, decanting and drying to constant weight.

The nitrate ion concentration in the supernatant was determined by a nitrate assay using photometric quantification (Spectroquant 109713, Merck, Darmstadt, Germany) with a fifth of the volumes listed in the supplier's manual.

Cell dry mass and nitrate ion data points were fitted (SigmaPlot, version 12.5, Systat Software Inc., Washington, USA) using a logistic model with four parameters (Zwietering et al. 1990). Results are plotted exemplarily for a single fermentation per bioreactor type.

Purification of surface active compounds

Supernatants of samples taken during the fermentation processes were extracted twice by mixing vigorously with 1.25 (v/v) ethyl acetate and centrifugation for 20 min at 4643 x g and 4 °C. Combined organic phases were evaporated at 40 °C, 50 mbar and 2000 rpm in a vacuum concentrator (ScanSpeed MiniVac Evaporator, Saur, Reutlingen, Germany).

After the fermentation process, the culture broth was transferred into glass bottles and cooled down at 4 °C until freezing of hexadecane and associated cell mass. The liquid phase was filtered to remove further residual hexadecane and cell mass, then stepwise repeatedly extracted, using 1.25 (v/v) ethyl acetate and shaking. The combined organic phases were concentrated to dryness using a rotary evaporator (Heidolph Laborota 4000, Schwabach, Germany) at 40 °C and 240 mbar followed by vacuum concentration at 40 °C, 2000 rpm m and 50 mbar. Total concentration of the crude extract per bioreactor was determined gravimetrically.

2.5 l bioreactor: Combined crude extracts were dissolved in ethyl acetate and further purified by medium-pressure-liquid-chromatography (MPLC, SepacoreX50, Büchi, Flawil, Switzerland) using 40-63 µm particle size prepacked silica stationary phase with 60 Å pore size (150 mm column length, 40 mm column diameter and 190 ml bed volume; Büchi, Flawil, Switzerland) with an isocratic chloroform / methanol (87:13 v/v) solvent system for 60 min at a flow rate of 40 ml min^{-1}. The eluate was collected in 40 ml fractions. Purified compounds were combined from fractions 13-17 and the solvent evaporated.

5 l bioreactor: Crude extracts from each fermentation process were purified separately. Extract were dissolved in 15 ml chloroform / methanol (87:13 v/v) and further purified by MPLC using a smaller column (150 mm column length, 12 mm column

diameter and 17 ml bed volume; Büchi, Flawil, Switzerland) of the same 60 Å pore silica stationary phase with a chloroform / methanol solvent system at a flow rate of 8 ml min^{-1} (gradient: 5 min 100 %-100 % $CHCl_3$; 40 min 100 %-70 % $CHCl_3$; 5 min 70 %-0 % $CHCl_3$, .5 min 0 %-0 % $CHCl_3$). The eluate was collected in 8 ml fractions. Purified compounds from fractions 22-30 were combined and the solvent evaporated. Purified extracts from all 3.315 l fermentations were dissolved in 20 ml ultrapure water / methanol (9:1 v/v) (Milli-Q, Merck-Millipore, Darmstadt, Germany) and further purified using a prepacked 40-63 µm particle size reverse phase C18ec column (Büchi, Flawil, Switzerland) with a H_2O / methanol solvent system at a flow rate of 10 ml min^{-1} (gradient: 15 min 100 %-100 % H_2O; 40 min 100 %-0 % H_2O; 30 min 0 %-0 % H_2O). The eluate was collected in 10 ml fractions. Fractions 2-6 were combined and used for structural characterisation.

Structural analysis

Functional staining: Produced metabolites were detected qualitatively and analysed towards functional groups by various TLC methods using the stationary phase 60 Å silica (Alugram Xtra SIL G, Macharey-Nagel, Düren, Germany) and the solvent system chloroform / methanol / acetic acid (70:10:2 v/v/v). Hydroxyl groups were indicated using anis aldehyde (acetic acid / p-anisaldehyde / sulphuric acid (100:1:2 v/v/v)) directly resulting in a transient bright orange spot after staining that was intensified after short development under 150 °C air stream and using cer-ammonium molybdate (1.05 g $(NH_4)_6MoO_{24} \cdot 4H_2O$, 0.5 g $Ce(SO_4)_2$, 250 ml H_2O, 15.5 ml H_2SO_4) resulting in a blue spot after development under 150 °C air stream for 5 min. Primary amines were stained with a ninhydrin / methanol solution (2:1 w/v) and developed under 110 °C air stream. Secondary amines were stained with a nitroprussid-ferrycyanide solution (solution A: 10% NaOH, sodium-nitroprussid and potassium-ferrycyanide mixed 1:4 v/v with H_2O. Solution A and acetone were mixed 1:1 v/v shortly before staining). Tertiary amines were stained using Dragendorff solution (solution B containing per litre: 42.5 g $BiNO_4$, 500 g tartaric acid ,mixed for 1h and solution C containing per liter: KI 400 g were combined and filtered. The retentate was washed with 1 l of H_2O and added to the filtrate. A 1:4 (w/v) aqueous solution of the mixture was used for staining). The TLC plate was developed at 100 °C air stream. Phosphorous compounds were stained using molybdenum blue (solution

D: 40 g l^{-1} MoO_3 dissolved in boiling 12.5 M H_2SO_4, boiled for 1h and cooled down. Solution E: 500 ml of solution D was mixed with 1.77 g Mo and heated for 15 min. A 2:4:9 (v/v/v) mixture of solution D, E and H_2O was used for staining.

Spectral analyses in ultraviolet (UV) and visual light: For the acquisition of UV spectra the purified compounds were separated by high-performance thin-layer chromatography (HPTLC). Purified extract from the 1 l fermentations was applied onto a 60 Å silica gel plate (1.05641, 10 x 20 cm, Merck, Darmstadt, Germany) as 8 mm bands by an automatic TLC sampler (ATS 4, CAMAG, Muttenz, Switzerland) and developed to a migration distance of 60 mm in an automatic developing chamber (ADC 2, CAMAG) with chloroform / methanol / acetic acid (65:15:2 v/v/v). Densitometry was performed in the absorbance mode at 200 nm and 254 nm (Hg lamp), and in the fluorescence mode (366 nm/K400), using a TLC scanner (TLC Scanner 4, CAMAG). Absorbance spectra of detected zones were recorded from 200 to 350 nm.

Infrared spectroscopy: Attenuated total reflection infrared (ATR-IR) spectra were measured using a Bruker Tensor 27 FTIR-spectrometer (Bruker Optics, Ettlingen, Germany) equipped with a deuterated triglycine sulfate (DTGS) detector and a Bruker Platinum ATR-accessory (diamond crystal, with one internal reflection). For measurements as well as spectra evaluation Bruker OPUS ® software Ver. 7.2 was used. Spectra were recorded from 4000 cm^{-1} to 370 cm^{-1} against an air background. Thirty two scans were co-added.

High-performance thin-layer chromatography - mass spectrometry (HPTLC_MS): For HPTLC-MS, samples were doubly applied (ATS 4) onto a 20 cm x 10 cm silica gel plate. After development with chloroform / methanol (70:10 v/v), half of the plate was stained with p-anisaldehyde. The exact positions of stained compounds were transferred with a pencil to the second half of the plate. Marked zones of interest were aimed by a red laser beam facilitated by an *x-y* coordinate crosshair on the TLC-MS interface equipped with an oval-shaped elution head (CAMAG). The zones were eluted with acetonitrile / 10 mM ammonium formate buffer, pH 4 (80/20). A flow of 0.2 ml min^{-1} was provided by an HPLC pump HP 1100 (Agilent Technologies, Waldbronn, Germany). The TLC-MS interface was coupled to a single-quadrupole mass spectrometer (G1956B

MSD, Agilent) with an electrospray ionization interface (ESI). The mass spectrometer was operated with the following settings: drying temperature 250 °C; drying gas rate 10 l min^{-1}; capillary voltage 4.0 kV; nebulising gas 30 bar; fragment or voltage 100 V; gain 1; threshold 1; step-size 0.05; time filter off; scan data storage full. Measurements were carried out in ESI positive mode. For data acquisition and processing, LC / MSD Chemstation B.02.01-SR1(260) software (Agilent) was used.

Nuclear magnetic resonance spectroscopy (NMR): For NMR spectroscopy, approximately 15 mg of the purified compounds were dissolved in 0.6 ml CD_3OD (Sigma-Aldrich; Germany). One dimensional ^1H NMR spectroscopy and two dimensional ^1H-^1H correlation spectroscopy (COSY) were recorded on a Bruker AVANCE II+ 600 MHz spectrometer (Bruker AG, Rheinstetten, Germany) equipped with a BBI probe head and interpretated using a spectral analysis software (Topspin 3.2, Bruker AG and Spinworks 3.1.8 (Marat)).

Characterization of the biosurfactant: Critical micelle concentration of the purified biosurfactant dissolved in demineralised water was determined by monitoring the surface tension against air at room temperature using the Du Noüy (1919) ring method on a Tensiometer (Lauda TD1, Lauda-Königshofen, Germany) according to the supplier's manual.

Results and Discussion

Extracellular compounds with surface active properties have been detected during growth of *Tsukamurella* spp. (*Tsukamurella spumae* DSM44113, DSM44114, DSM44115, DSM44116 and *Tsukamurella pseudospumae* DSM44117 and DSM44118) in a mineral medium containing n-hexadecane as carbon source. A striking orange spot was detected in supernatants of the *Tsukamurella* spp. by TLC and p-anisaldehyde staining. The compound was also detected when replacing the carbon source n-hexadecane to sunflower seed oil in the cultivation medium. It was investigated, that the production of the compounds is not due to the carbon source, as the same medium of Choi et al. (1999) with sunflower seed oil instead of n-hexadecane let to identical compounds.

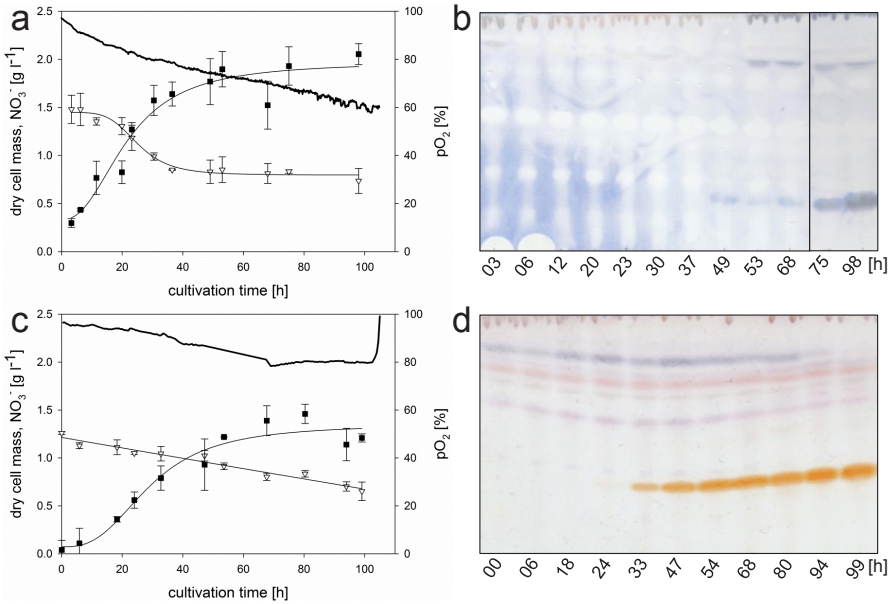

Figure 2.11: Time course of dry cell mass (black square), pO_2 (black line) and nitrate (inverted triangle) for 1 l (a) and 3.3 l (c) batch fermentations of *Tsukamurella pseudospumae* DSM44118. Thin-layer chromatographic qualitative and semi-quantitative detection of the surface active compound produced during fermentation in 1 l (b) and 3.3 l (d) scale

The production must thus be initiated by other differences in the cultivation media e.g. tryptone or $NaNO_3$ as nitrogen source that are not present in the media used in studies of Vollbrecht et al. (1998) and Kügler et al. (2014). Further, the coproduction of trehalose lipids as known to occur in sunflower seed oil by both strains did not occur in the media applied.

Production of the biosurfactant

Tsukamurella pseudospumae DSM44118 was cultivated in two different bioreactor types, a smaller one filled with 1 l and a larger one filled with 3.3 l medium. The cultivation broth changed from slight yellow in the beginning of the fermentation to dark orange at the end of the cultivation. Bacterial cells tended to accumulate in the hydrophobic hexadecane phase and attached to the cultivation device's glass surface in late cultivation phases. Only a slight layer of foam was observed at the end of all fermentation processes. Growth of cells in the bioreactor were neither nitrogen

(Figure 2.11) nor carbon limited (latter observed visually by solid hexadecane/cell pellets after centrifugation at 4 °C). During cultivation in the 1 l scale the cells showed a more intense growth resulting in a total dry cell mass of 1.93 ± 0.14 g l^{-1} compared to 1.50 ± 0.75 g l^{-1} during growth in the 3.3 l scale. An enhanced proliferation of the cells could as well be observed by the absolute pO_2 values that decreased continuously until it reached a value of approximately 60 % in the fermentation broth of the 1 l scale whereas it only decreased to approximately 80 % in the 3.3 l scale (Figure 2.11c). Nitrate ions were consumed more quickly and to a slightly higher extend in the smaller scale fermentation, final residual nitrate ions measured in the fermentation broth after nearly 100 h of cultivation are with 0.645 ± 0.091 for the 1 l scale and 0.744 ± 0.039 for the 3.3 l scale similar (Figure 2.11). Depending on the bioreactor type used differences within oxygen availability were observed. In the smaller scale bioreactor of 1 l working volume an approximately 25 % higher maximum of dry cell mass was observed and absolute pO_2 values were lower, the crude extract yield higher at the end of the fermentation process (Figure 2.11).

The production rate of the surface active compound was verified throughout the process via staining of the compounds after planar chromatographic separation of the extracts (Figure 2.11b and 2.11d). The beginning of product formation was first observed in late growth phase after 49.0 hours in the 1 l and 32.8 hours in the 3.3 l scale. The quantity of the product increased throughout the fermentation processes until the end of the fermentation observed by increased density of the spots stained in TLC (Figure 2.11 b and 2.11d). Total concentration of the crude extracts was in average 137.2 mg l^{-1} in the 1 l and 99.1 mg l^{-1} in the 3.3 l scale. A growth associated production of the biosurfactant intensified in a late growth phase (Figure 2.11) harmonize with the production behaviour of trehalose- and oligosaccharide lipids of *Tsukamurella tyrosinolsolvens* described by Vollbrecht et al. (1998).

Detection of the biosurfactant

A bright orange spot appeared in TLC of the extract of the fermentation supernatant after staining with p-anisaldehyde (even without heating), a blue spot after staining with cer-ammonium molybdate (Figure 2.12). The surface active compound isolated significantly differed to a product produced in the same media by the reference strain *Tsukamurella tyrosinosolvens* that appeared as a green spot when stained with

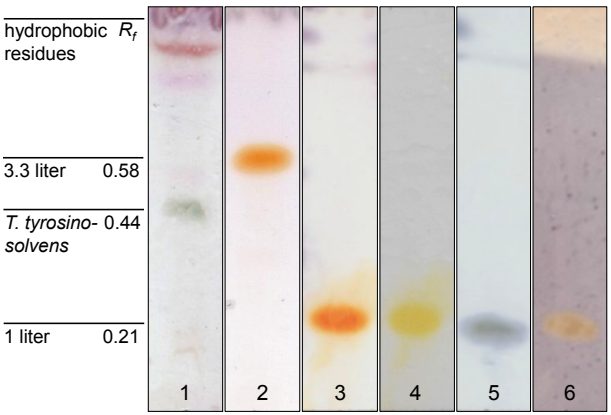

Figure 2.12: Thin-layer chromatography of the surface active compounds produced by *Tsukamurella pseudospumae* DSM44118 in 1 l (lane 2) and 3.3 l (lane 3) batch fermentations. Products produced by *Tsukamurella tyrosinosolvens* DSM44370 in an equal media (lane1). Functional staining of the surface active compound with: p-anisaldehyde (lane 3 and lane 4 before and after plate heating, respectively), cer-ammonium molybdate (lane 5) and nitroprussidferrycyanide (lane 6).

p-anisaldehyde (Figure 2.12 lane 1). It also differed to other surface active compounds so far described within *Tsukamurella* spp. such as trehalose (Kügler et al. 2014; Pasciak et al. 2010) and oligosaccharide lipids (Vollbrecht et al. 1998) that have been produced in a mineral medium with sunflower seed oil as carbon source. Differences have been determined in structural characteristics and surface active properties, retardation factor R_f) and staining behaviour of the compound by TLC.

The stained compound was significantly more polar in the smaller scale fermentation processes with R_f 0.21 compared to 0.58 in the larger scale process (Figure 2.12 lane 2 and 3) when the TLC plate was developed with chloroform / methanol / acetic acid (70 / 10 / 2 v/v/v). Causative to R_f differences of the compounds produced could be an alteration in oxygen supply, typically due to the implementation of different hydrophobic parts to the surfactant molecule. However typical long chain fatty acid residues as hydrophobic moieties were not detected. The occurrence of cell associated surface active molecules, as often reported to occur within actinobacteria (Philp et al. 2002) was investigated by two phase extraction of the cell broth with various solvents but could not be observed.

Characterisation of the biosurfactant

For structural analysis, the crude extract was separated by silica gel column and reversed phase (C18) column middle pressure liquid chromatography from by-products and other medium components. The purified product, dissolved in demineralised water reduced the surface tension to 28.7±0.2 mN m^{-1} at a critical micelle concentration

(CMC) of 2.5 g l^{-1}.

TLC analyses indicated that the compounds produced feature hydroxyl groups, detected by p-anisaldehyde and cer-ammonium molybdate staining (Figure 2.12 lane 2-5). No indication for primary and tertiary amines (ninhydrin and Dragendorff staining), as well as phosphorous compounds (molybdenum blue staining) could be observed. Solely functional staining of either aliphatic bound nitrogen or other alkaloids was observed when stained with a mixture of sodium-nitroprusside / potassium-ferrycyanide (Figure 2.12 lane 6), suggesting the presence of aliphatic bound nitrogen.

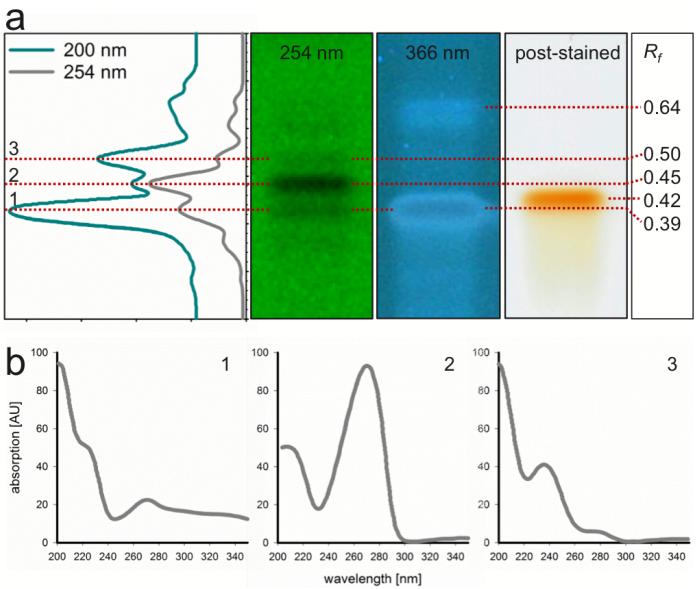

Figure 2.13: Spectral analyses in ultraviolet (UV) and visual light. (a) Densitograms and HPTLC plate images under UV (254 nm, 366 nm) and visual light illumination. (b) UV spectra recorded from the 3 main HPTLC zones at R_f 0.39, 0.45 and 0.50

UV absorption (200 and 254 nm) and fluorescent emission (λ_{ex} 366 nm) were recorded, revealing no absorbance for the stainable product; but three main zones in close vicinity were detected, one of which was fluorescent (Figure 2.13a). UV spectra of the three zones were recorded, with the fluorescent compound (1) at R_f 0.39 providing a broad absorbance without distinct maxima. Different absorption spectra were obtained for the compounds at R_f 0.45 and 0.50 (2 and 3) that each exhibited two maxima at 210 and 270 nm for (2) and 200 and 240 nm for (3) (Figure 2.13b). The UV spectra clearly demonstrated that the stainable product is, despite chromatographic

separation procedures not present in a pure form but in direct vicinity of UV absorbing and fluorescent compounds that show a similar amphiphilic balance in HPTLC (Figure 2.13). This coexistence hinders complete structural elucidations; however characteristic features of the compound mixtures could be demonstrated.

A direct colour development after dipping into p-anisaldehyde staining solution (Figure 2.12 lane 4) and both, infrared (Figure 2.14) and NMR (Figure 2.16) spectroscopic measurements suggest the presence of aromatic moieties in at least one of the compounds produced.

Infrared spectroscopic measurements bares typical hydroxyl patterns with a distinct and shouldered peak at 1142 cm^{-1} a as well as OH stretching vibration in the range of

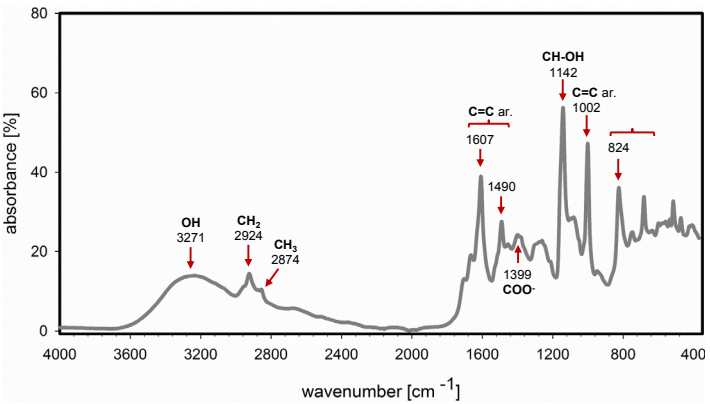

Figure 2.14: Infrared spectroscopy. Attenuated total reflection infrared (ATR-IR) spectra of the compounds produced and assigned bands at determined wavenumbers.
ar., aromatic compound

Band assignment	Wavenumber experimental [cm^{-1}]	Wavenumber literature [cm^{-1}]	References
tri substiuted aromatic CH	824, 681	840, 690 800-850, 675-730	Günzler and Gremlich 2002 Pretsch 2009
substiuted aromatic CH	1002	1000-1100	Günzler and Gremlich 2002
CH-OH	1142	1100-1210	Pretsch 2009
C=O of COO^-	1399	~1400	Gremlich and Yan 2000
aromatic C-C	1490, 1607	1450-1525, 1575-1625	Pretsch 2009
CH_3	2874	~2870 2856	Gremlich and Yan 2000 Heydt et al. 2008
CH_2	2924	~2930 2923	Gremlich and Yan 2000 Heydt et al. 2008
OH stretching	3271	~3500 3369	Gremlich and Yan, 2000 Heydt et al. 2008

Table 2.13: Determined wavenumbers from attenuated total reflection infrared (ATR-IR) spectra, band assignments and correlating values from literature

3000 to 3500 cm^{-1} (Figure 2.14 and Table 2.13). Furthermore, CH_3 and CH_2 groups were observed at 2874 cm^{-1} and 2924 cm^{-1}, respectively. Possible is the existence of a carboxyl group present at 1399 cm^{-1}. The coexistence of aromatic carbon atoms that typically show two distinct IR bands in a region of 1450-1525 cm^{-1} and 1575-1625 cm^{-1} (Pretsch 2009) appeared at 1607 cm^{-1} and 1490 cm^{-1}. Characteristic stretching of the carbon-carbon bonds in the ring structure at frequencies of 1607 cm^{-1}, 1002 cm^{-1}, 824 cm^{-1} and 681 cm^{-1} corroborate to tri-substituted aromatic moieties when compared to literature values (Günzler and Gremlich 2002). These distinct patterns were not observed in infrared spectra of biosurfactants produced by *Tsukamurella* sp. 26A in the same media (Choi et al. 1999).

Spectra of 1D ^1H (Figure 2.16a) and 2D ^1H COSY (Figure 2.16b) NMR measurements revealed CH_2 and CH_3 groups. Chemical shifts for OH groups of glycolipids that typically occur in the range of 3.3 to 4 ppm could not be assigned or were only present in traces. Two different moieties show chemical shifts that are typical for aromatic compounds (Figure 2.16c). Characteristic doublets at 7.28, 7.29, 7.46 and 7.47 and a quartet at 7.40 ppm have direct correlation to each other opposed to a distinct doublet at 7.23 (Figure 2.16c red arrow) as can be seen by chemical shifts 2D ^1H COSY spectra. Glycolipids that bear aromatic compounds are known to be produced within actinobacteria so are phenol-phtiocerol glycosides also termed phenolic glycolipids produced by *Mycobacteria* spp. (Brennan 1989).

The compounds produced were difficult to be ionized in mass spectrometric

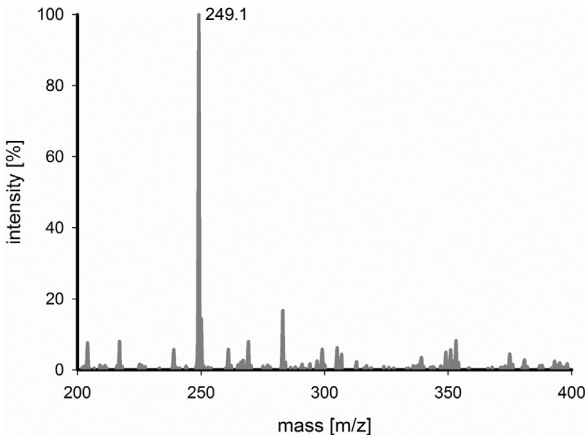

Figure 2.15: Mass spectrum of the extracellular biosurfactant produced by *Tsukamurella pseudospumae* DSM44118 and stainable by p-anisaldehyde

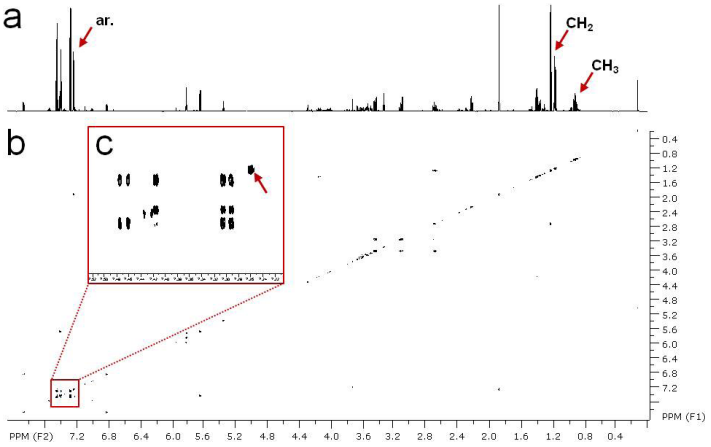

Figure 2.16: Nuclear magnetic resonance spectroscopy (NMR). (a) 1D ^{1}H and (b) 2D ^{1}H COSY NMR spectrum of the surface active compounds produced by *Tsukamurellapseudospumae* DSM44118. (c) Closeup of the COSY spectra for units with typical aromatic features. Red arrow indicates non correlating moiety. *ar., aromatic compound*

measurements. During HPTLC_MS measurements of the compound at the exact R_f of the orange stained zone on the HPTLC plate, one low molecular weight mass signal of m/z 249.1 was detected (Figure 2.15).

Conclusion

This study reveals the production of surface active compounds detected within growth of *Tsukamurella pseudospumae* and *T. spumae* but not *T. tyrosinosolvens*. The compounds produced are most likely to contain aromatic moieties and hydrophobic units that differ from typical fatty acid residues reported to occur within *Tsukamurella* spp. (Choi et al. 1999; Kügler et al. 2014; Pasciak et al. 2010; Vollbrecht et al. 1998). Production of the compounds was achieved during cultivation with the hydrophobic carbon sources n-hexadecane or sunflower seed oil. The amphiphilic performance of the compounds varies within the production device used, most likely due to oxygen occurrence.

Surfactant producing procaryotes from soil

3a Who eats all that seal fat? - Biosurfactant producing soil communities found underneath seal carcasses in Antarctica and characterization of surface active compounds produced by *Pseudomonas* sp. SCS1-O4

3b Biosurfactant producing prokaryotes inhabiting raised bog peat soil

3c Glycolipids produced by *Rouxiella* sp. DSM 100043 and isolation of the biosurfactants via foam-fractionation

This study has been performed within Extremophiles & Enzymes project (ExtEnz) funded by the International Bureau, Federal Ministry of Education and Research, Germany and was partly conducted at the University of the Western Cape, Bellville, South Africa.

Who eats all that seal fat? - Biosurfactant producing soil communities found underneath seal carcasses in Antarctica and characterization of surface active compounds produced by Pseudomonas sp. SCS1-O4

Kügler JH, Sanden ASJM, Pöhnlein M, Cowan DA, Syldatk C, Hausmann R

Contribution to this study:

JK has concepted and written this study, isolated microorganisms and performed screening experiments, collected and interpretated the relevant data used. AS contributed to screening, analysis and discussion within his Bachelor Thesis (Sanden 2013). MP contributed within strain maintenance, DC within sampling. All authors have fruitfully discussed content and structure of this study.

3a Who eats all that seal fat? - Biosurfactant producing soil communities found underneath seal carcasses in Antarctica and characterization of surface active compounds produced by *Pseudomonas* sp. SCS1-O4

Introduction

White is the predominant perception of Antarctica as it is, to its greatest extent, covered in ice. On and off the coastal margins are ice free regions known as Dry Valleys. They comprise around 0.4 % of the continental land mass and its extreme environment is not only due to harsh-cold fluctuating temperatures but also to high salinity and ultraviolet (UV) exposure. Although being surrounded by solid ice the Dry Valleys are, with less than 2 % mass water content, as dry as hot deserts (Cary et al. 2010). Their nutrient values are low compared to ornithogenic fed or freezing and thawing cycle exposed coastal or sub-Antarctic regions. Nevertheless molecular–based phylogenetic studies revealed the microbial diversity to be surprisingly high (Cowan et al. 2002; Smith et al. 2006). The amount of soil organic carbon is, with 0.02-0.4 g kg^{-1} around 100 times lower than those of high Arctic deserts in north eastern Greenland (Cary et al. 2010) and nutrients mainly arise from wind dispersal. Resident biota, mainly hypolithic or cryptoendolithic adapted to these harsh environments by populating in niches where they are protected from desiccation, e.g. underneath rocks where temperature is stabilized and UV radiation alleviated.

A sudden change in nutrient availability can be caused by external influences, e.g. marine derived organic matter. Seals sporadically get lost in ice storms, fall off cliffs and get mummified in the Dry Valleys of Antarctica. Although decomposition of the carcasses takes place over a very long timescale, a microclimate is build up when the carbon rich fatty skin of a seal's carcass slowly drips into the soil thus serving as a natural enrichment of the habitat. This sudden appearance of nutrients is the cause of an accumulation of communities of microorganisms whose metabolic versatility enables them to convert hydrophobic carbon sources into either small droplets of fat or fatty acids hence making them bioavailable for the cell.

In order to access these hydrophobic compounds, microorganisms adapted to their environment by the release of mediators. Amongst are lipases, that cleave these hydrophobic substances into smaller components and/or released biosurfactants. Biosurfactants are microbial produced amphiphilic molecules whose structural mutuality is the coexistence of hydrophilic and hydrophobic moieties within one molecule. As the uptake of nutrients mainly takes place in aqueous systems, biosurfactants serve the cell by occupying the interphase of seal fat with its proximity and, via its surface active properties, disperses or emulsifies the hydrocarbon until it can be used by the cells. Further, access to hydrocarbons is supported by cell associated biosurfactants that alter the cell wall's hydrophobicity and serves as a mediator of the cell towards its substrate. It is amongst the hydrocarbon degrading microorganisms, where most of the biosurfactant producing communities have been found (Perfumo A. 2010).

Surfactants act as foaming agents, emulsifiers and dispersants and use of it is made in cleaning associated products, cosmetics, food processing and pharmaceutical- as well as petroleum industry. Surfactants mark a growing market with an annual global production of 13 million tons in 2008 (Reznik et al. 2010). Mainly being synthesized from mineral oil, or derived from palm and coconut oil (Marchant and Banat 2012) current production strategies mainly involve either the use of valuable natural resources or those that constitute a threat to the environment. The great diversity of unique biosurfactant structures that can be build up by microorganisms has turned their production to a focal point of interest of surfactant related industry in the last decades. An ecological production based on renewable and sustainable resources as microbial feedstock holds further advantages of biosurfactants towards their chemical synthesized counterparts.

The variety of biosurfactant structures produced by microorganisms ranges from different sugar or peptide groups as hydrophilic backbone linked to variable amounts of hydrophobic side chains that differ in lengths and type and can either be non ionic or anionic in its nature. The structures of umpteen biosurfactants are described but theoretic combinations of hydrophilic and hydrophobic moieties produced in nature are countless. Within microbial strains isolated from Antarctic samples, several are described to be producing strains of biosurfactants. Among them are mannosylerythritol lipids produced by *Pseudozyma* (previously *Candida*) *antarctica* (Morita et al. 2007), *P. parantarctica* (Morita et al. 2008) and other glycolipids produced by *Rhodococcus* sp. (Gesheva et al. 2010), *Nocardioides* sp. (Vasileva-Tonkova and Gesheva 2005), *Halomonas* sp. (Pepi et al. 2005) and *Pantoea* sp. (Vasileva-Tonkova and Gesheva 2007).

Most likely, not yet discovered novel biosurfactant structures are more likely to be found within strains not yet described as surfactant producing organism. In this study the exceptional habitat of hydrophobic hydrocarbons derived from seal skin in the extreme environment of Antarctica is studied and directed towards surfactant producing communities present within.

Figure 3.1: Schematic area map of the dry valley sampling sites in proximity of Ross Sea, Antarctica. White: permanent ice; light grey: land / dry valley; dark grey: water. Soils were sampled in direct proximity of seal carcasses. One sampling site in Shangri-La (1; 78.062 S 163.780 E), three sampling sites at Seal Cliff (2; 78.063 S 163.858 E, 3; 78.067 S 163.862 E, 4; 78.067 S 163.864 E) and one sampling site at Miers Valley (5; 78.097 S 163.756 E)

Materials and Methods

Sampling:
Several grams of soil were taken in direct proximity of five different seal carcasses in Antarctic Dry Valleys in the area of McMurdo Sound and the Ross Sea, approximately 3500 km south of New Zealand. Soil was transferred aseptically into sterile polyethylene bags, stored at 4 °C and used for isolation assays within two weeks. All samples were taken in January of austral summer 2012. Three soils were sampled at Seal Cliff (SC S1, SC S2 and SC S3 for Seal Cliff Seal 1; 78.063 S 163.858 E, Seal Cliff Seal 2; 78.067 S 163.862 E and Seal Cliff Seal 3; 78.067 S 163.864 E. One sample was taken from Shangri-La (SL S1; 78.062 S 163.780 E) and one from Miers Valley (MV S1; 78.097 S 163.756 E). See Figure 3.1 for a schematic area map and Figure 3.2 as representative isotopes of the seal carcasses soil sampled.

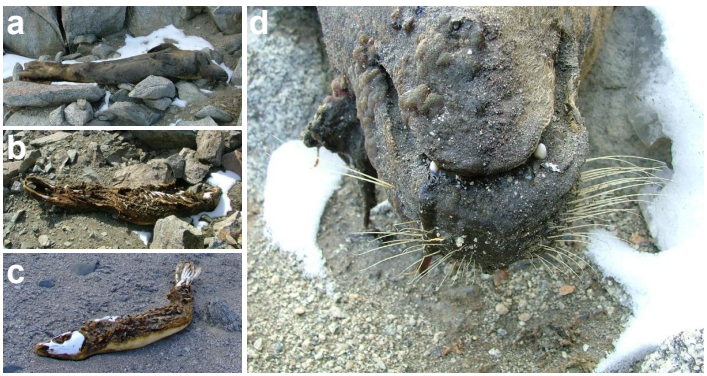

Figure 3.2: Seal carcasses directly neighboring the soil sampled. a) Seal Cliff Seal 2 (SC S2), b) Seal Cliff Seal 3 (SC S3) and c) Shangri-La Seal 1 (SL S1). d) Close up of the head of SC S2. Fat-rich components drip off the skin into the soil and serve microorganisms as a marine derived carbon source. *Photographs courtesy of Don A. Cowan*

Isolation of microorganisms
Enrichment of soil samples: 0.5 g of each soil sample was shaken vigorously in 5 ml 0.9 % NaCl solution for 1 min using a vortex. The mixture was kept at 4 °C. After settling of the soil the solution was seeded into four different enrichment cultures: Half strength nutrient broth (0.5 NA) containing per litre 2.5 g peptone and 1.5 g meat extract and three different enrichments based on a mineral media (SBH) supplemented and modified from Bushnell and Haas (1941) containing per litre: 1.0 g K_2HPO_4, 1.0 g KH_2PO_4, 0.8 g NH_4Cl, 0.8 g $NaNO_3$, 0.1 g yeast extract, 0.2 g $MgSO_4$, 0.26 g

$CaCl_2$ • 2 H_2O and 1 ml of a trace element solution containing per litre 2 g Na-citrate• 2 H_2O, 0.28 g $FeCl_3$ • 6 H_2O, 1.4 g $ZnSO_4$ • 7 H_2O, 1.2 g $CoCl_2$ • 6 H_2O, 1.2 g $CuSO_4$ • 5 H_2O, 0.8 g $MnSO_4$ • H_2O. The carbon content was altered and either 10 g l^{-1} glucose (SBH-G), 20 ml l^{-1} sunflower seed oil (SBH-O) or 10 g l^{-1} liquid paraffin was used. The pH was set to 7.0 using NaOH and H_3PO_4. 1 ml from each soil sample was inoculated into 50 ml of each enrichment culture (250 ml shake flasks). Cells were grown for three days at 15 °C and 130 rpm.

Isolation of colony forming units (cfu): Different dilutions of each enrichment culture were plated on agar plates with equal medium to the enrichment culture and 15 g l^{1} agar. Microorganisms were grown at 15 °C for four days or until colonies were clearly visible. Each morphological different single colony (*cfu*) was picked with a sterile toothpick and streaked out onto fresh agar plates of the appropriate enrichment culture. Cells were repeatedly grown and picked for at least three times or until visible purity. Isolates were named with the first two digits according to its soil source, and the second two digits according to its seal origin followed by tag and one digit assigning the enrichment type N for 0.5 NA, G for SBH-glucose, O for SBH-oil and P for SBH-paraffin and the number of the *cfu* picked. Additionally, *cfu*s were picked from the hydrophobic phases of SC S3-O and SC S1-P. These isolates were additionally marked by the letter "L" and the digits one or two (e.g. SC S3-L2O3). Purified single colonies were grown in the appropriate enrichment culture for three days, 15 °C, and 130 rpm, subsequently spiked with sterile 15 % glycerol and stored as cryo-stocks at 80 ° C.

Other microorganisms

Pseudomonas aeruginosa PAO1 (DSM 22644) and *Pseudomonas putida* mt-2 KT2440 (DSM 6125) were used as reference strains.

Genetic characterization of isolated strains:

For prokaryotes the 16S rRNA decoding DNA sequence was amplified by direct-colony polymerase chain reaction (PCR) in a 96well plate. Each well contained a master mix of 0.75 U polymerase (HotStar TaqTM, Qiagen, Hilden, Germany), 0.5 µL desoxyribonucleotide triphosphate mix (dNTPs; 10 mM of each dNTP: Qiagen, Hilden, Germany), 1 µl of oligonucleotide 27F (100 pmol μl^{1};

5'-AGAGTTTGATCCTGGCTCAG-3') and 1 µl of oligonucleotide 1385R (100 pmol µl^{1}; 5'-CGGTGTGTRCAAGGCCC-3' whereas R is A or G) (both Biomers, Ulm, Germany), 2.5 µl of a PCR reaction buffer (10 x, Qiagen, Hilden, Germany) filled up to a total volume of 25 µl per sample with nuclease free water (Carl Roth GmbH, Karlsruhe, Germany). Each well was seeded with tiny bits of cell mass from a single colony of each strain using a sterile tip. For amplifications resulting in no or uncertain signal during sequencing, the PCR reaction was repeated using extracted genomic DNA. Therefore, 10 ml overnight cultures of wild type strains grown in 0.5 NA were centrifuged for 20 min at 4643 x g and 4 °C. The supernatant was discarded and the genomic DNA (gDNA) of each cell pellet was extracted using PureLink Genomic DNA Mini Kit (Life Technologies GmbH, Darmstadt, Germany) according to the supplier's manual. gDNA was eluted in nuclease free water and 5 µl were used to seed the PCR master mix.

For eucaryotes, gDNA was extracted from the cellmass of 0.5 NA grown overnight cultures using the Isolate II gDNA extraction kit (Bioline GmbH, Luckenwalde, Germany) according to the supplier's manual. gDNA was eluted in nuclease free water and 5 µl were used to seed the PCR master mix containing the nucleotides ITS 1 (100 pm µl^{1} 5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (100 pm µl^{1} 5'-TCCTCCGCTTATTGATATGC-3') (both Biomers, Ulm, Germany).

The reaction took place in a thermocycler (Master Cycler Gradient, Eppendorf, Hamburg, Germany) programmed as follows: single activation step 15 min at 95 °C followed by 30 cycles comprising: 1) initial denaturation 1 min at 94 °C, 2) annealing 1 min at 55 °C, 3) elongation 1 min at 72 °C, followed by a terminating elongation step for 10 min at 72 °C with a subsequent storage temperature of 4 °C. Amplification of DNA was checked by gel electrophoresis. 5 µl of each sample was mixed with 1 µl loading dye and loaded onto a roti-safe (Carl Roth GmbH, Karlsruhe, Germany) stained 1 % agarose gel in tris base boric acid EDTA buffer (TBE; containing per liter 10.8 g tris base, 5.5 g boric acid, 20 mM EDTA) and migrated for approximately 1 h at 90 V. For visualizing DNA fragments the gel was irradiated with 312 nm UV light and the size of the amplified ~1.4 kb sized fragments was compared with a comigrated 0.110 kb DNA ladder (QuickLoad 2log, New England Biolabs, Frankfurt/Main, Germany).

DNA fragments were sequenced (GATC, Konstanz, Germany) from both sides,

submerged and after exclusion of each ends flanking approximately 30 base pairs compared with the 16S rRNA sequences of culturable species using the National Center for biotechnology Information (NCBI) MEGABLAST tool and database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were checked for chimeras using DECIPHER search tool (Wright et al. 2012). 16S rRNA sequences were submitted to NCBI GenBank (see appendix Table A-3a-1).

Phylogenetics:

Strains that performed best in the screening experiments were compared to their closest relative type strain using the NCBI MEGABLAST tool. 16S rRNA sequences of the selected strains and closest type strain relatives were aligned using the multiple alignment tool ClustalW (Thompson et al. 1994). A phylogenetic tree was constructed and evolutionary analysis were conducted using the molecular evolutionary genetics analysis tool MEGA6 (Tamura et al. 2013). The evolutionary history was inferred by using the Maximum Likelihood method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 28 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1075 positions in the final dataset.

Cultivation:

Cultivation for initial screening: All isolates were grown in SBH media at pH 7.0 using glucose and sunflower oil as carbon source as described in the isolation section. Strains were cultivated in autoclave sterilised 2 ml 96 deepwell plates (Riplate, Carl Roth, Karlsruhe, Germany) sealed with sterile cling film for cell cultures (Rotilabo, Carl Roth, Karlsruhe, Germany). For each strain a cryo-stock was directly seeded into one well containing a volume of 800 µl media using a sterile tip. Cells were grown for three days at 15 °C and 400 rpm using an incubation shaker (Multitron II, Infors, Bottmingen, Switzerland). After cultivation, 100 µl of each culture was transferred into a fresh flat-bottom 96 well plate and viability of cells observed by reading the optical density at 600 nm (OD_{600}). The deepwell plates were centrifuged for 20 min at 4,643 x g and 4 °C, 600 µl supernatant transferred into fresh deepwell plates and subsequently used for the screening methods.

Cultivation for secondary screening: Detailed screening methods were conducted for one

selected member of each genus of positively screened microorganisms. Strains were cultivated in SBH-G and SBH-O in 20 ml scale (100 ml baffled conical flasks) for 24 h at 15 °C and 130 rpm, the supernatant was harvested (20 min, 4,643 x g, 4 °C) and used for biosurfactant screening.

Production of biosurfactants: One selected strain isolated *Pseudomonas* sp. SC S1O4, was used for the production of biosurfactants. An LB overnight culture was seeded into several 2 l baffled conical flasks filled with 400 ml LB-glycerol medium containing per litre 5 g yeast extract, 10 g tryptone, 10 g NaCl and 10 % glycerol. The strain was inoculated with a starting optical density (OD_{600}) of 0.6 at 600 nm. Bacteria were cultivated for two days at 25° C and 110 rpm. The cultivation processes were sampled at 7 time points and checked for growth (OD_{600}), pH value and the production of biosurfactants via two phase extraction and thin layer chromatography (see screening section). After the cultivation, crude extract was gained by mixing cell free supernatant twice with ethyl acetate (1:1.25 v/v) in a separating funnel and subsequent evaporation of the solvent (Heidolph Laborota 4000, Schwabach, Germany).

Screening

All isolates were initially screened for the production of biosurfactants using quick qualitative screening methods followed by more extensive screening methods for the surfactants producers examined in larger scales.

Quick Surface tension grid assay: Method was applied according to Cottingham et al. (2004). For each strain 100 µl of supernatant was placed into one well of a flat-bottom 96 well plate. A paper with approximately 1 mm^2 black and white chess pattern was placed 2 cm beneath the 96 well plate. Seen through from above the tension dependent concave surface of the liquid in the well changes the grid pattern proportionally and was evaluated with (++) for small grids, (+) for slightly smaller grids and (-) for no serious change in the surface tension.

Surface tension values: Alteration in the surface tension of the culture supernatant or water dissolved extracts were monitored against air at room temperature using the Du Noüy (1919) ring method on a Tensiometer (Lauda TD1, Lauda-Königshofen, Germany) according to the supplier's manual.

Atomized oil assay: Extracellular biosurfactants were detected by a method described by Burch et al. (2010). Sterile liquid paraffin was sprayed onto 1 mm^2 sized two days

old microbial colonies on 0.5 NA agar plates using an air brush (Conrad Electronics, Hirschau, Germany). Extracellular surfactants were detected if paraffin drips off into small droplets.

Small scale emulsification assay: The ability of the supernatant to emulsify kerosene was measured by a method modified from Bicca et al. (1999). 500 µl supernatant and 500 µl kerosene (purum, SigmaAldrich; Germany) were mixed with a vortex for 1 min in 2 ml screw cap glas vials at room temperature. After 24 hours, the glas vial for each strain was checked for stable emulsified layers and classified into strong (++), weak (+) or no (-) emulsification.

Larger scale emulsification assay: The emulsification index (E_{24}) was determined by a method similar to the one described above using 2 ml cell free supernatant and 2 ml kerosene in 12 ml screw cap glas vials. After 24 hours, the E_{24} index was determined as percentage of the height of emulsified layer by the total height of the liquid column.

Hemolysis assay: Surfactant caused lysis of blood cells was screened with a method described by Mulligan et al. (1984). Centrifuged cellmass from each well was replicated using a 96 needle replicator (Bartelt, Graz, Austria) onto blood agar (BA) plates containing per litre 15 g agar, 40 g blood agar base and 50 ml sterile defibrinated sheep blood (Oxoid, Basingstoke, UK). Plates were incubated at 15 °C for 72 hours before being evaluated for lysis-halos surrounding the colonies formed.

Thin layer chromatography (TLC): The supernatant was harvested (20 min, 4,643 x g, 4 °C) and amphiphilic molecules were extracted by two times vigorously mixing 2 ml ethyl acetate (1:1.25 v/v) in 12 ml screw cap glass vials and centrifugation for 10 min at 4643 x g and 4 °C. The organic phases were combined and evaporated to dryness at 40 °C, 50 mbar and 2000 rpm in a vacuum concentrator (ScanSpeed MiniVac Evaporator, Saur, Reutlingen, Germany). Dry organic extracts were resuspended in ethyl acetate and the adequate amount to 300 µl supernatant was spotted onto a 60 Å silica TLC plate (Alugram Xtra SIL G, Macharey-Nagel, Düren, Germany) as stationary phase and a mobile phase of chloroform / methanol / acetic acid (65:5:2 v/v/v).

Purification of biosurfactants

Medium-pressure-liquid-chromatography (MPLC): 381 mg dried ethyl acetate crude extract from 400 ml cultivations of *Pseudomonas* sp. SC S1-O4 was dissolved in 20 ml chloroform / methanol (90:10, v/v) and purified by MPLC (SepacoreX50, Büchi,

Flawil, Switzerland) using 40-63 µm particle size silica stationary phase with 60 Å pore size (150 mm column length, 12 mm column diameter and 17 ml bed volume) and an isocratic chloroform / methanol (90:10, v/v) solvent system at a flow rate of 5 ml min^{-1}. The eluate was collected in 5 ml fractions and examined towards amphiphilic compounds using TLC. Fractions 6-11, 15-32 and 33-55 were combined, the solvent was evaporated and lyophilized (Christ beta 1 – 8, Martin Christ GmbH, Osterode, Germany). The lyophilized fractions were weighed, dissolved in ultra-pure water and the surface tension of the solution determined.

TLC staining of functional groups:
Anis aldehyde: Microbially produced surfactants were detected by dipping the plate into the general staining anis aldehyde (p-anis aldehyde / acetic acid / sulphuric acid, 100:1:2 v/v/v) developed under 150 °C air stream for 25 min.
Iodine vapour: Unsaturated and aromatic compounds were stained by placing the TLC plate two centimetres above iodine crystals in a closed chamber. The chamber was saturated with the crystals for 10 min, the plate incubated for another 10 min.
Ninhydrin: Primary amines were stained with a ninhydrin / methanol solution (2:1 w/v) and developed under 110 °C air stream.

Results

Growth could be observed in all enrichment cultures of each soil sample from both, homogenously and pellet-associated growing microorganisms. A total of 168 different *cfu*s were isolated from 20 enrichment approaches (five soil samples each in four enrichment cultures). Of these 168 *cfu*s, 12 were isolated from the hydrophobic phase of SC S3-O where growth with the morphology of yellow pellets was observed. One prokaryote was isolated from white mucus-ridden clouds in the paraffin phase of SC S1-P. All other strains were isolated from the aqueous phases of the enrichment cultures. Five *cfu*s isolated could be assigned to the kingdom of fungi. For a detailed list of all *cfu*s see appendix Table A-3a-1. With four different enrichment media used approximately 30 morphologically different colonies were isolated per seal-soil sample. In the soils of SL S1, SC S1 and SC S3 a generally higher diversity of morphologic differences

in cultivable colonies was detected for the hydrophobic enrichments (sunflower oil and paraffin), whereas the soils SC S1 and MV S1 show a higher diversity of colonies isolated from the hydrophilic enrichment experiments (glucose and 0.5 NA). The highest diversity of one soil (48) was observed for SC S3 with 20 distinct colonies in the oil enriched samples (see Figure 3.3).

All strains were screened for the alteration of surface tension and emulsification ability of their supernatant after growth in mineral medium with a hydrophobic (sunflower seed oil) and a hydrophilic (glucose) carbon source. A total number of 98 *cfu*s (58%) lowered the surface tension in at least one cultivation media, 51 *cfu*s (30%) lowered the surface tension strongly. The supernatant of 24 *cfu*s (14%) showed significant emulsification of kerosene, 6 strains exhibited halos of haemolytic activity on blood agar plates. A majority of the isolated strains that have been identified belong to species of the genus *Arthrobacter* spp. (46), followed by *Pseudomonas* spp. and *Psychrobacter* spp. (10 each) (for detailed results of the screening experiments see appendix Table A-3a-1).

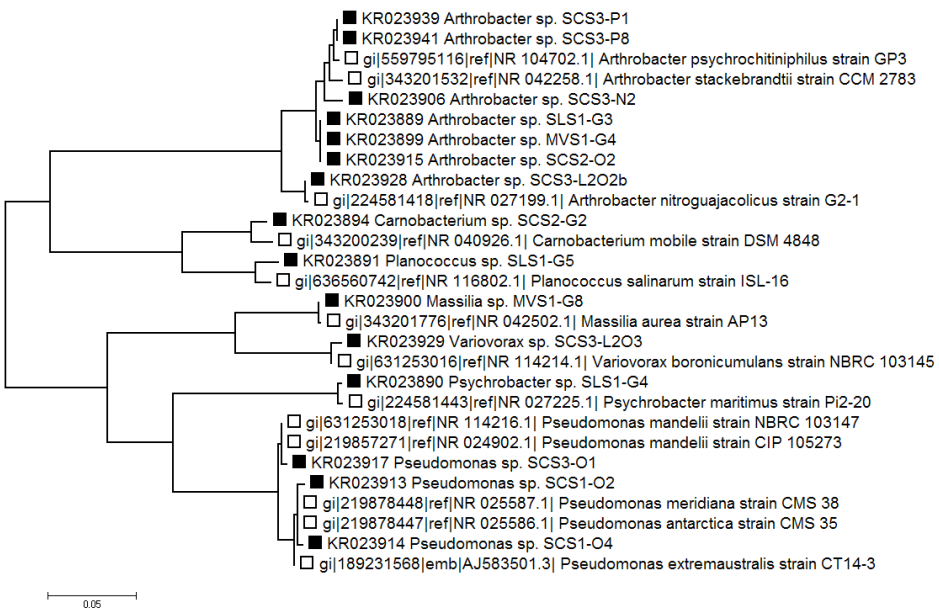

Figure 3.3: Molecular phylogenetic analysis of 15 selected procaryotic biosurfactant producing strains isolated from different Antarctic seal carcass soils (filled square) and their closest relative type strain (open square). Isolated strains and type strains are affiliated with their NCBI accession numbers

Fifteen prokaryotic strains and one eukaryote were carefully selected according to best performances in the screening methods and novelty of the strain related to biosurfactant production (Table 3.2). Most *Arthrobacter* spp. and *Pseudomonas* spp. and the fungi *Thelebolus* sp. were able to lower the surface tension and showed emulsification of kerosene in supernatants of both cultivation media. *Massilia* sp. MVS1-G8 and *Planococcus* sp. SLS1-G5 only grew in deep well cultivations with glucose as carbon source, *Variovorax* sp. grew only in the sunflower seed oil containing deep well cultivation. All three strains produced compounds that lowered the surface tension; additionally *Planococcus* sp. produced an emulsifying compound. *Carnobacterium* sp. SCS2-G2; *Psychrobacter* sp. SLS1-G4 and *Thelebolus* sp. SLS1-G8 exhibited haemolytic activity indicated by halos sourrounding the colonies growing in blood agar.

Compared to type strains, the phylogeny of the selected strains show, that they are closely related to *Arthrobacter* spp. of *A. psychrochitinophilus* for SCS3-P1 and SCS3-P8; *A. stackebrandtii* for SCS3-N2, SLS1-G3, MVS1-G4 and SCS2-O2, as well as *A. nitroguajacolicus* for SCS3-L2O2b. Strains related to *Pseusomonas* spp. showed highest similarity to type strains of *P. mandelii* for SCS3-O1; *P. meridiana* or *P. antactica*

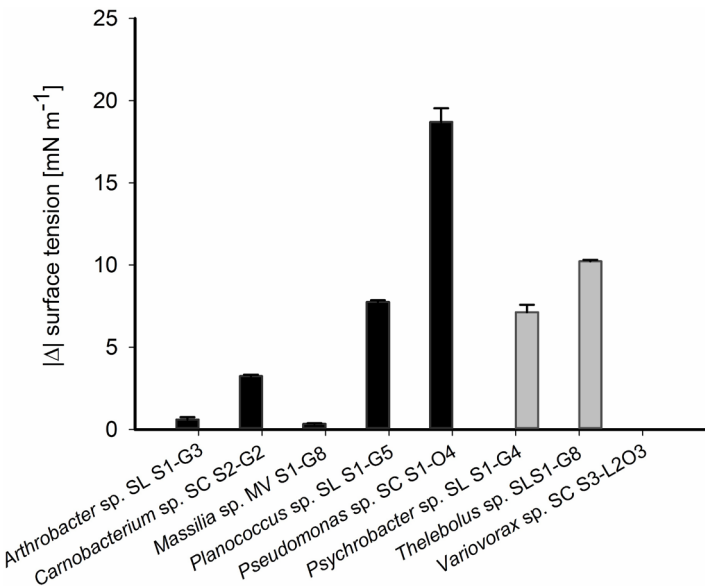

Figure 3.4: Alteration of the surface tension after 24h shake flask cultivation from one member of each positively screened genus in SBH media with glucose (black) and oil (grey) as carbon source

as well as *P. extremaustralis* for SCS1-O2 and SCS1-O4. Members of other genera were closest related to the type strains *Massilia aurea* for MVS1-G8, *Psychrobacter maritimus* for SLS1-G4 and *Variovorax boronicumulans* for SCS3-L2O3. More distantly related were strains SCS2-G2 with 98% similarity to *Carnobacterium mobile* and SLS1-G5 with 97% similarity to *Planococcus salinarum* (for detailed results, strain number and gene accession numbers, see Figure 3.3).

Six strains, one selected potential biosurfactant producer from each genus was used for a more detailed screening approach and cultivated in 20 ml shake flask cultivation in SBH glucose and sunflower seed oil media. All strains showed growth after a cultivation time of 24h. Exact measurements of the surface tension values of the supernatant after the cultivation revealed a decrease of 18.7 mN m^1 to an absolute value of 55.0±0.8 mN m^1 of the supernatant from *Pseudomonas* sp. SCS1-O4 in cultivations with glucose as carbon source. For *Thelebolus* sp. the surface tension in cultivations with sunflower seed oil showed a decrease of 10.2 mN m^1 to an absolute value of 39.5±0.1 mN m^1. The surface tension of the supernatant of *Psychrobacter* sp. SLS1-G4 in sunflower seed oil containing media and the surface tensions of *Planococcus* sp. SLS1-G5 as well as *Carnobacterium* SCS2-G2 in glucose mineral media were only slightly lowered (see Figure 3.4). Emulsification of kerosene was observed in supernatants derived from shake flask cultivations of *Pseudomonas* sp. SCS1-O4 in glucose containing medium and *Psychrobacter* sp. SLS1-G4 in sunflower seed oil containing medium, both with values of 20%.

Dispersal of atomized drops of liquid paraffin was observed in an approximately 3 mm^2 surrounding zone of a two day old colony of *Pseudomonas sp.* SCS1-O4 (see Figure 3.5).

The surfactant produced by *Pseudomonas* sp. SCS1-O4 was further characterized. In order to produce a suitable amount of crude extract, the strain was, after further evaluation of a suitable production medium cultivated in glycerol enriched LB medium four times 400 ml shake flasks and at a higher temperature of 25 °C. After cultivation, four different amphiphilic compounds that were not present in the production medium were detected using various TLC staining solutions. One of them (coumpound d) was positively stained in ninhydrine solution indicating the presence of primary amines. Compounds a to c feature both, a lipidic moiety visualized with iodine vapours and hydroxyl groups stained by anisaldehyde (see Figure 3.6). The compounds were

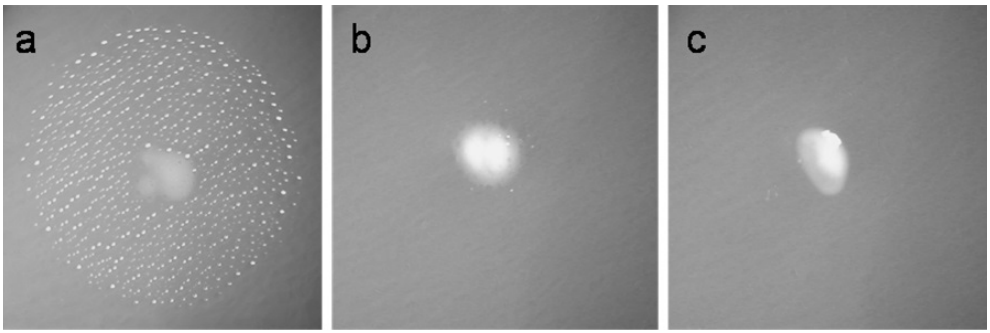

Figure 3.5: Atomized oil assay of 24h cultures of *Pseudomonas* spp. on LB agar. Dispersion of liquid paraffin into small droplets indicates the production of an extracellular biosurfactant (Burch et al. 2010). (a) Strong dispersion in vicinity of a colony from Antarctic seal carcass isolated strain *Pseudomonas* sp. SCS1-O4, (b) slight dispersion around a colony of rhamnolipid producing *Pseudomonas aeruginosa* PAO1, (c) no visible dispersion around a colony of *Pseudomonas putida* KT2440

separated using silica column chromatography. Neighbouring eluents containing different compounds were combined to three different fractions (see Figure 3.7). Measurements of the surface tension of the fractions revealed that the compounds exhibiting surface activity were present in the fraction combined from eluents 6 to 11 as the surface tension of these fractions were lowered to 29 mN m^1. Fraction 2 (65 mN m^1) containing compound c and fraction 3 (55 mN m^1) containing compound d showed no or only limited reduction of the surface tension thus the surface active metabolite produced is limited to compound a or b that is present in fraction 1 only.

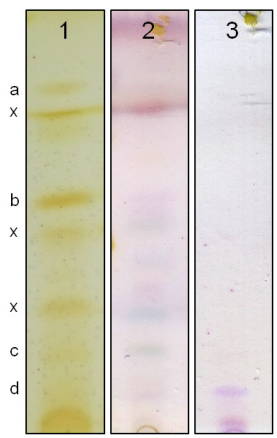

Figure 3.6: Functional staining of extracted amphiphiles from *Pseudomonas* sp. SCS1O4. Lane 1: iodine vapor for the detection of lipidic components. Lane 2: anisaldehyde for the detection of hydroxyl groups. Lane 3: ninhydrine staining of primary amines. Compounds a-d are produced during cultivation, compounds marked as x corroborate to medium components

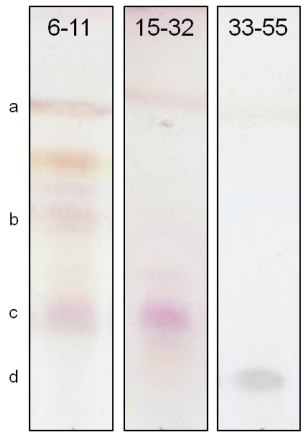

Figure 3.7: Thin layer chromatography of combined eluents from silica gel column chromatographic purification of crude extract produced by *Pseudomonas* sp. SCS1-O4 in LB and glycerol. Fractions 6 to 11 contain surface active compounds

Discussion

Colony forming units were detected from all Antarctic soils sampled and enriched. The variation in number of *cfu*s isolated only differed to a small extent of the soils and enrichment media chosen. The slightly higher amount of strains isolated after the enrichment in hydrophobic carbon sources indicate the ability of microbial communities inhabiting the soil to utilize hydrophobic carbon sources, generally speaking of the fatty skin of the seal carcasses in its proximity.

Out of the 168 *cfu*s isolated it cannot be ruled out that isolated strains were found in more than one soil examined (see also Table 3.1). Further those strains isolated could well be growing in several enrichment cultures and therefore be repeatedly examined within this study.

The selected strains (Table 3.2) that were positively screened for a production of surface active compounds mainly correspond to the genera of *Arthrobacter* spp. and

Table 3.1: Morphologically different colony forming units (cfu) isolated from seal carcass exposed soil in enrichment cultures with different carbon sources

Soil sample / Carbon source	Glucose	0.5 NA	Seed oil	Paraffin	Total *cfu*
SL S1	8	6	6	14	34
SC S1	14	6	4	5	29
SC S2	3	4	6	14	27
SC S3	10	10	20	8	48
MV S1	11	6	6	7	30
total *cfu*	46	32	42	48	168

Table 3.2: Selected strains from deep well plate cultivations in SBH with glucose (SBH G) and sunflower seed oil (SBH O) as carbon source as well as blood hemolysis agar (BA). Displayed are isolates and their performance in lowering the surface tension (grid assay) and emulsification indices (E24) as well as hemolytic activity (hem.) and the NCBI accession number of their 16S rRNA sequence

Isolated strain	SBH G			SBH O			BA		GenBank accession number
	growth	Grid	E 24	growth	Grid	E 24	growth	Hem	
Arthrobacter sp. SLS1-G3	x	++	+	x	+	-	x	-	*KR023889*
Arthrobacter sp. MVS1-G4	x	++	-	x	++	-	x	-	*KR023899*
Arthrobacter sp. SCS3-N2	x	++	+	x	-	+	x	-	*KR023906*
Arthrobacter sp. SCS2-O2	x	++	+	x	-	+	x	-	*KR023915*
Arthrobacter sp. SCS3-L2O2b	x	+	+	x	+	+	x	-	*KR023928*
Arthrobacter sp. SCS3-P1	x	++	+	x	+	-	x	-	*KR023939*
Arthrobacter sp. SCS3-P8	x	+	+	x	++	-	x	-	*KR023941*
Pseudomonas sp. SCS1-O2	x	++	+	x	++	+	x	-	*KR023913*
Pseudomonas sp. SCS1-O4	x	++	+	x	++	+	x	-	*KR023914*
Pseudomonas sp. SCS3-O1	x	++	+	x	-	+	x	-	*KR023917*
Carnobacterium sp. SCS2-G2		no growth			no growth		x	+	*KR023894*
Massilia sp. MVS1-G8	x	++	-		no growth		x	-	*KR023900*
Planococcus sp. SLS1-G5	x	+	+		no growth		x	-	*KR023891*
Psychrobacter sp. SLS1-G4		no growth			no growth		x	+	*KR023890*
Variovorax sp. SCS3-L2O3		no growth		x	++	-	x	-	*KR023929*
Thelebolus sp. SLS1-G8	x	++	+	x	++	+	x	+	

Pseudomonas spp. which is not surprising as both genera inherit species that are known biosurfactant producing strains. Different trehalose lipids (Passeri et al. 1990; Suzuki et al. 1969), fructose- (Itoh and Suzuki 1974) and sucrose lipids (Suzuki et al. 1974) are described to occur within *Arthrobacter* spp. as well as dimannosyl glycerol, galactosyl glycerol (Pasciak et al. 2010) and corynexanthin mono- and diglycosides (Arpin et al. 1972). Within *Pseudomonas* spp. rhamnose containing glycolipids (Bergström et al. 1947) and the lipopeptides viscosin (Neu et al. 1990) and viscosinamide (Nielsen et al. 1999) are described.

Little is known about surface active compounds of isolated strains that belong to other genera. A bioemulsifier has previously been described to be produced by *Variovorax* sp. (Franzetti et al. 2012), several non defined biosurfactants are reported to be produced by *Planococcus* spp. (Ebrahimipour et al. 2014; Jacobucci et al. 2009; Kumar et al. 2007). A surface active compound produced by *Psychrobacter* sp. has been detected, but not characterized by Malavenda et al. (2010). Both, *Pseudomonas* sp. and *Psychrobacter* sp. are also known producer of cold-active extracellular lipases (Joseph et al. 2008), that could play a distinct role in the brake down of hydrophobic carbon sources.

Phylogenetic studies reveal the closest relative type-strains to the described biosurfactant producer are related to species of *psychrochitiniphilus*, *stackebrandtii* and *nitroguajacolicus* for the genus *Arthrobacter* and *meridiana*, *antarctica* as well as *extremaustralis* for the genus *Pseudomonas* (Figure 3.3). The production of a biosurfactant in biofilms has been reported for *P. extremaustralis* (Tribelli et al. 2012), no biosurfactant production is known within the others. However the degradation of polycyclic aromatic hydrocarbons was recently described for *A. nitroguajacolicus* (Mansur et al. 2015).

Pseudomonas sp. SCS1-O4 as a close relative to the above mentioned *Pseudomonas* spp. type strains was studied in more detail and the type of biosurfactant produced clearly is of extracellular nature (Figure 3.5) and its production is not necessarily biofilm dependent. During its cultivation on LB medium enriched with glycerol, four different compounds (a to d) were detected. Whereas compound d showed the presence of primary amines, the other compounds seems to inherit hydroxyl and lipidic moieties (Figure 3.6). The surface active compounds, either compound a, b or both reduced the surface tension of water to 29 mN m^{1}. A ionization for mass spectral analysis of the compounds was not successful, however the production of rhamnolipids by *Pseudomonas sp.* SCS1-O4 can be excluded as shown by TLC and genetic analysis of potential homologies to the known rhamnolipid synthesis genes *rhl a* and *rhl c* (Sanden 2013).

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Biosurfactant producing prokaryotes inhabiting raised bog peat soil

Kügler JH, Hansen SH, Völp AR, Syldatk C, Hausmann R

Contribution to this study:

JK has concepted and written this study, performed experiments, collected and interpretated the relevant data used. SH and AV have sampled the soil and contributed to screening and discussion within their Master Thesis (Hansen 2013; Völp 2013). All authors have fruitfully discussed content and structure of this study.

3b Biosurfactant producing prokaryotes inhabiting raised bog peat soil

Introduction

Raised bog is a particular habitat that is only fed by rainwater. Poor drainage of the peatland soil and absence of contact to groundwater causes nutrient deficiency and the availability of nutrients is limited to transfer from adjacent ecosystems. Permanent saturation with water generates a lack of oxygen that result in incomplete decomposition of mainly plant organic matter. Bog mosses, mainly of the genus *Sphagnum* bind minerals and release hydrogen ions that cause an acidic environment of approximately pH 4 (Kamal and Varma 2008). Uronic acids and polyphenols are produced resulting in a strong inhibitory effect on microbial activity (Bragazza et al. 2006). Dead plant material accumulates over thousands of years and exceeds its decomposition rate, thus serving as an important carbon sink. This imbalance by input of mainly photosynthesis derived organic matter and its losses through decomposition is named ombrothrofication, a raising formation of bog and accumulation of peat, brown to black soil that is rich in humic substances. The carbon to nitrogen ratio is about three times higher than in humus soils (Kuhry and Vitt 1996). The dark peat heats up in summer and its low conductivity impedes thermal transfer to lower soil layers. This result in an extreme

habitat with temperature fluctuating up to 70 °C within one sunny day and a cold night.

Microbiota in peat lands have developed physiological and metabolic adaptations to cope the above mentioned conditions. Decomposition is carried out by a consortia of microorganisms that, influenced by environmental circumstances interact together with complementary enzymatic activities (Andersen et al. 2013). In the acrotelm, the oxic upper layer of the peat, nitrogen-fixing prokaryotes such as *Bukholderia* but also *Proteobacteria*, *Bacteroides*, *Actinobacteria*, *Acidobacteria* and *Plantomycetes* are predominately described to occur (Andersen et al. 2013). In proportion to its vertical stratification, environmental constraints as oxygen availability, pH and the accumulation of complex polymers increase (Turetsky et al. 2000). The number of bacteria decreases with depth, microbial communities change from aerobic bacteria to obligate anaerobes in deeper zones. The mesotelm, interface between oxic and anoxic layers is predominately inhabited by methanothrophic bacteria. The abundance of yeast is reported to remain stable in the different zones, whereas the amount of spores of filamentous fungi decreases (Golovchenko et al. 2005).

Enzymes or other secondary metabolites from microorganisms adapted to living conditions in the acrotelm of peat bog areas may produce surface active or emulsifying compounds as tool for an accession of nutrients, swarming or defense of their habitat. In this study, the upper layers of a pristine raised bog in the region of Kaltenbronn, northern Black Forest are used for the isolation of microorganisms and a study of their ability to produce surface-active or emulsifying compounds.

Materials and Methods

Peat-bog soil samples

Sampling: Samples were taken out of four different spots in a raised peat region of the northern Black Forest near Kaltenbronn, Baden Württemberg, Germany, previously described for the isolation of oleaginous yeasts (Schulze et al. 2014). Several hundred grams of bog was transferred with a sterile spatula into 50 ml sampling tubes. All samples were taken in summer time at a sea level between 880 and 920 m. Sample one was taken from a bark-rich ground underneath conifer trees (47.720°N, 8.471°E),

sample two was taken from a swampy meadow (48.719°N, 8.464°E), sample three was taken from grassland close to a small lake (48.719°N, 8.459°E) and sample four was taken from soil at the intersection of swamp to forest (48.716°N, 8.456°E). All samples were taken at a depth of approximately 2 cm and stored at 20 °C.

Microorganisms

Serratia marcescens DSM 30121 and DSM 12481 as well as *S. rubidea* DSM 4480 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Strains were stored in cryo-stocks at 80 °C in 15 % glycerol and lysogeny-broth (LB) media containing per liter 5 g yeast extract, 10 g tryptone and 10 g NaCl. The isolate *Rouxiella* sp. strain 323 was submitted to the DSMZ and assigned as *Rouxiella* sp. DSM 100043 strain 323.

Isolation of microorgansims: Approximately 10 g of each soil sample was resuspended in 1 ml sterile demineralised water and 100 µl aliquots of different dilutions were streaked onto yeast-malt (YM) agar plates containing per liter: 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose and 20 g agar. The pH was set to 7.0 using NaOH and H_3PO_4. Microorganisms were grown at 20 °C until colonies were clearly visible. Each morphological different colony forming unit (*cfu*) was picked with a sterile tip and streaked out onto fresh YM agar plates, repeatedly grown and picked for at least three times or until visible purity. Isolates were named with a three digit code, the first digit according to its soil sample spot, the second for the agar plate originating and the third for number of the colony picked. Isolated microorganisms were stored in cryo-stocks at 80 °C in YM containing 15 % glycerol.

Gram test: A drop of 3 % potassium hydroxide was mixed with a loop of a single colony on a glass surface by stirring with a needle for 1 min. Bacteria were determined to be gram negative when the formation of threads was observed after lifting the stirring device.

Genetic characterization: 10 ml overnight culture of wild type strains grown in YM media were centrifuged for 20 min at 4643 x g and 4 °C. The supernatant was discarded and the genomic DNA of each cell pellet was extracted using PureLink Genomic DNA Mini Kit (Life Technologies GmbH, Darmstadt, Germany) according to the supplier's manual. Genomic DNA (gDNA) was eluted in nuclease free water (Carl Roth GmbH, Karlsruhe, Germany). The 16S rRNA decoding DNA sequence

was amplified by polymerase chain reaction (PCR) with each sample containing 5 µl of 1:10 diluted DNA template in nuclease free water, 0.75 U polymerase (HotStar TaqTM, Qiagen, Hilden, Germany), 0.6 µL desoxyribonucleotide triphosphate mix (dNTPs; 10 mM of each dNTP: Qiagen, Hilden, Germany), 1 µl of oligonucleotide 27F (100 pmol μl^{-1}; 5'-AGAGTTTGATCCTGGCTCAG-3') and 1 µl of oligonucleotide 1385R (100 pmol μl^{-1}; 5'-CGGTGTGTRCAAGGCCC-3' whereas R is A or G) (both Biomers, Ulm, Germany), and 3 µl of a PCR reaction buffer (10 x, Qiagen, Hilden, Germany) filled up to a total volume of 25 µl per sample with nuclease free water. Reaction took place in a thermocycler (Master Cycler Gradient, Eppendorf, Hamburg, Germany) programmed as follows: single activation step 15 min at 95 °C followed of 30 cycles comprising: 1) initial denaturation 1 min at 94 °C, 2) annealing 1 min at 55 °C, 3) elongation 1 min at 72 °C, followed by a terminating elongation step for 10 min at 72 °C with a subsequent storage temperature of 4 °C. Amplification of DNA was checked by gel electrophoresis. 5 µl of each sample was mixed with 1 µl loading dye and loaded onto a roti-safe (Carl Roth GmbH, Karlsruhe, Germany) stained 1 % agarose gel in tris base boric acid EDTA buffer (TBE; containing per liter 10.8 g tris base, 5.5 g boric acid, 20 mM EDTA) and migrated for approximately 1 h at 90 V. For visualizing DNA fragments the gel was irradiated with 312 nm UV light and the size of the amplified ~1.4 kb sized fragments was compared with a comigrated 0.110 kb DNA ladder (QuickLoad 2log, New England Biolabs, Frankfurt/Main, Germany). DNA fragments were sequenced (GATC, Konstanz, Germany) from both sides, submerged and after exclusion of each ends flanking 30 base pairs compared with the 16S rRNA sequences of culturable species using the NCBI MEGABLAST tool and databse (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Mismatches to the sequences of the most similar type strains were checked manually in the sequence spectrograms. Sequences were checked for chimeras using DECIPHER search tool (Wright et al. 2012). 16S rRNA sequences were submitted to NCBI GenBank and an accession number assigned (see Table 3.3 and appendix Table A3b1).

Phylogenetics: 16S rRNA sequences of isolated strains and their closest relative type strain according to the NCBI MEGABLAST tool were aligned using the multiple alignment tool ClustalW (Thompson et al. 1994). A phylogenetic tree was constructed and evolutionary analysis were conducted using the molecular evolutionary genetics analysis tool MEGA6 (Tamura et al. 2013). The evolutionary history was inferred by

Table 3.3: Biosurfactant producing peat-bog soil isolated strains and their performance in lowering the surface tension (grid assay) in SBH-G and SBH-O medium as well as emulsification indices (E24) in SBH-G medium and the NCBI accession number of their 16S rRNA sequences. Strains marked bold were studied in more detail.

Isolated strain	grid SBH-G	grid SBH-O	E_{24} SBH-G	GenBank accession n°
Citrobacter sp. 322	+	-	+	KP642160
***Janthinobacterium* sp. 112A**	+	+	++	KP642150
***Janthinobacterium* sp. 112C**	-	++	++	KP642151
***Pseudomonas* sp. 230**	++	++	+	KP642158
***Pseudomonas* sp. 423**	++	-	-	KP642165
***Rouxiella* sp. 213**	++	+	++	KP642153
Rouxiella sp. 223	+	-	-	KP642157
***Rouxiella* sp. 323 DSM 100043**	++	++	-	KP642161
***Rouxiella* sp. 421**	++	++	-	KP642164
***Serratia* sp. 210**	++	+	++	KP642152
***Serratia* sp. 214**	+	+	++	KP642154
Serratia sp. 221	+	-	+	KP642155
Serratia sp. 324	+	+	-	KP642162
***Serratia* sp. 411**	+	-	-	KP642163

using the Maximum Likelihood method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 26 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 566 positions in the final dataset.

Screening

Cultivation: Isolated strains were grown in YM or in a medium (SBH) supplemented and modified from Bushnell and Haas (1941) containing per litre: 1.0 g K_2HPO_4, 1.0 g KH_2PO_4, 0.8 g NH_4Cl, 0.8 g $NaNO_3$, 0.1 g yeast extract, 0.2 g $MgSO_4$, 0.26 g $CaCl_2$ • 2 H_2O and 1 ml of a trace element solution containing per litre 2 g Na-citrate• 2 H_2O, 0.28 g $FeCl_3$ • 6 H_2O, 1.4 g $ZnSO_4$ • 7 H_2O, 1.2 g $CoCl_2$ • 6 H_2O, 1.2 g $CuSO_4$ • 5 H_2O, 0.8 g $MnSO_4$ • H_2O. For SBH media either glucose (10 g l^{-1}) (SBH-G) or sunflower seed oil (20 ml l^{-1}) (SBH-O) was used as carbon source. For comparison with other *Serratia* sp. strains for the production of serrawettin W1, all strains were cultivated in LB medium supplemented with 10% glycerol. For all media, the pH was set to 7.0 using NaOH and H_3PO_4. Cells were grown for three to five days at 30 °C and 130 rpm. For the initial screening, test glas tubes were used (12 ml sterile screw cap glass tubes, 4 ml broth volume) and directly centrifuged. Detailed screening was conducted in shake flasks (100 ml shake flask, 20 ml broth volume), 10 ml culture broth was centrifuged for 20 min at 4643 x g and 4 °C. The supernatant was kept for the screening of extracellular biosurfactants:

Surface tension microplate assay: Method was applied according to Cottingham et al. (2004). For each strain 100 µl of supernatant was placed into one well of a flat-bottom

96 well plate. A paper with approximately 1 mm^2 black and white chess pattern was placed 2 cm beneath the 96 well plate. Seen through from above the tension dependent concave surface of the liquid in the well changes the grid pattern proportionally.

Bleeding droplet assay: Oil subilization method was modified from Maczek et al. (2007). The supernatant was stained with 10 % (v/v) of 1 g l^{-1} crystal violet solution. 5 µl of the stained supernatant was planted into 10 µl sunflower seed oil placed in the middle of a 96 well plate well. The plate was sealed to prevent desiccation and ceased for 24 h before the coloured droplets were observed for bleeding.

Emulsification assay: The emulsification index (E_{24}) was measured by a method modified and downscaled from Bicca et al. (1999). 500 µl cultivation supernatant and 500 µl kerosene (purum, SigmaAldrich, Germany) were mixed vigorously with a vortex for 1 min in 2 ml screw cap glas vials at room temperature. After 24 hours, the E_{24} index was determined as percentage of the height of emulsified layer by the total height of the liquid column.

Thin layer chromatography (TLC): Amphiphilic molecules were extracted by two times vigorously mixing 4 ml of a strains's supernatant with 5 ml ethyl acetate (1:1.25 v/v) in 12 ml screw cap glass vials with subsequent centrifugation for 20 min at 4643 x g and 4 °C. The organic phases were combined and evaporated to dryness at 40 °C, 50 mbar and 2000 rpm in a vacuum concentrator (ScanSpeed MiniVac Evaporator, Saur, Reutlingen, Germany). The extract was resuspended in ethyl acetate and the adequate amount to crude extracts gained from 250 ml supernatant was spotted onto a 60 Å silica TLC plate (Alugram Xtra SIL G, Macharey-Nagel, Düren, Germany) as stationary phase. A mobile phase of chloroform / methanol / acetic acid (70:10:2 v/v/v) was used for migration of the compounds. For comparison to serrawttin and rubiwettin producing strains and its detection a running solution of chloroform / methanol / 25% ammonia (80:25:1.36 v/v/v), modified from Matsuyama et al. (1986) was used. Glycolipids were detected by dipping the plate into the general staining solution of anisaldehyde (panisaldehyde / acetic acid / sulphuric acid, 100:1:2 v/v/v) with subsequent development under 150 °C air stream for 25 min. Serrawettin W1 was detected by spraying the plate with 50% H_2SO_4 and subsequent development under 220°C air stream.

Results

A total of 26 morphologically distinct peat sample derived *cfu*s were isolated of which, 14 *cfu*s were screened positively for the production of surface-active or emulsifying compounds as tested in three different media: YM, SBH-G and SBH-O (for results see Table 3.3 and appendices Table A3b1).

Phylogenetic analyses of the isolated 14 biosurfactant producing strains revealed, that all prokaryotic strains isolated belong to the phylum *Proteobacteria* and a majority of the strains is closely related to the family *Enterobacteriaceae*. Two strains (isolates 230 and 423) belonged to the family of *Pseudomonadaceae* and two strains (112A and 112C) to the family of *Oxalbacteraceae*. Within enterobacteria isolated, only *Citrobacter* sp. 322 could clearly be affiliated to its genus. The other enterobacteria are, according to

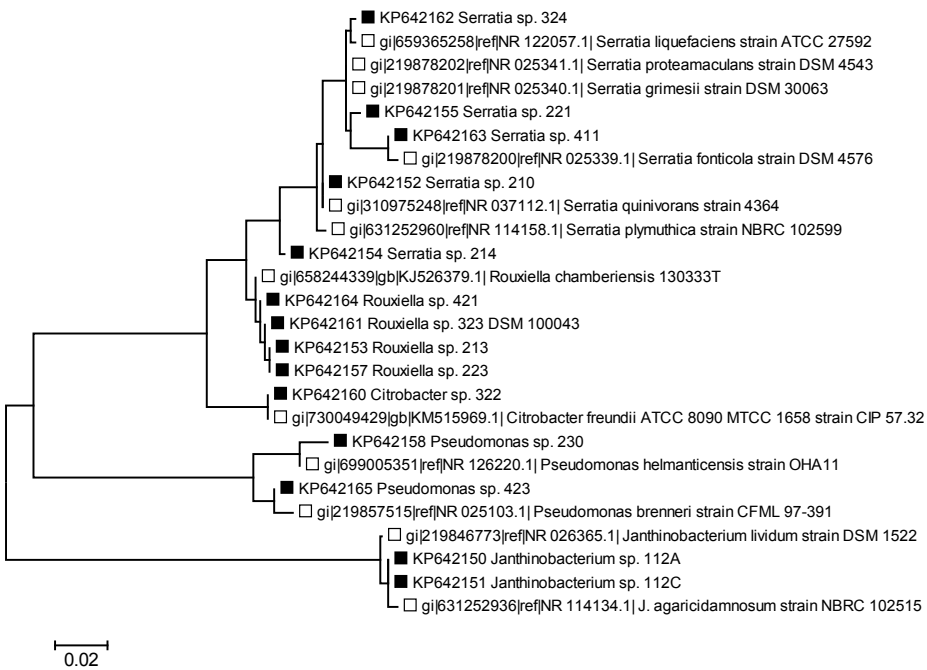

Figure 3.8: Molecular phylogenetic analysis of 14 procaryotic biosurfactant producing strains isolated from raised peat-bog soils (filled square) and their closest relative type strains (open square). Distances were calculated from aligned sequences by Maximum Likelihood method. Branch lengths indicate the number of substitutions per site. Isolated strains and type strains are affiliated with their NCBI accession numbers

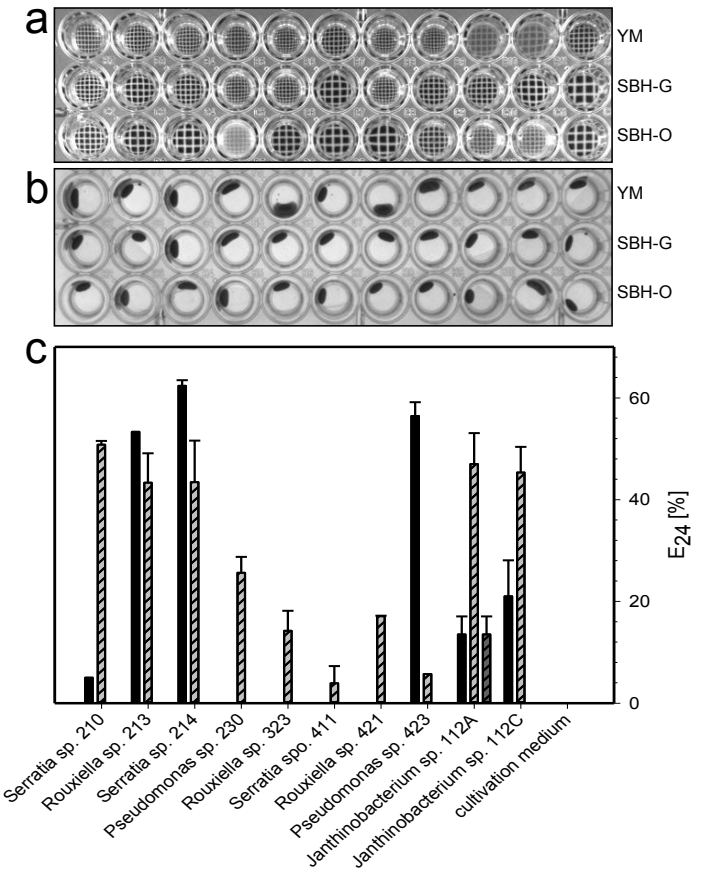

Figure 3.9: Results of screening assays for biosurfactant production of the top ten peat-bog soil strains: (a) grid test revealing variation in surface tension, (b) bleeding droplet assay and (c) E_{24} values for the emulsification of kerosene of culture supernatants in YM (black), SBH-G (light grey) and SBH-O (dark grey)

their 16S rRNA coding sequences closest related to the newly described (Le Fleche-Mateos et al. 2015) genus *Rouxiella* (for isolates 213, 223, 323 and 421) and *Serratia* (for isolates 210, 214, 221, 222, 324, 411) (Figure 3.8) but also in close proximity to other enterobacter as *Rahnella* sp., *Yersinia* sp., *Ewingella* sp. and *Hafnia* sp.. Strain 323 did not show growth at 37 °C, as also reported for its closest 16S rRNA relative (Le Fleche-Mateos et al. 2015) and was therefore assigned as *Rouxiella* sp. 323 and submitted as *Rouxiella* sp. strain 323 DSM100043.

The ten best performing biosurfactant producers were cultivated in shake flasks and screened in more detail. The production of potential surface active compounds (Figure 3.9a and 3.9b) as well their emulsifying properties was investigated (Figure 3.9c). Extracted amphiphilic compounds were visualized in TLC (Figure 3.10).

Differences were observed within the production of biosurfactants depending on nutrient availability in the media tested. Seven out of the ten strains tested exhibited a decrease of surface tension in complex medium and glucose containing mineral medium, only five in the media containing oil as substrate (Figure 3.9a). Reduction of the surface tension was clearly visible in all media by supernatants of *Serratia* sp. 210 as well as *Pseudomonas* spp. 230 and 423. Interestingly, *Janithobacterium* spp. 112A and 112C only showed reduction of the surface tension in supernatants derived from oil containing medium, and slightly lower surface tension for strain 112A in glucose containing mineral medium. For *Serratia* sp. 214, *Rouxiella* spp. 323 and 421 the surface tension is reduced in both, complex and glucose containing media. Solely *Rouxiella* sp. 411 performed poorly in terms of all secondary screening methodologies applied.

The bleeding droplet assay visualizes the effect of a surfactant to solubilise oil droplets by diffusing into them thus lowering the interfacial tension between the stained surfactant itself and the oil surrounding. For supernatants from different cultivation media used within the raised bog peat isolates this effect can only be observed poorly and for few strains, namely *Rouxiella* spp. 323, 421 and *Pseudomonas* sp. 423 in complex media (M) and slightly for *Janthinobacterium* sp. 112C in SBH-O (Figure 3.9b).

Emulsifying properties were observed for a whole range of the isolates tested. Supernatants derived fromYM cultivation of all strains showed with less than 10% slight emsulification in case of *Serratia* sp. 411 and *Pseudomonas* sp. 423 to strong emulsification in case of *Serratia* spp. 210 and 214, *Rouxiella* sp. 213 and *Janthinobacterium* spp. 112A and 112C with more than 40%. *Rouxiella* sp. 213, *Serratia* sp. 214 and *Pseudomonas* sp. 423 showed, with more than 50% high emulsification indices when grown in mineral medium containing glucose. Supernatants from SBH-O grown strains only emulsified kerosene in case of *Janthinobacterium* 112A (Figure 3.9c).

Two phase extractions of the supernatants revealed a wide range of amphiphilic molecules when visualized with anisaldehyde that unspecifically stains a wide range of functional groups (chromatograms shown in Figure 3.10). A striking yellow pigment has been observed inYM derived extracts of *Serratia* sp. 411, pink spots were stained in extractions of *Serratia* sp. 214 and *Rouxiella* sp. 323. With less hydrophobic and two different retardation factors (R_f), further brown spots were visible within extracts of *Rouxiella* spp. 323 and 421. Both strains also exhibited an orange stained spot with an R_f inbetween the above. Red to brown spots were observed in extracts of *Rouxiella* sp.

213 as well as *Serratia* spp. 214 and 411. A spot with a relatively hydrophilic R_f was observed in extracts of *Pseudomonas* sp. 230. None of the spots observed from YM extracts can clearly be affiliated to a genus, although the brown twin spots and the orange stained spots seem to be exclusive for *Rouxiella* spp. 323 and 421 but not 213 (Figure 3.10a).

Within extracts derived from mineral medium containing glucose, blue spots with hydrophilic R_fs are stained from extracts of all strains except *Rouxiella* sp 213 and *Serratia* sp. 411. These extracts are not visible in control samples of non-inoculated

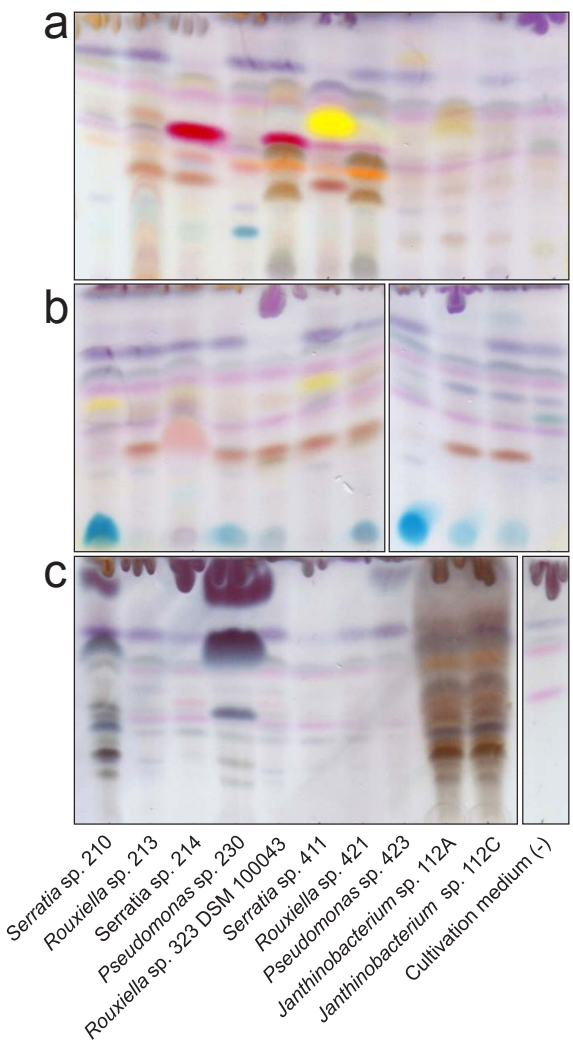

Figure 3.10: Thin layer chromatographic separation of ethyl acetate extracts derived from the top ten peat-bog soil biosurfactant producing strains. Extracts derived from supernatants of (a) YM, (b) SBH glucose and (c) SBH sunflower seed oil medium. All plates were stained with anisaldehyde.

medium. Noticeable are further brown spots present in extracts of most strains except *Serratia* spp. 210 and 213 as well as *Pseudomonas* sp. 423. Again the production of these compounds does not seem to be affiliated to a certain genus, neither was it present in control extracts of the medium. Two yellow spots were stained in extracts of *Serratia* spp. 201 and 411 that could, from its R_f confer to the yellow pigment produced in YM media supernatant of strain *Serratia* sp 411. A salmon stained compound is produced as the major extract of *Serratia* sp. 214 (Figure 3.10b).

Janthinobacterium sp 112A and 112C produced a minimum of 14 distinguishable amphiphilic compounds in cultivations with sunflower seed oil as carbon source. Further major extracts were stained for *Serratia* sp. 210 and *Pseudomonas* sp. 230. Of these, the two most hydrophobic substances could, according to colour and R_f be identical (Figure 3.10c)

Most of the isolated biosurfactant producing strains are closely related to the genus *Serratia*. This genus inherit known biosurfactant producing species (Matsuyama et al. 2011), most notably the cyclic lipopeptides serrawettin and glycolipids rubiwettin. A selection of *Serratia* sp. affiliated peat-bog isolates were again screened and compared to the known serrawettin and rubiwettin producing strains *S. marcescens* DSM 30121 and DSM 12481 and *S. rubidea* DSM 4480. For its production, cells were cultivated in LB medium containing 10% glycerol and cultivated at 30 °C as described for the production of serrawettin (Matsuyama et al. 2011). Sulphuric acid stained chromatograms of extracted compounds revealed the brown spots for serrawettin clearly visible in case of the two type strains *S. marcescens* DSM 30121 and DSM 12481 (indicated by blue arrow, Figure 3.11a). Compounds similar to serrawettin were stained for extracts of *Rouxiella* sp. 213 in even higher concentrations (blue arrow, Figure 3.11a) as well as in *S. rubidea* DSM 4480, *Serratia* spp. 210, 214, 221 and 222 in slightly lower concentrations. It is further noticeable, that a pink compound is stained in extracts from *Serratia* spp. 214 and 411 as well as *Rouxiella* spp. 223, 323 and 421. Yellow spots are present in extracts of the three type strains as well as *Serratia* spp. 210, 214, 221, 222 and 411. This spot is not present in *Rouxiella* spp and therefore seems to be genus dependent. A strongly hydrophobic spot is revealed in type strain *S. rubidea,* its retardation factor is too hydrophobic to be rubiwettin (Figure 3.11b). For *Rouxiella* spp. 323 and 421 a remarkable black spot is revealed by spraying with sulphuric acid (Figure 3.11a).

Discussion

The samples taken from peat-bog soils revealed a high number of enterobacteria, especially in those that did not rely to conifer forest associated area but to swampy regions or humid grassland. Species such as *Serratia* spp. are known to inhabit soil samples and grasslands (Hejazi and Falkiner 1997) and it's occurrence at the three different areas sampled most likely excludes external factors such as animal faecal as a reason for its presence. The utilization of YM medium, a complex substrate, for the isolation of the species might have favoured the growth of the strains isolated towards more nutrient specific soil microbes.

Even more surprising is the outcome that the majority of the strains isolated produce compounds that act surface-active. Bodour et al. (2003) compared several undisturbed and hydrocarbon or metal contaminated soil sampling sites and concluded an average of 3.4% of all isolates from undisturbed sampling sites to produce biosurfactants, and 8.4% of those isolates derived from contaminated sites. Although statisticaly not proven, with more than 50% of all raised bog isolated *cfu*s it comes naturally to mind, that the production of biosurfactants might be environmentally driven due to the habitat present in the peat. Peat-bog soil is, besides extreme fluctuation of temperature and an acidic environment a typical carbon rich soil with deficiency in nitrogen concentrations. A great majority of the carbon is present in form of humic substances, caused by insufficient composting of plant material. These mainly consist in humic acids that, to their greatest extend are composed of aromatic and polyketide moieties (Zipper et al. 2003). Microorganisms evolved mechanisms for the brake down of these substances. One of these mechanisms could be the production of biosurfactants to either facilitate the accessibility of excreted enzymes or enhance the attachment of the microorganism to its substrate. Biosurfactants could further serve as a carbon reservoir, thus exhibit another functional characteristic that is reported for cells in carbon rich environments (Schulze et al. 2014). The acidic pH of the peat is mainly caused by humic and fluvic acids present. Similar to surfactants they have an amphiphilic character and form macromolecular micelles (Kuznetsova et al. 2014). The excretion of microbial surfactants could also serve as a mediator between the cell and its acidic environment.

Identification of the microorganisms that were screened positively for the production

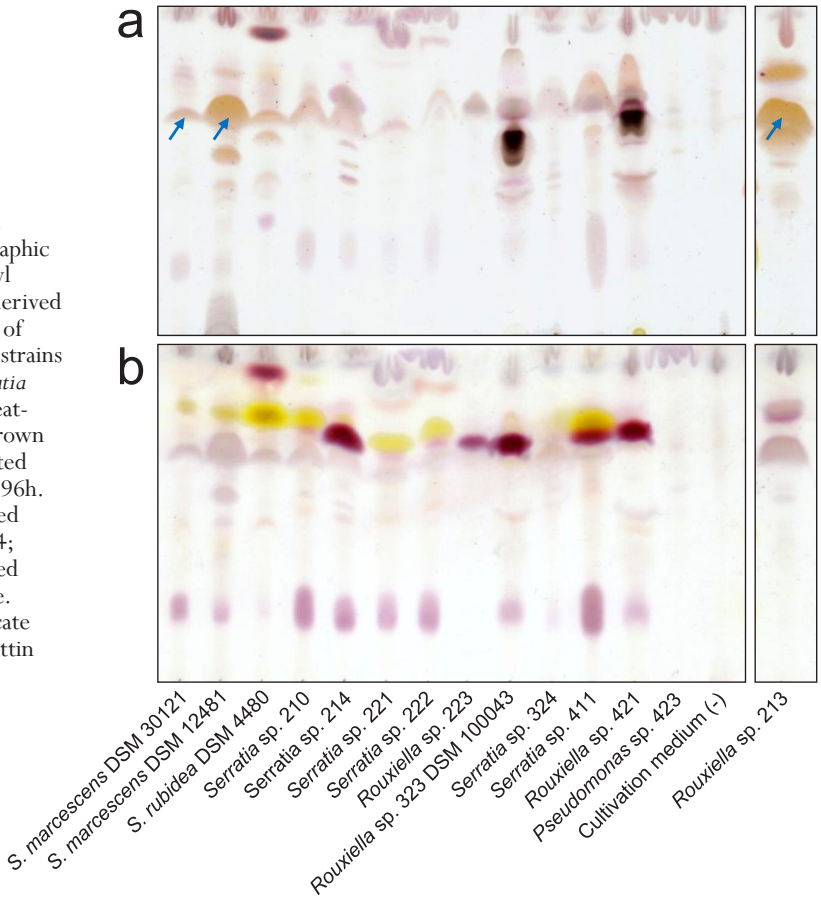

Figure 3.11: Thin layer chromatographic separation of ethyl acetate extracts derived from cultivations of *Serratia* spp. type strains and selected *Serratia* spp. associated peat-bog soil strains grown in LB supplemented with glycerol for 96h. (a) Extracts stained with 50% H2SO4; (b) extracts stained with anisaldehyde. Blue arrows indicate potential serrawettin W1

of biosurfactants was accurately assigned for *Pseudomonas* spp. 230 and 423 with closest affiliations to the type strains of *P. helmaticensis* and *P. brenneri* that are described to occur in soil samples (Ramírez-Bahena et al. 2014) and mineral water (Baida et al. 2001). Both have not yet been described within the production of biosurfactants.

The genus *Janthinobacterium*, closest related to the isolates 112A and 112C, is known for production of bio-active violacein, a purple pigment as well as for the production of different janthinocins (Johnson et al. 1990) and jagiricin, peptide lactones with antifungal and antibiotic properties (O"Sullivan et al. 1990). Other members of its order: *Burkholderiales,* such as *Burkholderia plantarii* are reported within the production of rhamnolipid, a glycolipid biosurfactant (Hörmann et al. 2010). The isolated strains,

Janthinobacterium spp. 112A and 112C did not produce the purple pigment violacein in the cultivation media used. However affiliation to its genus is likely, as the production of violacein is limited to an adequate amount of tryptophan or glycerol present in the cultivation medium and non-pigmented *Janthinobacterium* spp. have also been reported (Gillis and De Ley 2006). As a known producer of valuable secondary metabolites, and a relative to known biosurfactant producing strains, it is most likely, that some of the vast consortia of amphiphilic molecules produced by isolates *Janthinobacterium* spp. 112A and 112C in sunflower seed oil containing medium are surface-active, however separation and characterization of the numerous amounts of compounds produced might be tedious.

The genera of *Sarratia*, *Rouxiella* and some other enterobacteria were, due to high similarity in their 16S rRNA coding region difficult to be assigned by genetic approaches as typically reported for these strains (Le Fleche-Mateos et al. 2015; Spröer et al. 1999). Different *Serratia* spp. are known for the production of a diverse range of biosurfactants. The opportunistic human pathogen *S. marcescens* synthesizes three different cyclic lipopeptides named serrawettin (Matsuyama et al. 2011) but also glycolipids are reported to be produced by *S. marcescens* (Dusane et al. 2011) and *S. rubidea* (Matsuyama et al. 1990). The depsipeptide serrawettin W1, originally termed serratamolide is reported to exhibit antimicrobial, antitumor and plant protecting properties (Thies et al. 2014). Strain 323 could be assigned as a newly reported genus *Rouxiella* (Le Fleche-Mateos et al. 2015) as it can be distinguished to relative *Serratia* spp. by limitation in growth temperature. Further its inability to grow at 37° C makes this strain unlikely to be pathogenic. A non pathogenic biosurfactant producing strain would, in opposite to several biosurfactant producing *Serratia* spp. markedly reduce productions costs in industrial scale. The comparison within serrawettin and rubiwettin production of *Serratia* afilliated and relative strains isolated revealed a potential novel serrawettin producer strain: *Rouxiella* sp. 213. TLC comparison of the products extracted remains hypothetical and further analysis needs to be conducted in order to clearly identify the compounds. Same applies to strains that show TLC bands at R_fs close to serrawettin (Figure 3.11). The production of rubiwettin could not be identified within isolated strains nor within the described producer *S. rubidea* DSM 4480.

Within screening results for biosurfactants, solely *Rouxiella sp*. 411 performed poorly

in screening experiments of the more detailed screening (Figure 3.9) which stands in contrast to the preliminary results shown in Table 3.3. For all other strains tested, results of the methods applied fall within both cultivation devices used, glass vials and shake flasks. A direct link between the screening procedures applied, e.g. the reduction in surface tension and emulsification capability cannot generally be drawn. *Pseudomonas* sp. 230 and *Rouxiella* sp. 323 are examples that show severe reduction in the surface tension of YM and SBH-G cultivation, but none to poor emulsification capabilities. This stands in accordance with literature (Uzoigwe et al. 2015) as good bioemulsifier can, but must not act surface-active and vice versa. Emuslification of oil based mineral medium was only achieved in cultivation supernatants of *Janthinobacterium* sp. 112A, most likely due to residual oil acting demulsifying.

An assignment of hydrophilic compounds detected in TLC to the performance of the strains in the other screening methodologies can be drawn for the oil based cultivations, as four out of the five strains that lower the surface tension in this media show extended compounds in TLC. For *Pseudomonas sp* 230 this can be narrowed down to three main products stained (Figure 3.10c). The number of compounds detected in cultivations made in SBH-G and YM medium is too numerous to conclude to surface activity. The yellow pigment produced by *Serratia* sp. 411 (Figure 3.10a) can be excluded as a potential biosurfactant.

Conclusion

The raised bog peat sampled contains an astonishingly high percentage of prokaryotes that produce surface-active and emulsifying compounds. Therefore it appears likely, that the production of biosurfactants and bioemulsifiers is environmentally and thus nutrient driven. The extraordinary habitat of peat-lands can be added as a new and promising source for the isolation of biosurfactant producing microorganisms.

The producer strains isolated were mainly identified to be closely affiliated to genera of *Serratia*, *Rouxiella*, *Pseudomonas* and *Janthinobacterium*. Some of the newly detected producer strains are relatives of known biosurfactant producers, such as the serrawettin producing *Serratia marcescens*, the glycolipid producing *Serratia rubidea* and several

known glycolipid and liopopeptide producing *Pseudomonas* spp. Screening results and comparative studies suggest *Rouxiella* sp. 213 to belong to a new serrawettin producing genus, however the structure of the compound has not yet been verified. Besides *Rouxiella* as a new surfactant producing genera, close relatives that have not yet been described as biosurfactant producing strains were found among *Serratia* spp. and *Pseudomonas* spp.. The large amount of amphiphilic molecules produced in different complex, glucose and seed oil containing media suggest a wide range of potential novel biosurfactants that remain to be characterized.

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Glycolipids produced by *Rouxiella* sp. 323 DSM 100043 and isolation of the biosurfactants via foam-fractionation

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JK has concepted and written this study, performed experiments, collected and interpretated the relevant data used. SH and AV have sampled the soil and contributed to screening and discussion within their Master Thesis (Hansen 2013; Völp 2013). CMG and BL contributed within aquiring and interpreation of NMR data. FK, BK and GBW contributed within mass spectrometry. All authors have fruitfully discussed content and structure of this study.

3c Glycolipids produced by *Rouxiella* sp. DSM 100043 and isolation of the biosurfactants via foam-fractionation

Introduction

A great variety of surfactants occur as metabolites synthesized by various microorganisms. Their structures are versatile and many different hydrophilic and hydrophobic moieties are described in literature (Hausmann and Syldatk 2014; Kügler et al. 2015). Within glycolipids, the hydrophilic moieties usually are composed of one or more sugar components, mainly present in their ring form. Glucose, sophorose, rhamnose, mannose and the disaccharid trehalose are best studied as hydrophilic moieties of glycolipid biosurfactants. Linked to these are a variety of different lipophilic moieties, largely described are fatty acids of variable length.

Microbial surfactants can, besides differences in their structural composition, as well be different in their physiological characteristics such as foaming. The formation of foam that builds up pressure in bioreactors is a challenge within the production of biosurfactants. Foam-fractionation, the separation of foam via an outlet of the reactor during the cultivation process has been successfully applied within the production of biosurfactants (Chen et al. 2006; Davis et al. 2001; Willenbacher et al. 2014) and not only hinders the increase of pressure in the reactor but also displays a first step of

product removal.

The strain *Rouxiella* sp. DSM 100043 is an isolate of the upper layer of a pristine raised bog, a habitat rich in carbon sources such as humic substances but deficient in other nutrients. The production of surface active or emulsifying compounds as secondary metabolites and the release of enzymes envolved may serve as a tool for an accession of nutrients, swarming, or defense of habitat and displays an adaption to living conditions in the acrotelm of peat-bog areas.

This study characterises amphiphiles produced by *Rouxiella* sp. DSM 100043, describes a production method using glycerol as carbon source as well as the extraction and purification of glycolipids from fractionated foam. The utilization of two dimensional NMR septroscopy is used for structural characterization of the glycolipids produced.

Methods

Microorganism

Peat was sampled in a raised bog of the northern Black Forest near Kaltenbronn, Germany (48.719°N, 8.459°E) at a depth of approximately 2-5 cm and stored at 20 °C. The soil was resuspended in sterile demineralised water and dilutions were streaked onto yeast-malt (YM) agar plates containing per liter: 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose and 20 g agar set to a pH of 7.0 using NaOH and/or H3PO4. Agar plates were incubated at 20 °C until colonies were clearly visible. Morphological different colony forming units (cfu) were picked with a sterile tip and streaked out onto fresh YM agar plates, repeatedly grown and picked for at least three times or until visible purity. Isolates were stored in cryo-stocks at -80 °C in YM containing 15 % glycerol and used as inoculate for all experiments. The isolated strain *Rouxiella* sp. 323 was submitted to the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and assigned as *Rouxiella* sp. DSM 100043.

Gram characteristics of the strain was determined by mixing a drop of 3 % potassium hydroxide with a loop of a single colony on a glass surface by stirring with a needle for

1 min. Bacteria were determined to be gram negative when the formation of threads was observed after lifting the stirring device.

Phylogenetic affiliation was determined genetically. 10 ml YM overnight cultures from a single colony of *Rouxiella* sp. DSM 100043 was centrifuged for 20 min at 4643 x g and 4 °C. The genomic DNA of each cell pellet was extracted using PureLink Genomic DNA Mini Kit (Life Technologies GmbH, Darmstadt, Germany) according to the supplier's manual. Genomic DNA (gDNA) was eluted in nuclease free water (Carl Roth GmbH, Karlsruhe, Germany). The 16S rRNA decoding DNA sequence was amplified by polymerase chain reaction (PCR) containing 5 µl of 1:10 diluted DNA template in nuclease free water, 0.75 U polymerase (HotStar TaqTM, Qiagen, Hilden, Germany), 0.6 µL desoxyribonucleotide triphosphate mix (dNTPs; 10 mM of each dNTP: Qiagen, Hilden, Germany), 1 µl of oligonucleotide 27F (100 pmol µl 1; 5' AGAGTTTGATCCTGGCTCAG 3') and 1 µl of oligonucleotide 1385R (100 pmol µl 1; 5' CGGTGTGTRCAAGGCCC 3' whereas R is A or G) (both Biomers, Ulm, Germany), and 3 µl of a PCR reaction buffer (10 x, Qiagen, Hilden, Germany) filled up to a total volume of 25 µl per sample with nuclease free water. Reaction took place in a thermocycler (Master Cycler Gradient, Eppendorf, Hamburg, Germany) programmed as follows: single activation step 15 min at 95 °C followed of 30 cycles comprising: (1) initial denaturation 1 min at 94 °C, (2) annealing 1 min at 55 °C, (3) elongation 1 min at 72 °C, followed by a terminating elongation step for 10 min at 72 °C with a subsequent storage temperature of 4 °C. Amplification of DNA was checked by gel electrophoresis. 5 µl of each sample was mixed with 1 µl loading dye and loaded onto a roti-safe (Carl Roth GmbH, Karlsruhe, Germany) stained 1 % agarose gel in tris base boric acid EDTA buffer (TBE; containing per liter 10.8 g tris base, 5.5 g boric acid, 20 mM EDTA) and migrated for approximately 1 h at 90V. For visualizing DNA fragments the gel was irradiated with 312 nm UV light and the size of the amplified ~1.4 kb sized fragments was compared with a co migrated 0.1 10 kb DNA ladder (QuickLoad 2 log, New England Biolabs, Frankfurt/Main, Germany). DNA fragments were sequenced (GATC, Konstanz, Germany) from both sides, submerged and after exclusion of each ends flanking 30 base pairs compared with the 16S rRNA sequences of culturable species using the National Center for Biotechnology Information MEGABLAST tool and databse (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Mismatches to the sequences of the most similar type strains were checked manually in the sequence spectrograms.

Sequences were checked for chimeras using DECIPHER search tool (Wright et al. 2012). 16S rRNA sequence of *Rouxiella* sp. DSM 100043.was submitted to NCBI GenBank [GenBank: KP642161].

Production

For the production of glycolipids a glycerol basal media (GBM3) adapted from Roldán-Carrillo et al. (2011) was used containing per litre: 3.0 g $(NH_4)_2SO_4$, 50 g glycerol, 1.1 g KCl, 1.1 g NaCl, 1.0 g $MgSO_4$, 2.33 mg $FeSO_4$ • 7 H2O and a phosphate buffer of 4.4 g K_2HPO_4, 3.4 g KH_2PO_4 for shake flask experiment respectively 1.1 g K_2HPO_4, 0.85 g KH_2PO_4 for bioreactor cultivation. The medium was enriched with 5 ml of a trace element solution containing per litre 0.29 g $ZnSO_4$ • 7 H2O, 0.19 g $CaCl_2$ • 2 H_2O, 0.25 g $CUSO_4$ • 5 H_2O, 0.17 g $MnSO_4$ • 7 H_2O. 20 µl of *Rouxiella* sp. DSM 100043 cryo-stock were inoculated in 20 ml GBM3 (100 ml baffled conical flasks) and cells were grown over night at 30 °C and 130 rpm then transferred into 100 ml GBM3 (1000 ml baffled conical flasks) and again grown for approximately 35 h until an optical density at λ=600 nm (OD600) of 6 was reached.

For batch fermentation stirred 2.5 l bench-scale bioreactors (Minifors, Infors, Bottmingen, Switzerland) were used, each equipped with a double foam trap consisting of switchable single use bags for foam fractionation (Figure 3.12). Bioreactors were filled with GBM3 media and inoculated from shake flasks to a starting optical density of 0.1 (OD600) in an operating volume of 1 l. The processes were run for approximately 75h at a controlled temperature of 30 °C. Physiological activities were monitored by internal pO2 electrodes and pH 7.0 was controlled and adjusted by internal pH-electrodes using 4M H_3PO_4 and 4M NaOH. Airflow of 0.1 vvm was kept constant throughout the process; dissolved oxygen was maintained between 8 and 18 % by varying stirring speeds between 300 and 1,200 rpm. The fermentation process was controlled and recorded using a bioprocess software (Iris 5, Infors, Bottmingen, Switzerland). Foam formed during the cultivation process was collected via the exhaust cooler in single use bags that were cooled on ice to prevent further growth of foamed microorganisms.

During the fermentation processes, 12 ml culture samples were taken as duplicates at different time points for the analysis of growth characteristics. Optical density

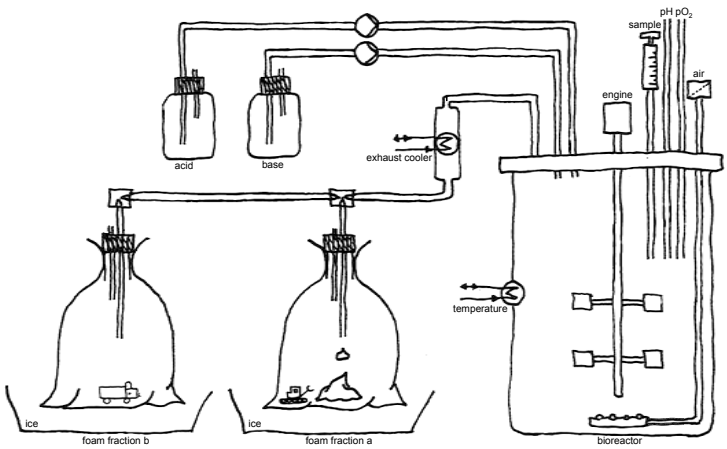

Figure 3.12: Scheme of the bench-scale bioreactor with switchable double foam trap devices used for the production and fractionation of glycolipids produced by *Rouxiella* sp. DSM 100043

was determined using concentration dependent dilutions with 0.9 % (w/v) NaCl. For gravimetric determination of dry cell mass 10 ml of the culture broth from each sampling point was transferred into dry-weighed 15 ml sampling tubes and centrifuged for 20 min at 4643 x g and 4 °C. The supernatant of each sample was transferred into a new tube and stored at -20 °C prior to the determination of ammonia ions, glycerol content and surface tension. The remaining cell pellet was washed with 5 ml 0.9 % (w/v) NaCl followed by centrifugation (10 min at 4643 x g and 4 °C), decanting and drying to constant weight in a drying closet at 100 °C.

Foam bags were replaced five times during the process, liquid and foam from each bag was wringed into weighed 50 ml sampling tubes and centrifuged at 4643 x g and 4 °C for 20 min or until foam was fluidified. Spun down cell masses were carefully solubilised and OD600 was measured with adequate dilutions in 0.9 % (w/v) NaCl. Samples were again centrifuged (14,000 rpm, 20 min, 4 °C), the supernatant transferred into a fresh tube and stored at -20 °C. For gravimetric determination of dry cell mass in the foam fractions, the remaining cell pellets were washed and dried until constant weight as described.

The ammonium ion concentration in the supernatant was determined by an ammonia assay using photometric quantification (Spectroquant 109713, Merck,

Darmstadt, Germany) downscaled to a fifth of the volumes listed in the supplier's manual, spectrophotometric measurements were conducted in a microtiter plate and concentrations were determined using an ammonia ion standard curve.
Glycerol content in the supernatant was determined using a nicotinamide adenine dinucleotide (NAD^+) coupled enzymatic test kit with photometric quantification (Boehringer-Mannheim/R-Biopharm, Darmstadt, Germany) by downscaling to a twentieth proportion of the volumes listed in the supplier's manual and quantification via glycerol standard curves in a microtiter plate.
Dry cell mass, glycerol and ammonia ion data points were fitted (SigmaPlot, version 12.5, Systat Software, Inc., Washington, USA) using a logistic model with four parameters (Zwietering et al. 1990). All fermentation results are plotted as mean values of two fermentation processes with each data point measured as duplicate for dry cell mass and triplicate for glycerol content and ammonia ion concentration.
Alteration in the surface tension of samples taken from the fermentation supernatant as well as of foam trap samples were monitored against air at room temperature using the Du Noüy (1919) ring method on a Tensiometer (Lauda TD1, Lauda-Königshofen, Germany) according to the supplier's manual. Trend of the surface tension values in the bioreactor was fitted using a linear equation.

Extraction and isolation of glycolipids from foam

Supernatants from the fluidifized foam were acidified until neutral pH using H_3PO_4 and subsequently extracted twice using 1.25 volumes of ethyl acetate (v/v) in 12 ml screw cap glass vials with subsequent centrifugation (10 min at 4643 x g, 4 °C). The combined organic phases were concentrated using a rotary evaporator (Heidolph Laborota 4000, Schwabach, Germany) at 40 °C and 240 mbar followed by vacuum concentration at 40 °C, 2000 rpm and 50 mbar (ScanSpeed MiniVac Evaporator, Saur, Reutlingen, Germany) to gain crude extract. Qualitative detection of the glycolipids was performed by thin layer chromatography (TLC) using 60 Å silica TLC plates (Alugram Xtra SIL G, Macharey-Nagel, Düren, Germany) as stationary phase and a mobile phase of isopropyl acetate / methanol / acetic acid (100:10:1 v/v/v). Glycolipids and fatty acids were detected by dipping the TLC plate into 10 % (v/v) H_2SO_4 and development under 180 °C air stream for 4-5 min.

The crude extract was dissolved in two times 20 ml 10 % (v/v) methanol in ultrapure water and further purified for structural analysis by medium-pressure-liquid-chromatography (MPLC; SepacoreX50, Büchi, Flawil, Switzerland) using prepacked 40-63 µm particle size reverse phase C18ec columns (RP18ec; 150 mm column length, 12 mm column diameter and 17 ml bed volume; Büchi, Flawil, Switzerland) with a ultrapure H_2O / methanol gradient solvent system for 90 min at a flow rate of 10 ml min^{-1} (gradient: 15 min 100 %-100 % H2O; 45 min 100 %-0 % H_2O; 30 min 0 %-0 % H_2O). The eluate was collected in 10 ml fractions. From each separation fractions 60-61, 64-65 and 67-69 were combined and the solvent was evaporated, the sample lyophilized (Beta 2-16, Martin Christ GmbH, Osterode, Germany) and used for structural analysis of the fatty acids. Fractions containing the glycolipids (63-65) were combined and again purified to remove residual fatty acids before structural analysis.

To further elucidate the sugarsystems, the fraction was dissolved in 20 ml isopropyl acetate / methanol (1:1 v/v) and further purified using 40-63 µm particle size silica stationary phase with 60 Å pore size (150 mm column length, 12 mm column diameter and 17 ml bed volume; Büchi, Flawil, Switzerland) and manually eluted isocratically with isopropyl acetate / methanol (24/1 v/v). The eluate was collected in 10 ml fractions, fraction 2-3 contained fatty acids, the other fractions were combined to samples 63-65 A (fractions 4-6), 63-65 B (fraction 7), 63-65 C (fractions 8-10), 63-65 D (fractions 11-16) and 63-65 E (fractions 17-23), the solvent evaporated and the samples again lyophilized.

Structural analysis

For nuclear magnetic resonance (NMR) spectroscopy fractions containing the fatty acids (60-61 and 67-69) as well the glycolipids (64-65 and subfraction 63-65 E) were dissolved in 0.6 ml $CDCl_3$ / CD_3OD (both 7:3 v/v) (Sigma Aldrich; Germany). One dimensional ^1H NMR spectroscopy and two dimensional ^1H ^1H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), nuclear Overhauser enhancement spectroscopy (NOESY), ^1H ^{13}C heteronuclear single quantum coherence spectroscopy (HSQC) and heteronuclear multiple-bond correlation spectroscopy (HMBC) were recorded on a Bruker AVANCE II+ 600 MHz spectrometer (Bruker AG, Rheinstetten,

Germany) equipped with a BBI probe head. Spectra were analyzed with Topspin 3.2 (Bruker AG) and Spinworks 3.1.8 software (Marat, University of Manitoba, USA). Intensities were measured from a one dimensional 1H spectrum acquired with a single scan. Chemical shifts are referenced to the ^{1}H and ^{13}C resonance of the residual $CHCl_3$ signal.

Mass determination of subfractions 63-65 A to 63-65 E was performed using ESI-Q-ToF (Q-Star Pulsar, AB SCIEX, Darmstadt, Germany). Small amounts of dried fractions were dissolved in methanol/H_2O/acetic acid (v/v/v 500:500:1) containing 5 ppm LiCl. Samples were continuously infused via a syringe pump at a flow of 10 µl min^{-1}. The system was operated in the positive mode with a heater temperature of 300 C. The spray tip voltage was set to 5000 V, the declustering potential was 30 V and the focusing potential was 60 V. The Nebulizer gas and the curtain gas was nitrogen 5.0. Spectra were recorded in in a mass range from m/z 50 to m/z 1200 in the activated "enhance all" mode at an accumulation time of 1 s. The ESI-Q-ToF was calibrated using a calibration standard (M600, Applied Biosystems) and the measuring accuracy was determined to be ±0.05.

Results

The strain *Rouxiella* sp. DSM 100043 has been chosen for the production of glycolipids due to results in screening experiments, novelty of the genera in terms of biosurfactant production and the product portfolio revealed using functional staining in TLC. According to its 16S rRNA coding sequence strain *Rouxiella* sp. DSM 100043 was, besides the genus *Rouxiella* in close proximity to a range of other enterobacteria such as *Serratia*, *Rahnella*, *Yersinia*, *Ewingella* and *Hafnia* spp.. The isolated strain did not show growth at 37 °C, as also reported for its closest 16S rRNA relative (Le Fleche-Mateos et al. 2015) and was therefore assigned to the genus *Rouxiella* and submitted as *Rouxiella* sp. DSM 100043.

Glycolipids of strain *Rouxiella* sp. DSM 100043 were produced as duplicates in 1 litre bench scale bioreactors and fractionated in foam traps during the cultivation. The fermentation processes took place under nitrogen limiting conditions in a mineral medium with glycerol as carbon source; glycolipids were extracted from fluidifized

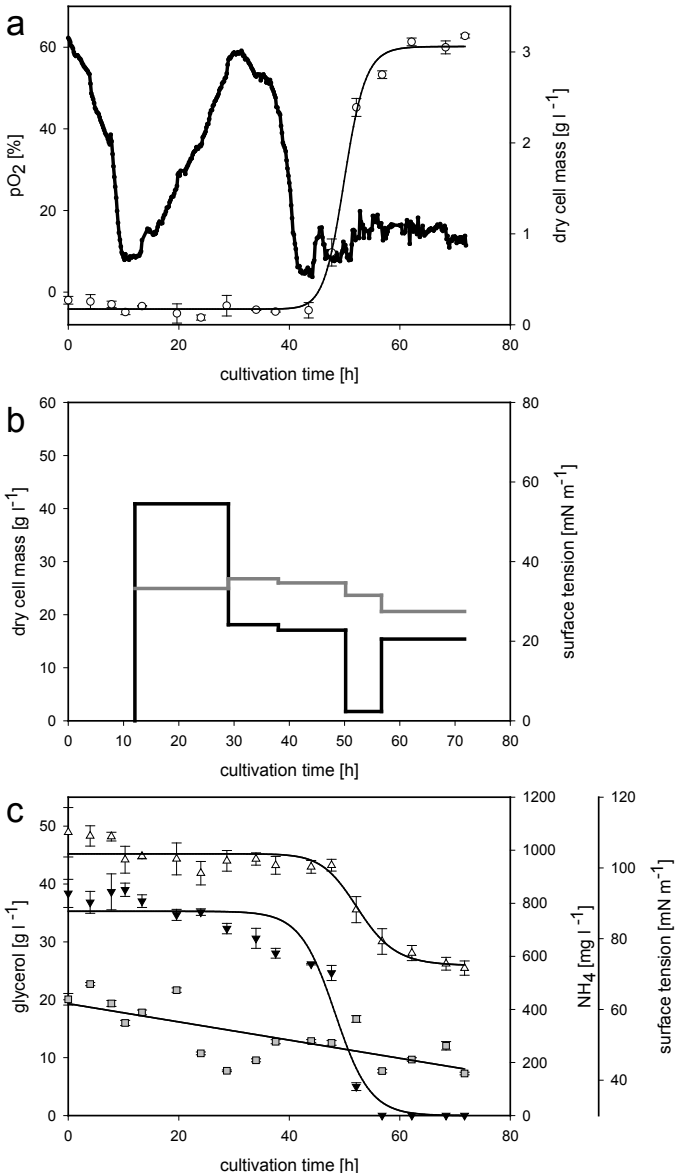

Figure 3.13: Growth parameters of *Rouxiella* sp. DSM 100043 during biosurfactant production. Time course of (a) dissolved oxygen (solid line) and dry cell mass (open circle) in the bioreactor system and (b) of dry cell mass (black line) and surface tension (grey line) examined from fractionated foam. (c) Time course of the surface tension (grey square) in the reactor as well as depletion of glycerol (open triangle) and ammonium (filled inverted triangle).

foam trapped in the foam bags. Figure 3.13a and 3.13c show physiological conditions present within the bioreactor system. Remote consumption of carbon and nitrogen within the first 10 hours of cultivation, as well as a decrease of dissolved oxygen indicates growth of *Rouxiella* sp. in the beginning of the fermentation. An increase of

optical density and dry cell mass in the bioreactor (Figure 3.13a) was not observed within this time period. The formation of foam started about two hours after inoculation, filling up the reactor void and exiting via the exhaust gas cooler until being captured in the foam bag traps after 10 hours of cultivation (Figure 3.13b). Between 10 and 40 hours of cultivation, nutrients were consumed steadily and biomass formed continuously led into the foam traps where a concentration of up to 40 g l^{-1} dry cell mass was reached. Between hours 40 to 60, growth of *Rouxiella* sp. approaches an exponential phase, so is the decrease of carbon and nitrogen concentrations in the cultivation media (Figure 3.13c). With low pO_2 values reached after 60 hours of cultivation an increase of stirring speed was regulated and cells accumulate in the cultivation media (Figure 3.13a). The foaming off of cells decreases during that period

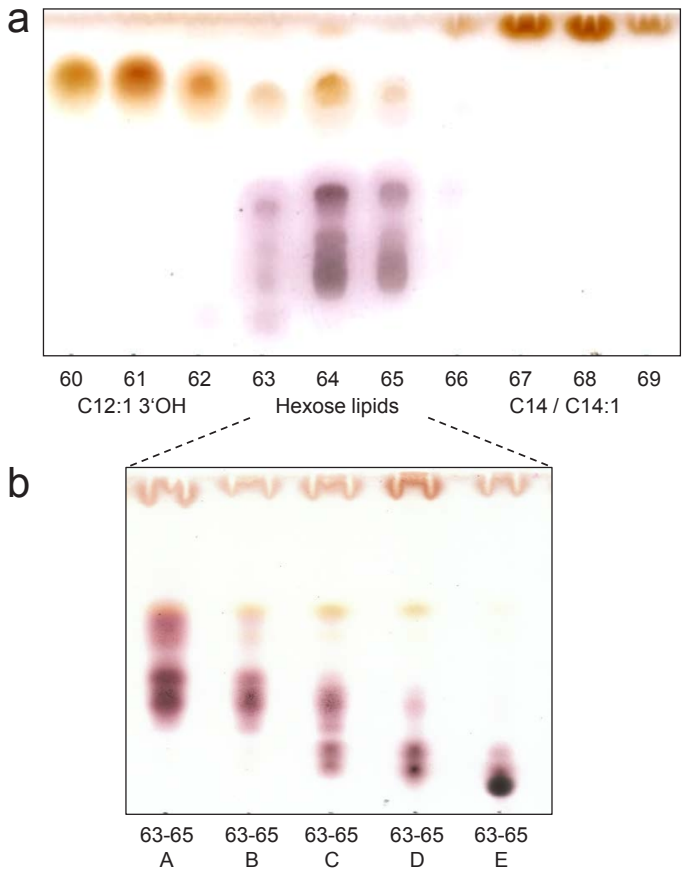

Figure 3.14: Thin layer chromatography (TLC) of glycolipids extracted and purified from foam fractionated during cultivation. TLC plates are stained with sulphuric acid. (a) 3' hydroxyl lauroleic acid present in fractions 60-62, glycolipids present in fractions 63-65 and myristic as well as myristoleic acids present in fractions 67-69. (b) TLC of further purified glycolipids from fraction 63-65 resulting in subfractions 63-65 A to 63-65 E with the most hydrophilic glycolipids in 63-65 E containing talose as carbohydrate moieties.

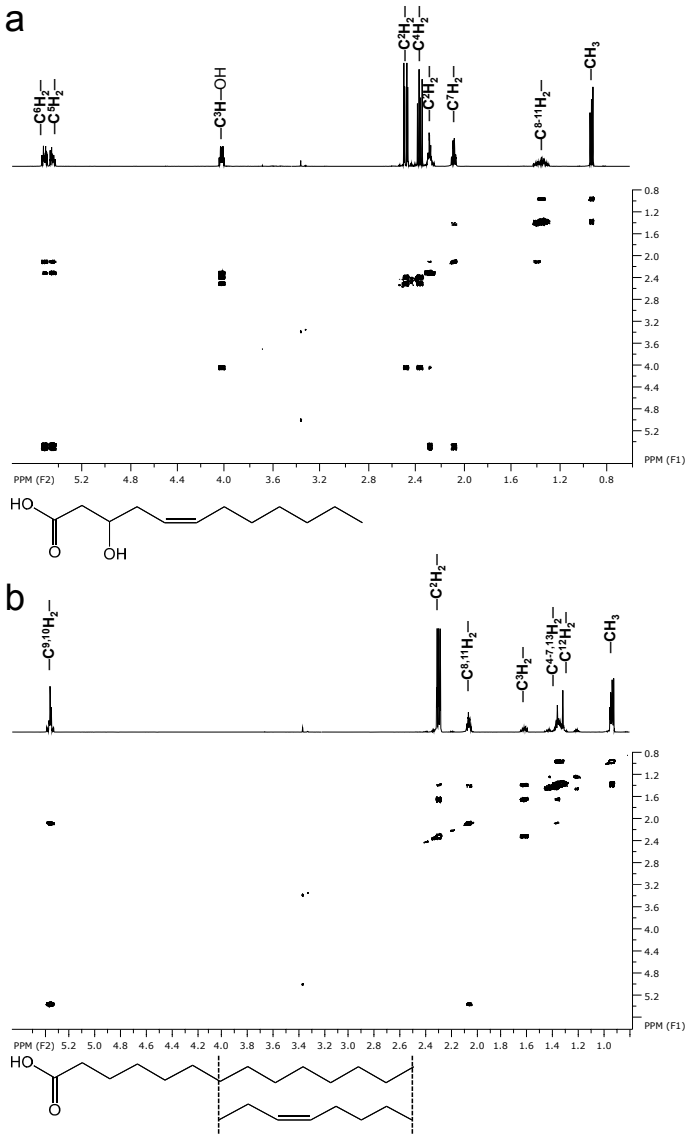

Figure 3.15: Assigned spectra of two dimensional $^{1}H/^{1}H$ correlated spectroscopy (COSY).
(a) Derived from fractions 60-61 elucidated as 3' hydroxy lauroleic acid and (b) derived from fractions 67-69 elucidated as a mixture of myristic and myristoleic acids. Both are shown as molecular structures.

(Figure 3.13c). Surface tensions in supernatants from the reactor medium are fluctuating with a decreasing trend indicated by fitting of the data but remain above 40 mN m^{-1} throughout the process (Figure 3.13c). Surface tension values steadily remained below 28 mN m^{-1} in fluidified foam collected from all traps (Figure 3.13b).

A total of 145 ml fluidified foam was collected per batch cultivation with a total

3' OH C12:1 FA	C Shift [ppm]	H Shift [ppm]	Multiplicity	Coupling [Hz]
O=C^1–OH	175.95			
–C^2H_2–	42.39	2.46, 2.24	dd,dd	15.4, 8.25, 4.6
–C^3H–OH	69.6	4.00	m	
–C^4H_2–	35.8	2.26	m	
–C^5H=	125.7	5.43	m	
–C^6H=	133.5	5.50	m	
–C^7H_2–	28.3	2.05	q	7.1
–C^8H_2–	30.7	1.37	m	
–C^9H_2–	30.1	1.34	m	
–$C^{10}H_2$–	32.9	1.31	m	
–$C^{11}H_2$–	23.7	1.33	m	
–$C^{12}H_3$	14.4	0.90	t	6.8
C14 / C14:1 FA	**C Shift [ppm]**	**H Shift [ppm]**	**Multiplicity**	**Coupling [Hz]**
O=C^1–OH	177.8			
–C^2H_2–	35.1	2.27	t	7.4
–C^3H_2–	26.1	1.59	m	
–C^4H_2–	30.26	1.33		
–C^5H_2–	30.6	1.33		
–C^6H_2–	30.3	1.33		
–C^7H_2–	31.1	1.33	m	
–C^8H_2–	28.17	2.03	dd	12.3, 6.4
–C^9H_2– / –C^9H=	130.9	5.34		
–$C^{10}H_2$– / –$C^{10}H$=	130.9	5.34		
–$C^{11}H_2$–	28.17	2.03	dd	12.3, 6.4
–$C^{12}H_2$–	33.1	1.29	m	
–$C^{13}H_2$–	23.8	1.32	m	
–$C^{14}H_3$	14.6	0.89	t	6.6

Table 3.4: NMR data of fatty acids moieties. Chemical shifts of carbon and hydrogen nuclei, multiplicity of the peak observed and its coupling constant of 3' hydroxy lauroleic acid from fractions 60-61 (3'OH C12:1 FA) and potential myristic/ myristoleic acid from farctions 67-69 (C14 / C14:1 FA).

FA, fatty acid;
d, doublet;
t, triplet;
q, quartett;
m, multiplet

cultivation volume of 1 l. Glycolipids as well as fatty acids were detected in the foam that with pH 9 had a relatively alkaline character. The pH was neutralized prior to the extraction and purification of the components. 119.2 mg l^{-1} crude extract was yielded per batch process after triple extraction of the fluidifized foam. TLC and subsequent staining of the extracts revealed the presence of two different fatty acid molecules as well as a mixture of glycolipids that varies within their retardation factors. Reverse-phase chromatographic separation of 238.4 mg extract from both fermentations allowed an isolation of the fatty acids and the glycolipids (Figure 3.14a). The total yield of the fractions after purification steps was 19.9 mg of fractions 60-61, 27.9 mg of fractions 64-65 and 34.9 mg of fractions 67-69. The fatty acids could be unambiguously elucidated from the pattern of ^{1}H COSY, ^{13}C HSQC and ^{13}C HMBC as 3' hydroxyl lauroleic acid for the more hydrophilic fractions 60-61. Fractions 67-69 most probably contained a mixture of myristic and myristoleic acids deduced from ^{1}H COSY (Figure 3.15 and Table 3.4), ^{13}C HSQC and ^{13}C HMBC NMR spectroscopy and the ratio of intensities for C^xH_2, $C^\omega H_3$, C^2H_2 and C^3H_2 in ^{1}H 1D NMR spectrum.

The mixture of glycolipids present in the combined fractions 64-65 (Figure 3.14a) contained as hydrophilic moieties at least four different systems assigned as sugar A, B, C

Table 3.5: NMR data of sugar moieties. Chemical shifts of carbon and hydrogen nuclei, multiplicity of the peak observed and its coupling constant from four different glucose lipids (sugar A, B, C and D) present in fraction 64-65, and two talose units present in subfraction 63-65 E. Values are given for the dominant sugar conformations of each sugar moiety.

d, doublet;
m, multiplet;
n.d., not determinable

Fraction 64-65 (sugar A)	C Shift [ppm]	H Shift [ppm]	Multiplicity	Coupling [Hz]
–C^1H–O–	91.2	5.33	n.d	< 1.5
–C^2H– (acylated C' 173.1)	73.3	4.80	dd	7.8
–C^3H– (acylated C' 173.5)	74.2	5.46	dd	7.8, 9.3
–C^4H–	69.9	3.613	dd	9.3, 9.6
–C^5H–	72.5	3.93	m	9.6, 11.9, 5
–C^{6a}H–	62.5	3.83	m	12.0, 2.6
–C^{6b}H–		3.76	m	12.0, 5.2
Fraction 64-65 (sugar B)	**C Shift [ppm]**	**H Shift [ppm]**	**Multiplicity**	**Coupling [Hz]**
–C^1H–O–	93.8	5.17	d	3.9
–C^2H–	72.1	3.55	n.d.	>7
–C^3H– (acylated C' 174.0)	72.3	5.23	n.d.	> 7
–C^4H–	73.6	3.51	n.d.	>7, >7
–C^5H–	70.4	4.09	dd	10.1
–C^{6a}H– (acylated C' 173.7)	64.8	4.39	m	n.d.
–C^{6b}H–		4.30	m	n.d.
Fraction 64-65 (sugar C)	**C Shift [ppm]**	**H Shift [ppm]**	**Multiplicity**	**Coupling [Hz]**
–C^1H–O–	96.1	4.75	d	7.8
–C^2H–	74.6	4.83	dd	7.8, > 9
–C^3H– (acylated C' 173.5)	77.1	5.11	dd	> 9, > 9
–C^4H–	69.7	3.62	dd	> 9
–C^5H–	77.7	3.42	overlap	n.d.
–C^{6a}H–	62.6	3.73	overlap	n.d.
–C^{6b}H–		3.89	overlap	n.d.
Fraction 64-65 (sugar D)	**C Shift [ppm]**	**H Shift [ppm]**	**Multiplicity**	**Coupling [Hz]**
–C^1H–O–	98.0	4.60	d	7.5
–C^2H–	74.3	3.33	dd	7.5, > 8
–C^3H– (acylated C' 173.7)	79.2	4.97	dd	> 9, > 9
–C^4H–	69.9	3.52		> 8
–C^5H–	75.0	3.60		n.d.
–C^{6a}H– (acylated C' 173.7)	64.8	4.26		n.d
–C^{6b}H–		4.44		n.d
Subfraction 63-65 E (talose)	**C Shift [ppm]**	**H Shift [ppm]**	**Multiplicity**	**Coupling [Hz]**
–C^1H–O–	102.1	4.92	d	4.0
–C^2H–	77.5	4.03	dd (overlap)	4, 4.4
–C^3H–	76.3	4.26	dd	4.4, 4.4
–C^4H–	77.1	4.03	dd (overlap)	
–C^5H–	70.6	3.86	m	7.3, 6.0, 3.7
–C^{6a}H–	63.2	3.63	dd	11.5, 6.0
–C^{6b}H–	63.2	3.76	dd	11.5, 3.7
Subfraction 63-65 E (acylated talose)	**C Shift [ppm]**	**H Shift [ppm]**	**Multiplicity**	**Coupling [Hz]**
–C^1H–O–	109.0	4.76		< 1
–C^2H–	79.7	4.05		< 2
–C^3H–	75.9	4.12		overlap
–C^4H–	81.2	4.13		overlap
–C^5H–	70.5	3.91	m	
–C^{6a}H–	63.8	3.68	dd	11.6, 6.0
–C^{6b}H–	63.8	3.82	dd	11.6, 3.3

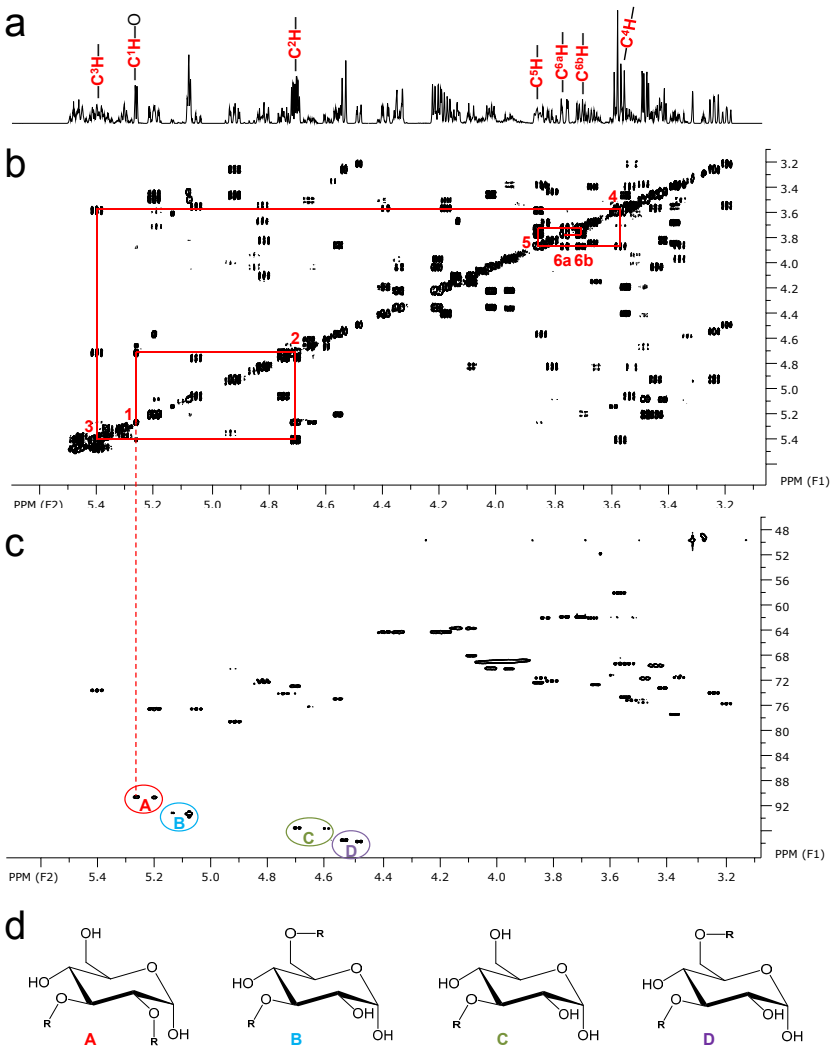

Figure 3.16: NMR spectra of *Rouxiella* sp. DSM 100043 glycolipids present in fractions 64-65. Close-up of the carbohydrate region is shown: (a) ^{1}H spectrum, (b) two dimensional $^{1}H/^{1}H$ COSY and (c) two dimensional $^{1}H/^{13}C$ HSQC spectrum. Anomeric C1 of the glucose moieties A to D in both, α and β configuration is shown in (c), molecular structures in (d). The more dominant form of sugar A, carrying acylation at C2 and C3 is exemplarily assigned in red in (a) ^{1}H spectrum and (b) as red lines in the COSY spectrum.

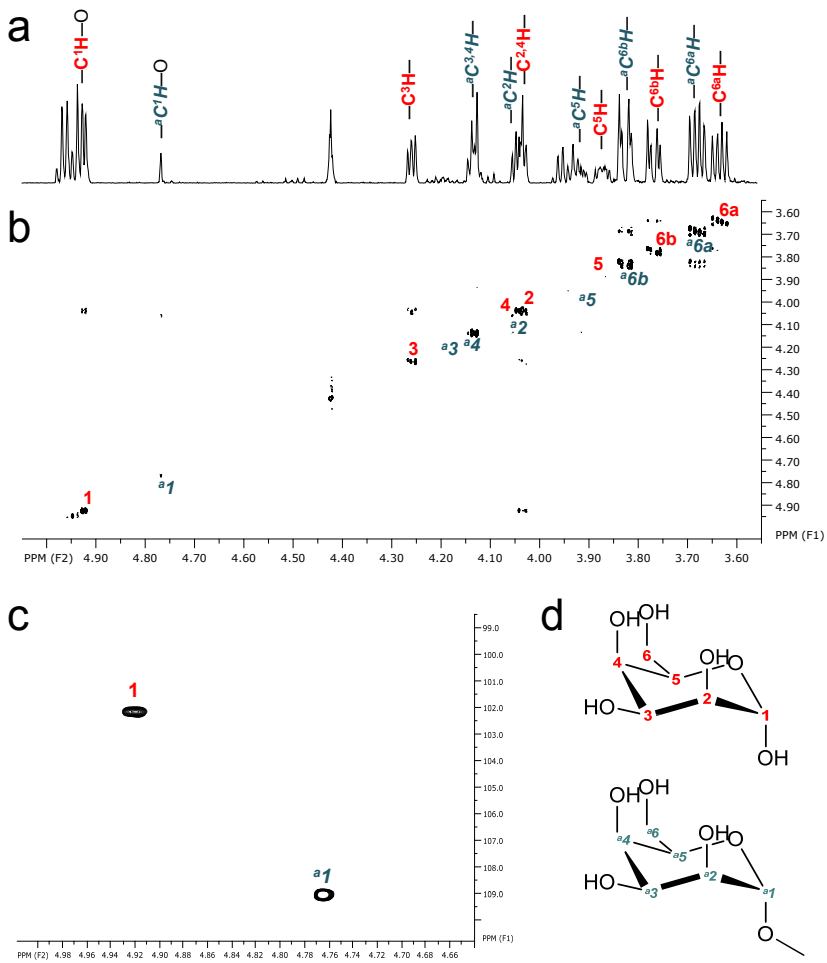

Figure 3.17: NMR spectra of glycolipids produced by *Rouxiella* sp. DSM 100043 present in subfraction 63-65 E.
Close-up of the carbohydrate region in (a) ^{1}H spectrum and (b) two dimensional $^{1}H/^{1}H$ COSY spectrum and assignment of signals for two sugar moieties C1-6 in red and aC1-6 in blue. (c) Two dimensional $^{1}H/^{13}C$ HSQC spectrum revealing two anomeric nuclei: C1 and aC1, the latter downshifted and indicating an acetylation. (d) Potentional molecular structures and assigned C atoms of talopyranose (red) and 1' acetyl talosepyranose (blue)

and D present in both diastereomers α and β. The sugar systems were assigned starting at the anomeric carbon atoms that are downshifted in $^{1}H/^{13}C$ two dimensional HSQC NMR spectra (Figure 3.16c). Neighboring carbon atoms of each sugar system were identified by tracing the spin systems in ^{1}H COSY and TOCSY spectra (Figure 3.16b and Appendices A-3c-5). Exchange cross-peaks between the corresponding anomers were determined using NOESY spectra and are also easily identified in the $^{1}H/^{13}C$ HSQC spectrum (Figure 3.16c). Due to their hydrogen coupling constants of mainly larger than ~ 7.5 or 9 Hz (Table 3.5), sugar ring protons could be determined to be all axial, which arranges the substituted hydroxy groups in an equatorial form and thus glucose is the dominant sugar in fractions 64-65. The glycolipids have different acylation patterns. The acylation position could be unambiguously determined from ^{13}C HMBC spectra. All sugars A-D are acylated at position C3 with additional acylation at C2 for sugar A and C6 for sugar B and sugar D (Table 3.5 and Figure 3.16d). Sugar system A in its more dominant form is exemplarily indicated with red lines in ^{1}H and COSY spectra of Figure 3.16a and Figure 3.16b. The lipophilic moiety could not completely be identified but carries variable double bonds.

Combined fractions 63-65 were further separated by silica column chromatography into subfractions 63-65 A to 63-65 E that resulted in a partial separation of the glycolipids (Figure 3.14b). The concentration in the most hydrophilic subfraction 63-65 E was high enough to conduct two dimensional NMR experiments that revealed two hexose forms as hydrophilic moiety shown by chemical shifts obtained from ^{1}H and two dimensional ^{1}H COSY (Figure 3.17a and Figure 3.17b). According to their coupling constants, these findings mostly refer to the presence of talopyranose (Snyder et al. 1989). The presence of 1' acetyl-talopyranose as the other sugar moiety in subfraction 63-65 E is indicated by a downshift of the talose C1 nuclei to 109 ppm indicating acetylation at C1 (Figure 3.17 and Table 3.5) in ^{13}C HMBC and HSQC NMR spectroscopy, respectively.

Mass spectrometric ESI-ToFMS analysis of lithium chloride supplemented subfractions 63-65 A to 63-65 E (see Appendices Table A-3c-1 and Figure A-3c-2 to Figure A-3c-4) revealed the presence of both Na^{+} and Li^{+} adduct ions of different m/z ratios that allowed concluding to the resulting neutral masses present (Appendices Table A-3c-1).

Discussion

Rouxiella can be assigned as a new surfactant producing genera. The glycolipid producing strain was identified to be closely related to other enterobacter, such as the genus *Serratia* that hold some known biosurfactant producing species. Examples are *Serratia marcescens* that produces different cyclic lipopeptides (Matsuyama et al. 2011) with antimicrobial, antitumor and plant protecting properties (Thies et al. 2014). Also glycolipids are reported to be produced by *S. marcescens* (Dusane et al. 2011) and *S. rubidea* (Matsuyama et al. 1990). *Rouxiella* sp. DSM 100043 could be distinguished to relative *Serratia* spp. by limitation in growth temperature. Its inability to grow above 37° C makes this strain unlikely to be pathogenic thus holding advantages as a potential industrial scale biosurfactant producer strain.

Several glycolipids were detected to be produced by *Rouxiella* sp. DSM 100043 in a mineral medium with glycerol as carbon source. The majority of the surface active amphiphiles produced expanded into foam that was formed during cultivation in a bioreactor as indicated by lower surface tension values present in the foam compared to the cultivation medium. Transition of the glycolipids into the foam makes foam-fractionation suitable as a tool for the isolation of the biosurfactants produced.

Reverse phase chromatographic purification of the foam derived extract revealed the presence of three main compound groups as represented in Figure 3.14a. Using H_2O and methanol as solvent system during reverse-phase chromatographic separation, elution of the products took place at high methanol concentrations. Interestingly in the order hydroxyl fatty acid - glycolipid - fatty acid with the most polar gylcolipids as second group and thus not according to their hydrophobicity. A delay of the hexose systems within the elution off the C18 reverse phase column must thus be due to other interactions than hydrophobic.

Two dimensional NMR spectroscopy measurements revealed the presence of 3' hydroxyl lauroleic acid, and potential myristic as well as myristoleic acid as free fatty acids (Figure 3.15 and Table 3.4). The fatty acids revealed in this study are known as common lipophilic moieties of biosurfactants, the shorter hydroxy fatty acid is present in both, glycolipids (Matsuyama et al. 1990) as well as lipopeptides (Matsuyama et al. 2011;Thies et al. 2014) of the relative enterobacteriaceae *Serratia* spp.. Glucose moieties with acylations at multiple carbon atoms of the sugar ring are observed (Table 3.5).

It could not be further determined whether an ester or ether bond is present at the acylation site. Mass spectrometric measurements (Appendices Table A-3c-1 and Figure A-3c-2 to Figure A-3c-4) hint to the presence of various double bonds that corroborates to the results observed from NMR experiments. Hydrophilic moieties of subfraction 63-65 E revealed the presence of two forms of talopyranose presenting a different hydrophilic moiety to the other glucose lipids present (Figure 3.17). Particular for the glycolipids is the absence of an acylation at the anomeric carbon C1 that is usually present in glycolipids (Hausmann and Syldatk 2014; Kügler et al. 2015). It remains unclear whether this absence is a unique property of the glycolipids revealed or due to hydrolysis caused during postprocessing of the glycolipids.

Concluding remarks

The production of microbial surfactants as extracellular metabolites is a common phenomena and due to the properties they inherit biosurfactants become increasingly interesting for divers applications. This thesis is dedicated to reveal not yet described amphiphilic structures of microbial origin. It emphasizes to seek them in two different areas: in the class *Actinobacteria*, known to encode for numerous secondary metabolites and in soils exposed to uncommon carbon sources, namely Black Forest raised peat bog and seal carcass exposed Antarctic soils.

The wide diversity of surfactants, derived from various actinomycetes is reflected in detail in chapter 2a. Information about the different properties and structural characteristics are used to establish and describe methods that are suitable to screen numerous microorganisms in relatively short time and with little complexity. Chapter 1a refers to the methods established and they are in detail tested and applied in screening approaches of microorganisms isolated from Antarctic seal fat exposed soils (chapter 3a) and raised bog peat (chapter 3b). Multiple production procedures of microbial surfactants were established and operated in chapters 2b, 2c, 3a and 3c for the production of different biosurfactants in quantities large enough to apply diverse purification procededures necessary for structural elucidations. Staining of functional groups as well as tandem mass spectrometry, nuclear magnetic resonance (NMR) and fourier transformed infrared (FTIR) spectroscopy as methods for the structural characterization of trehalose lipids and aromatic biosurfactants from the actinomycetes *Tsukamurella spumae* and *T. pseudospumae* as well as glucose and talose containing glycolipids produced by the peat isolate *Rouxiella* sp. DSM 100043 are applied. The results presented in this thesis include several microorganisms that are for the first

time described to produce biosurfactants. Further not yet known surfactant structures are unveiled.

The results obtained within examinations of the screenining habitats feature most astonishing insights. The sceening sites raised bog and seal carcass exposed soil, that were chosen in this study revealed the presence of numerous producer strains of amphiphilic compounds and much higher percentages of producers than expected. It thus can be assumed, that the production of surfactants by microorganisms seems to be omnipresent and not exclusive to certain genera. The bottleneck within the description of a novel biosurfactant turned out to be the yielding of enough quantities for the extensive purification procedures necessary and the complexity of structural characterization rather than the finding of suitable organisms for its production.

The description of novel producer strains and the surfactant structures revealed in this study pave the way for several research projects that can be established based on the knowledge acquired. This includes structure determinations of many more secondary metabolites either from strains isolated in this study (chapter 3a and 3b) or from one of the numerous strains that are reported to produce, but whose surfactant structures remain to be identified (chapter 2a).

Many surface active compounds show interesting properties as biotechnological products or additives, but often application based studies are lacking mostly due to low quantity producing strains. An optimization of production procedures as well as an establishement of methods for the quantification of the surfactants described can lead to high yields and thus to advanced knowledge about potential applications of the surfactant. A metabolomic approach can further upgrade production yields and flux analysis could identify enzymes involved in the synthesis as well as their genetic regulation. Information that are also required for the generation of heterologous production strains of the products revealed.

Amelioration of biosurfactant production yields is the main requesite for extensive testing and thus for potential applications of a novel compound. Striving toward a functional implementation of novel findings is a guarantee for success in white biotechnology and negates the efforts made with regards to production, purification and elucidation.

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Appendices

2b Trehalose lipid biosurfactants produced by the actinomycetes *Tsukmurella spumae* and *T.pseudospum*ae

A-2a-1

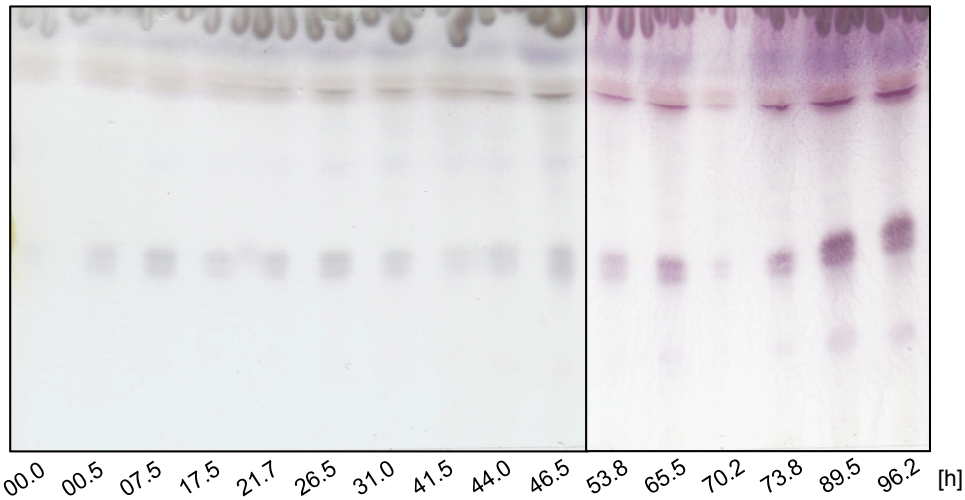

Figure A-2a-1: Ethyl acetate crude extracts of 800 µl fermentation broth before (00.0 h) and after inoculation (00.5 h – 96.2 h) of a bioreactor with *Tsukamurella spumae* DSM44113. TL A and TL B, stained as purple double spots were already present in the preculture and their concentration increased throughout the process. Results are shown for one bioreactor exemplarily

A-2a-2

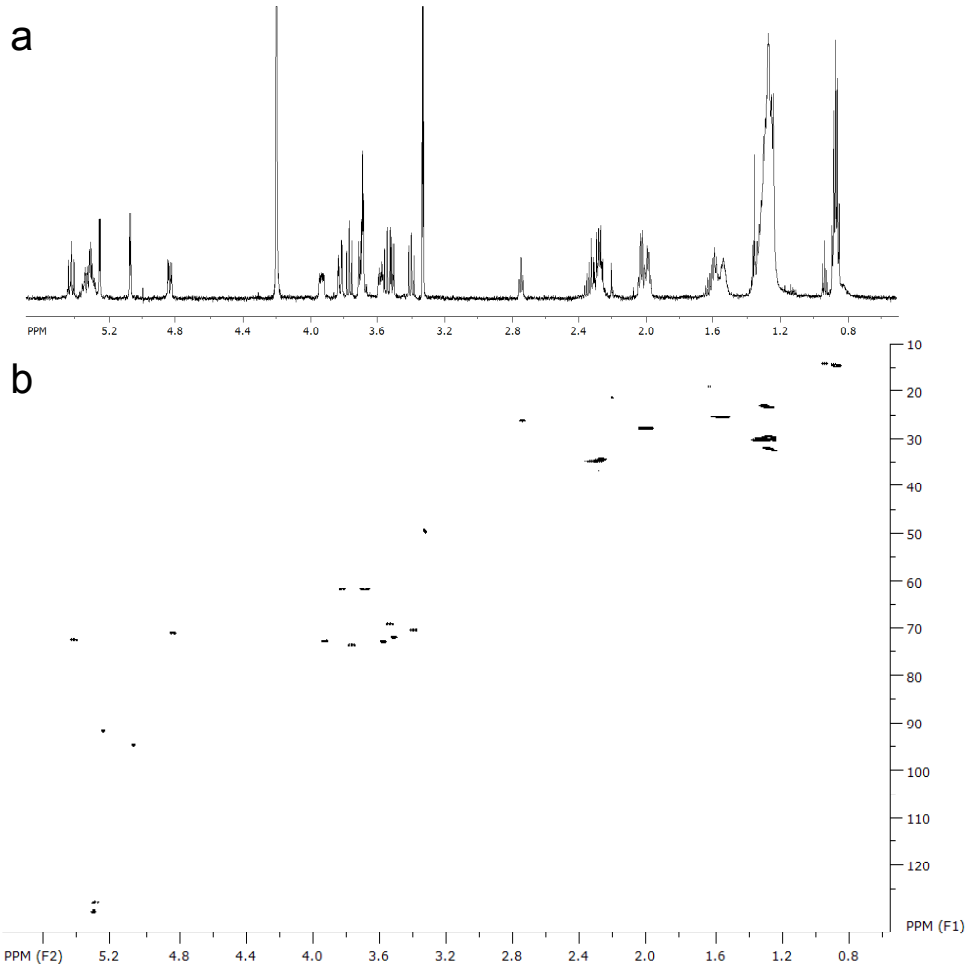

Figure A-2a-2: NMR of the purified fraction containing mainly trehalose lipid TL A. (a) 1H NMR, (b) two dimensional $^{1}H/^{13}C$ heteronuclear single quantum correlation spectroscopy (HSQC)

A-2a-3

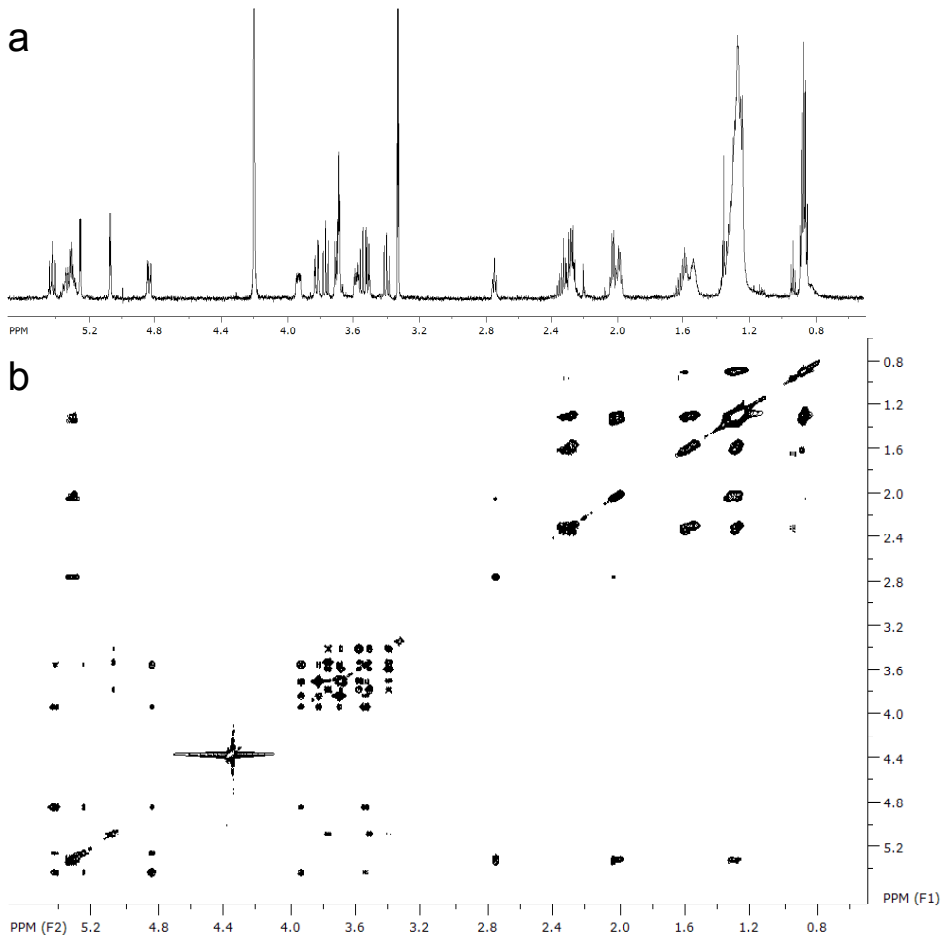

Figure A-2a-3: NMR of the purified fraction containing mainly trehalose lipid TL A. (a) 1H NMR, (b) two dimensional $^{1}H/^{1}H$ total correlation spectroscopy (TOCSY)

3a Who eats all that seal fat? - Biosurfactant producing soil communities found underneath seal carcasses in Antarctica and characterization of surface active compounds produced by *Pseudomonas* sp. SCS1-O4

A-3a-1 part 1 of 3

Table A-3a-1: List and screening results of Antarctic seal carcass soil isolated strains.

#	Strain ID	16S rDNA 130403/131031	NCBI Nr.	SBH-G growth	grid	emulsion	SBH-O growth	grid	emulsion	Blood growth	agar hemolysis
1	SLS1-G1	x		O	-	-	x	+	-	x	-
2	SLS1-G2	Arthrobacter sp.	KR023888	x	++	+	x	+	-	x	-
3	**SLS1-G3**	*Arthrobacer sp.*	KR023889	x	++	+	x	+	-	x	-
4	**SLS1-G4**	*Psychrobacter sp.*	KR023890	O	-	-	O	-	-	x	+
5	**SLS1-G5**	*Planococcus sp. (psychrotoleratus)*	KR023891	x	+	+	O	-	-	x	-
6	SLS1-G6			O	-	-	O	-	-	x	-
7	SLS1-G7			O	-	-	O	-	-	x	-
8	**SLS1-G8**	*Thelebolus sp. (globosus)*		x	++	+	x	++	+	x	+
9	SCS1-G1	x		x	++	+	x	+	-	x	-
10	SCS1-G2	x		x	++	++	x	+	-	x	-
11	SCS1-G3	*Arthrobacter sp. (psychrolactophilus)*		x	++	++	x	-	-	x	-
12	SCS1-G4			x	-	-	O	-	-	x	-
13	SCS1-G5			O	-	-	O	-	-	O	-
14	SCS1-G6	*Arthrobacter sp. (psychrolactophilus)*		x	+	-	x	++	-	x	-
15	SCS1-G7			O	-	-	O	-	-	O	-
16	SCS1-G8	*Psychrobacter sp. (maritimus)*	KR023892	x	++	+	x	-	-	x	-
17	SCS1-G9	*Bacillus* sp.	KR023893	x	++	-	x	-	-	O	-
19	SCS1-G11			x	+	-	x	++	-	O	-
20	SCS1-G12	x		x	++	-	x	++	-	x	-
21	SCS1-G13			O	-	-	O	-	-	O	-
22	SCS1-G14			x	-	-	x	+	-	x	-
23	SCS1-G15			x	+	-	x	-	-	x	-
24	SCS2-G1	x		x	+	-	x	++	-	x	-
25	**SCS2-G2**	*Carnobacterium sp. (mobile)*	KR023894	O	-	-	O	-	-	x	+
26	SCS2-G3			x	-	-	x	-	-	x	-
27	SCS3-G1			O	-	-	x	+	-	x	-
28	SCS3-G2			x	+	-	x	+	-	x	-
29	SCS3-G3	*Arthrobacter sp. (psychrolactophilus)*	KR023895	x	++	-	x	+	-	x	-
30	SCS3-G4	*Arthrobacter sp. (psychrolactophilus)*	KR023896	x	++	-	x	+	-	x	-
31	SCS3-G5	x		x	+	-	x	+	-	x	-
32	SCS3-G6			O	-	-	O	-	-	O	-
33	SCS3-G7			O	-	-	x	-	-	x	-
34	SCS3-G8	*Psychrobacter sp. (cibarius)*		O	-	-	x	++	-	x	-
35	SCS3-G9	*Psychrobacter sp. (urativorans)*		O	-	-	x	++	-	x	-
36	SCS3-G10	*Arthrobacter sp. (sulfureus)*		x	+	-	x	-	-	x	-
37	MVS1-G1	*Arthrobacter sp. (psychrochitinophilus)*	KR023897	x	++	+	x	-	-	x	-
38	MVS1-G2			O	-	-	O	-	-	O	-
39	MVS1-G3	*Arthrobacter sp. (stackebrandtii)*	KR023898	x	++	-	x	+	-	x	-
40	**MVS1-G4**	*Arthrobacter sp. (psychrolactophilus)*	KR023899	x	++	-	x	++	-	x	-
41	MVS1-G5			O	-	-	O	-	-	O	-
42	MVS1-G6			O	-	-	O	-	-	O	-
43	MVS1-G7			O	-	-	O	-	-	x	-
44	**MVS1-G8**	*Massilia sp (aurea)*	KR023900	x	++	-	O	-	-	x	-
45	MVS1-G9	*Arthrobacter sp.*	KR023901	x	++	+	x	+	-	x	-
46	MVS1-G10			O	-	-	O	-	-	x	-
47	MVS1-G11			x	+	+	x	+	+	O	-
48	SLS1-O0	*Arthrobacter sp. (psychrolactophilus)*	KR023911	x	++	-	x	-	-	O	-
49	SLS1-O1			O	-	-	O	-	-	O	-
50	SLS1-O2			x	-	-	O	-	-	x	-
51	SLS1-O3			O	-	-	O	-	-	x	-
52	SLS1-O4	*Psychrobacter sp. (maritimus)*	KR023912	O	-	-	x	+	-	x	-
53	SLS1-O5	*Psychrobacter sp.(aguimaris)*		O	-	-	O	-	-	x	-
54	SCS1-O1			O	-	-	O	-	-	x	-
55	**SCS1-O2**	*Pseudomonas sp. (antarctica)*	KR023913	x	++	+	x	++	+	x	-
56	SCS1-O3	*Pseudomonas sp. (antarctica)*		x	++	+	x	-	-	x	-
57	**SCS1-O4**	*Pseudomonas sp. (extremaustralis)*	KR023914	x	++	+	x	++	+	x	-
58	SCS2-O1			x	-	-	O	-	-	x	-

A-3a-1 part 2 of 3

59	**SCS2-O2**	*Arthrobacter sp. (psychrolactophilus)*	KR023915	x	++	+	x	-	+	x	-
60	SCS2-O3			x	-	-	x	-	-	x	-
61	SCS2-O4			x	-	-	x	-	-	x	-
62	SCS2-O5	*Pseudomonas sp. (antarctica)*		x	-	-	O	-	-	x	-
63	SCS2-O6	*Arthrobacter sp. (psychrolactophilus)*	KR023916	x	+	-	x	+	-	x	-
64	**SCS3-O1**	*Pseudomonas sp. (mandelii)*	KR023917	x	++	+	x	-	+	x	-
65	SCS3-O2	*Psychrobacter sp. (cryhalolentis)*		O	-	-	x	+	-	O	-
66	SCS3-O3	*Pseudomonas sp. (flourescens)*	KR023918	x	-	+	x	+	+	x	-
67	SCS3-O4	*Arthrobacter sp. (sulfureus)*	KR023919	x	+	+	O	-	-	x	-
68	SCS3-O5			x	++	-	O	-	-	x	-
69	SCS3-O6	*Arthrobacter sp. (psychrolactophilus)*	KR023920	x	+	-	x	++	-	x	-
70	SCS3-O7	*Arthrobacter sp. (psychrolactophilus)*	KR023921	x	+	-	x	++	-	x	-
71	SCS3-O8	*Arthrobacter sp. (psychrolactophilus)*	KR023922	x	+	-	x	+	-	x	-
72	MVS1-O1			x	-	-	O	-	-	x	-
73	MVS1-O2	*Arthrobacter sp. (sulfureus)*	KR023923	x	++	-	O	-	-	x	-
74	MVS1-O3	*Arthrobacter sp. (psychrolactophilus)*	KR023924	x	+	-	x	+	-	x	-
75	MVS1-O4	*Arthrobacter sp. (sulfureus)*	KR023925	x	++	-	O	-	-	x	-
76	MVS1-O5	*Arthrobacter sp. (psychrolactophilus)*	KR023926	x	+	-	x	+	-	x	-
77	MVS1-O6			x	-	-	O	-	-	x	-
78	SCS3-L1O1	*Pseudomonas sp. (mandelii)*		x	-	-	x	++	-	x	-
79	SCS3-L1O2	x		x	-	-	x	+	-	x	-
80	SCS3-L1O3	x		x	-	-	x	+	-	x	-
81	SCS3-L2O1			x	-	-	x	-	-	x	-
82	SCS3-L2O2a	*Arthrobacter sp. (cryotolerans)*	KR023927	x	-	-	x	+	-	x	-
83	**SCS3-L2O2b**	*Arthrobacter sp. (nitroguajacolicus)*	KR023928	x	+	+	x	+	+	x	-
83	**SCS3-L2O3**	*Variovorax sp. (paradoxus)*	KR023929	O	-	-	x	++	-	x	-
84	SCS3-L2O4	*Variovorax sp. (paradoxus)*		O	-	-	x	++	-	x	-
85	SCS3-L2O5	*Pseudomonas sp. (syringae)*		x	++	-	x	-	-	x	-
86	SCS3-L2O6a	x		O	-	-	x	-	-	x	-
87	SCS3-L2O6b	x		x	-	-	x	-	-	x	-
88	SCS3-L2O7			x	-	-	x	+	-	x	-
89	SLS1-P1			O	-	-	x	-	-	x	-
90	SLS1-P2			x	-	-	x	-	-	x	-
91	SLS1-P3			x	-	-	x	-	-	x	-
92	SLS1-P4			x	-	-	x	-	-	x	-
93	SLS1-P5			x	-	-	x	-	-	x	-
94	SLS1-P6			x	-	-	O	-	-	x	-
95	SLS1-P7	*Arthrobacter sp. (psychrolactophilus)*	KR023930	x	+	-	x	+	-	x	-
96	SLS1-P8	*Arthrobacter sp. (psychrolactophilus)*	KR023931	x	+	-	x	+	-	x	-
97	SLS1-P9			x	-	-	O	-	-	x	-
98	SLS1-P10	*Carnobacterium sp. (mobile)*	KR023932	O	-	-	O	-	-	x	+
99	SLS1-P11	*Arthobacter sp. (antarcticus)*	KR023933	x	-	-	x	-	-	x	+
100	SLS1-P12			x	-	-	x	-	-	x	-
101	SLS1-P13	*Arthrobacter sp. (psychrolactophilus)*	KR023934	x	-	-	x	++	-	x	-
102	SLS1-P14			x	+	-	x	+	-	x	-
103	SCS1-P1			x	-	-	O	-	-	x	-
104	SCS1-P2			x	-	-	O	-	-	x	-
105	SCS1-P3			x	-	-	O	-	-	x	-
106	SCS1-P4			x	-	-	O	-	-	x	-
107	SCS2-P1	*Arthrobacter sp.*	KR023935	x	+	-	x	++	-	x	-
108	SCS2-P2			x	-	-	x	-	-	x	-
109	SCS2-P3			x	+	-	x	-	-	x	-
110	SCS2-P4			O	-	-	O	-	-	O	-
111	SCS2-P5			x	+	-	x	+	-	x	-
112	SCS2-P6	*Arthrobacter sp. (psychrolactophilus)*	KR023936	x	+	-	x	+	-	x	-
113	SCS2-P7			x	+	-	x	-	-	x	-
114	SCS2-P8	*Arthrobacter sp. (psychrolactophilus)*	KR023937	x	+	-	x	-	-	x	-
115	SCS2-P9	*Arthrobacter sp. (gangotriensis)*		x	-	-	x	++	-	x	-
116	SCS2-P10			x	-	-	x	-	-	x	-

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117	SCS2-P11	x		O	-	-	O	-	-	x	-	
118	SCS2-P12	*Arthrobacter sp. (psychrolactophilus)*		x	-	-	x	+	-	x	-	
119	SCS2-P13	x		x	+	-	x	++	-	x	-	
120	SCS2-P14	*Arthrobacter sp. (psychrolactophilus)*	KR023938	x	+	-	x	+	-	x	-	
121	**SCS3-P1**	*Arthrobacter sp. (psychrochitiniphilus)*	KR023939	x	++	+	x	+	-	x	-	
122	SCS3-P2	*Psychrobacter sp. (cryohalolentis)*		O	-	-	x	+	-	x	-	
123	SCS3-P3	*Arthrobacter sp. (psychrolactophilus)*	KR023940	x	+	-	x	++	-	x	-	
124	SCS3-P4			O	-	-	x	-	-	x	-	
125	SCS3-P5			x	+	-	O	-	-	x	-	
126	SCS3-P6			O	-	-	O	-	-	x	-	
127	SCS3-P7			x	+	-	x	-	-	x	-	
128	**SCS3-P8**	*Arthrobacter sp. (psychrolactophilus)*	KR023941	x	+	+	x	++	-	x	-	
129	MVS1-P1	x		x	++	-	x	++	-	x	-	
130	MVS1-P2	*Arthrobacter sp. (psychrolactophilus)*		x	+	-	x	+	-	x	-	
131	MVS1-P3			x	-	-	O	-	-	x	-	
132	MVS1-P4	*Arthrobacter sp. (psychrochitinophilus)*	KR023942	x	+	-	x	++	-	x	-	
133	MVS1-P5			O	-	-	O	-	-	O	-	
134	MVS1-P6	*Arthrobacter sp. (psychrochitiniphilus)*		x	++	+	x	-	-	x	-	
135	MVS1-P7	*Thelebolus sp.*		x	+	-	x	+	-	x	+	
136	SCS1-L1P1	*Arthrobacter sp. (sulfureus)*	KR023943	x	++	-	O	-	-	x	-	
137	SLS1-N1			O	-	-	O	-	-	x	-	
138	SLS1-N2			x	-	-	x	-	-	x	-	
139	SLS1-N3			O	-	-	O	-	-	O	-	
140	SLS1-N4			O	-	-	O	-	-	O	-	
141	SLS1-N5			O	-	-	O	-	-	x	-	
142	SLS1-N6			x	++	+	O	-	-	O	-	
143	SCS1-N1			O	-	-	O	-	-	x	-	
144	SCS1-N2	*Pseudomonas sp. (gessardii)*		x	+	-	x	-	-	x	-	
145	SCS1-N3	*Arthrobacter sp.*	KR023902	x	-	-	x	-	-	x	-	
146	SCS1-N4	x		x	+	-	x	-	-	x	-	
147	SCS1-N5	*Pseudomonas sp. (antarctica)*	KR023903	x	++	-	x	+	-	x	-	
148	SCS1-N6			x	-	-	x	-	-	x	-	
149	SCS2-N1			x	-	-	x	-	-	x	-	
150	SCS2-N2	*Pseudomonas sp. (lini)*	KR023904	x	+	-	x	++	-	x	-	
151	SCS2-N3	*Arthrobacter sp. (psychrolactophilus)*	KR023905	x	+	-	x	+	-	x	-	
152	SCS2-N4			x	-	-	x	-	-	x	-	
153	SCS3-N1			x	-	-	x	-	-	x	-	
154	**SCS3-N2**	*Arthrobacter sp.*	KR023906	x	++	+	x	-	+	x	-	
155	SCS3-N3	*Psychrobacter sp. (cryohalolentis)*	KR023907	x	-	-	x	+	-	O	-	
156	SCS3-N4			x	-	-	x	-	-	x	-	
157	SCS3-N5	*Arthrobacter sp. (psychrolactophilus)*	KR023908	x	+	-	x	+	-	x	-	
158	SCS3-N6			x	-	-	O	-	-	x	-	
159	SCS3-N7	*Psychrobacter sp. (cryohalolentis)*	KR023909	O	-	-	x	+	-	x	-	
160	SCS3-N8			O	-	-	x	+	-	O	-	
161	SCS3-N9			x	-	-	x	+	-	x	-	
162	SCS3-N10			x	-	-	x	+	-	x	-	
163	MVS1-N1			x	-	-	x	-	-	x	-	
164	MVS1-N2	x		x	-	-	x	+	-	x	-	
165	MVS1-N3			x	-	-	x	-	-	x	-	
166	MVS1-N4			x	-	-	O	-	-	x	-	
167	MVS1-N5			x	-	-	x	-	-	x	-	
168	MVS1-N6	*Psychrobacter sp. (cryohalolentis)*	KR023910	x	-	-	x	+	-	O	-	

Annotation:

- no/bad sequence
- sequence from one primer or partial
- sequence from both 16S primers
- eucaryote

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Table A-3a-2: 16S ribosomal RNA gene partial sequence of soil communities found underneath seal carcasses in Antarcitca. Identifier numbers: gi=NCBI PropSet; gb=NCBI GenBank

>gi|816379761|gb|KR023888.1| Arthrobacter sp. SLS1-G2 16S ribosomal RNA gene, partial sequence
TGCAGTCGACGATGACCCCGCTTGCGGGGGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTTGGAAACGGGGTCTAATACTGGATATTGACTTT
TCCTCGCATGGGGTTTTGTTGAAAGATTTATTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGC
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCT
CTTTCAGTAGGGAACAAGGCCAGCATTTTTGTTGGTTGAGGGTACTTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGG
GCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACTCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCGG
TGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAA
ACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACA
AGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGAACTGGAAATACCTGGAAACAGGTGCCCCGCTTGCGGTCGGTTTACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTTATGGTGGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAAGAAGGTG
AGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATT
GGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCA

>gi|816379762|gb|KR023889.1| Arthrobacter sp. SLS1-G3 16S ribosomal RNA gene, partial sequence
GCAGTCGACGATGAACCCCGCTTGCGGGGGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTTGGAAACGGGGTCTAATACTGGATATTGACTTA
CTGCCGCATGGTGGAAAGTTGAAAGATTTATTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGC
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCT
CTTTCAGTAGGGAACAAGGCCAGTGTTTAGCTGGTTGAGGGTACTTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGGG
CGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCGGT
GAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAA
CGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAA
GCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGAACTGGAAACAGGTGCCCCGCTTGCGGTCGGTTTACAGGTGGTGCATGGTTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGAG
GACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGG
GGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGT

>gi|816379763|gb|KR023890.1| Psychrobacter sp. SLS1-G4 16S ribosomal RNA gene, partial sequence
TGCAGTCGAGCGGAACGATGATAGCTTGCTATCAGGCGTCGAGCGGCGGACGGGTGAGTAATACTTAGGAATCTACCTAGTAGTGGGGGATAGCTCGGGGAAACTCGAATTAATACCGCATACG
ACCTACGGGAGAAAGGGGGCAGTTTACTGCTCTCGCTATTAGATGAGCCTAAGTCGGATTAGCTAGATGGTGGGGTAAAGGCCTACCATGGCGACGATCTGTAGCTGGTCTGAGAGGATGATCA
GCCACACCGGGACTGAGACACGGCCCGGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAG
CACTTTAAGCAGTGAAGAAGACTCCATGGTTAATACCCATGGACGATGACATTAGCTGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCG
GAATTACTGGGCGTAAAGCGAGCGTAGGTGGCTTGATAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATCTGATACTGTTAGGCTAGAATAGGTGAGAGGAAGGTAGAATTCCA
GGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCTTCTGGCATCATATTGACACTGAGGTTCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGTCTACTAGTCGTTGGGTCCCTTGAGGACTTAGTGACGCAGCTAACGCAATAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATATCTAGAATCCTGCAGAGATGCGGGAGTGCCTTCGGGAATTAGAATACAGGTGCTG
CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTACCAGCGGGTTAAGCCGGGAACTCTAAGGATACTGCCAGTGACAAACTGGA
GGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGTAGGTACAGAGGGCAGCTACACAGCGATGTGATGCGAATCTCAAAAAGCCTATCGTAG
TCCAGATTGGAGTCTGCAACTCGACTCCATGAAGTAGGAATCGCTAGTAATCGCG

>gi|816379764|gb|KR023891.1| Planococcus sp. SLS1-G5 16S ribosomal RNA gene, partial sequence
TGCAGTCGAGCGGACCAGGAGAGCTTGCTCTTTCTGGTTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCCTGCAGATCGGGATAACTCCGGGAAACCGGTGCTAATACCGAATAGT
TTGGAGCCTCTCCTGAGGTTCCACGGAAAGGCGGCTTCGGCTGCCACTGCAGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGGGGTAATGGCCTACCAAGGCGACGATGCGTAGCCGACCTGA
GAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGCAAGTCTGACGGAGCAATGCCGCGTGAGTGATGACGAAGGTTTTC
GGATCGTAAAACTCTGTTGTAAGGGAAGAACAAGCCCCATTTAACTGATGGGGCCCTGACGGTACCTTACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA
GCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGGACTTGAGTGCAGAAGAGGAAAG
TGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCGCTGATCGGCGTAGAGATACGCTTTTCCTTCGGGGACAGCG
GTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGT
GACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGGGCTGCCAACCCGCGAGGGGGAGCCAATCCCAGAAA
ACCGTTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATC

>gi|816379765|gb|KR023892.1| Psychrobacter sp. SCS1-G8 16S ribosomal RNA gene, partial sequence
TGCAGTCGAGCGGAACGATGAGCTTGCTATCAGGCGTCGAGCGGCGGACGGGTGAGTAATACTTAGGAATCTACCTAGTAGTGGGGGATAGCTCGGGGAAACTCGAATTAATACCGCATACG
ACCTACGGGAGAAAGGGGGCAGTTTACTGCTCTCGCTATTAGATGAGCCTAAGTCGGATTAGCTAGATGGTGGGGTAAAGGCCTACCATGGCGACGATCTGTAGCTGGTCTGAGAGGATGATCA
GCCACACCGGGACTGAGACACGGCCCGGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAG
CACTTTAAGCAGTGAAGAAGACTCCATGGTTAATACCCATGGACGATGACATTAGCTGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCG
GAATTACTGGGCGTAAAGCGAGCGTAGGTGGCTTGATAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATCTGATACTGTTAGGCTAGAATAGGTGAGAGGAAGGTAGAATTCCA
GGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCTTCTGGCATCATATTGACACTGAGGTTCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGTCTACTAGTCGTTGGGTCCCTTGAGGACTTAGTGACGCAGCTAACGCAATAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATATCTAGAATCCTGCAGAGATGCGGGAGTGCCTTCGGGAATTAGAATACAGGTGCTG
CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTACCAGCGGGTTAAGCCGGGAACTCTAAGGATACTGCCAGTGACAAACTGGA
GGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGTAGGTACAGAGGGCAGCTACACAGCGATGTGATGCGAATCTCAAAAAGCCTATCGTAG
TCCAGATTGGAGTCTGCAACTCGACTCCATGAAGTAGGAATCGCTAGTAATCGCGATCAG

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>gi|816379766|gb|KR023893.1| Bacillus sp. SCS1-G9 16S ribosomal RNA gene, partial sequence
TGCAGTCGAGCGATCGATGGGAGCTTGCTCCCTGAGATTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATACGT
TCTTTTCTCGCATGAGAGAAGATGGAAAGACGGTTTACGCTGTCACTTATAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAG
AGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAACGAAGAAGGCCTTCG
GGTCGTAAAGTTCTGTTGTTAGGGAAGAACAAGTACCAGAGTAACTGCTGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA
GCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTCCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAAAG
TGGAATTCCAAGTGTAGCGGTGAAATGCGTAGAGATTTGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCCCTTCGGGGGACAGA
GTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGT
GACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACA

>gi|816379767|gb|KR023894.1| Carnobacterium sp. SCS2-G2 16S ribosomal RNA gene, partial sequence
TGCAGTCGAACGCTTCTTTTCTACCGGGTGCTTGCACCCACCAGAGAAGAAGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGTGGGGGATAACAGCCGGAAACGGCTGCTA
ATACCGCATAATTCCAGTGATCTCCTGATCGTTGGATGAAAGGTGGCTTCGGCTACCGCTTATGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAATGATACG
TAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAATGCCGCGTGAGTG
AAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGCTCATCCCCTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTCTTTAAGTCTGATGTGAAAGCCCCCAGCTCAACTGGGGAAGGTCATTGGAAACTGGGGAACTTGAGTG
CAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGGGGGTTTCCGCCCCTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCT
GAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACCTTAGAGATAGGGCTTTCCCT
TCGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCAGCATTCAGTTGGGCACTCTAGTG
AGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTCGCAAGACCGCGAGGTCAAGCT
AATCTCTTAAAGCCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGATC

>gi|816379768|gb|KR023895.1| Arthrobacter sp. SCS3-G3 16S ribosomal RNA gene, partial sequence
TGCAGTCGACGATGAACCCCGCTTGCGGGGGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTTGGAAACGGGGTCTAATACTGGATATTGACTT
ACTGCCGCATGGTGGAAAGTTGAAAGATTTATTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACC
TCTTTCAGTAGGGAACAAGGCCAGTGTTTAGCTGGTTGAGGGTACTTGCAGAAGAAGCCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGG
GCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCGG
TGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAA
ACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACA
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>gi|816379769|gb|KR023896.1| Arthrobacter sp. SCS3-G4 16S ribosomal RNA gene, partial sequence
TGCAGTCGACGATGAACCTCGCTTGCGGGGGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTTGGAAACGGGGTCTAATACTGGATATTGACTT
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GGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTG
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>gi|816379770|gb|KR023897.1| Arthrobacter sp. MVS1-G1 16S ribosomal RNA gene, partial sequence
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GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAAC
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CAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGAACTGGAAATACCTGGAAACAGGTGCCCCGCTTGCGGTCGGTTTACAGGTGGTGCATGGTT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTTATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGT
GAGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGAT
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>gi|816379771|gb|KR023898.1| Arthrobacter sp. MVS1-G3 16S ribosomal RNA gene, partial sequence
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GAGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGAT
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>gi|816379772|gb|KR023899.1| Arthrobacter sp. MVS1-G4 16S ribosomal RNA gene, partial sequence
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AGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGAACTGGAAACACCTGGAAACAGGTGCCCCGCTTGCGGTCGGTTTACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGA
GGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTG
GGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGAT

A-3a-2 part 3 of 9

>gi|816379773|gb|KR023900.1| Massilia sp. MVS1-G8 16S ribosomal RNA gene, partial sequence
TGCAGTCGAACGGCAGCGCGGGGCAACCTGGCGGCGAGTGGCGAACGGGTGAGTAATATATCGGAACGTACCCAAGAGTGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATACGATCTA
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GCCCTAAACGATGTCTACTAGTTGTCGGGTTTTAATTAACTTGGTAACGCAGCTAACGCGTGAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCG
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TGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCTACATTCAGTTGAGCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTACATACAGAGGGCCGCCAACCCGCGAGGGGGAGCTAATCCCAGAAAGTGTATCGTAGTCCGG
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>gi|816379774|gb|KR023901.1| Arthrobacter sp. MVS1-G9 16S ribosomal RNA gene, partial sequence
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CGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAA
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GGTGAGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGG
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>gi|816379775|gb|KR023902.1| Arthrobacter sp. SCS1-N3 16S ribosomal RNA gene, partial sequence
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CGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGTGAAGAAGGTCTTCGGATTGTAAAGC
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TGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTGCTAGATCGCCATAGAAATATGGTTTCCCCTTTGGGGCTGGTTCACAGGTGGTGCATGGTTGTCGTCAGCTCGT
GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTTATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCA
AATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAAC
TCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCA

>gi|816379776|gb|KR023903.1| Pseudomonas sp. SCS1-N5 16S ribosomal RNA gene, partial sequence
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GAAGGTGGG

>gi|816379777|gb|KR023904.1| Pseudomonas sp. SCS2-N2 16S ribosomal RNA gene, partial sequence
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TACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCACTATCCGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAG
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ACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGC
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>gi|816379778|gb|KR023905.1| Arthrobacter sp. SCS2-N3 16S ribosomal RNA gene, partial sequence
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GAGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGAT
TGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGAT

>gi|816379779|gb|KR023906.1| Arthrobacter sp. SCS3-N2 16S ribosomal RNA gene, partial sequence
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>gi|816379780|gb|KR023907.1| Psychrobacter sp. SCS3-N3 16S ribosomal RNA gene, partial sequence
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>gi|816379782|gb|KR023909.1| Psychrobacter sp. SCS3-N7 16S ribosomal RNA gene, partial sequence
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GCCACACCGGGACTGAGACACGGCCCGGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAG
CACTTTAAGCAGTGAAGAAGACTCTTCGGTTAATACCCGGAGACGATGACATTAGCTGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCG
GAATTACTGGGCGTAAAGGGAGCGTAGGTGGCTCGATAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGTTGAGCTAGAGTATGTGAGAGGAAGGTAGAATTCCA
GGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCTTCTGGCATAATACTGACACTGAGGCTCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGTCTACTAGTCGTTGGGTCCCTTGAGGACTTAGTGACGCAGCTAACGCAATAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATATCTAGAATCCTGCAGAGATGCGGGAGTGCCTTCGGGAATTAGAATACAGGTGCTG
CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTACCAGCGGGTTAAGCCGGGAACTCTAAGGATACTGCCAGTGACAAACTGGA
GGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGTAGGTACAGAGGGCAGCTACACAGCGATGTGATGCGAATCTCAAAAAGCCTATCGTAG
TCCAGATTGGAGTCTGCAACTCGACTCCATGAAGTAGGAATCGCTAGTAATCGCGGATCA

>gi|816379783|gb|KR023910.1| Psychrobacter sp. MVS1-N6 16S ribosomal RNA gene, partial sequence
TGCAGTCGAGCGGAACGATGATAGCTTGCTATCAGGCGTCGAGCGGCGGACGGGTGAGTAATACTTAGGAATCTACCTAGTAGTGGGGGATAGCACGGGGAAACTCGTATTAATACCGCATACG
ACCTACGGGAGAAAGGGGGCAGTTTACTGCTCTCGCTATTAGATGAGCCTAAGTCGGATTAGCTAGATGGTGGGGTAAAGGCCTACCATGGCGACGATCTGTAGCTGGTCTGAGAGGATGATCA
GCCACACCGGGACTGAGACACGGCCCGGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAG
CACTTTAAGCAGTGAAGAAGACTCTTCGGTTAATACCCGGAGACGATGACATTAGCTGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCG
GAATTACTGGGCGTAAAGGGAGCGTAGGTGGCTCGATAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGTTGAGCTAGAGTATGTGAGAGGAAGGTAGAATTCCA
GGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCTTCTGGCATAATACTGACACTGAGGCTCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGTCTACTAGTCGTTGGGTCCCTTGAGGACTTAGTGACGCAGCTAACGCAATAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATATCTAGAATCCTGCAGAGATGCGGGAGTGCCTTCGGGAATTAGAATACAGGTGCTG
CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTACCAGCGGGTTAAGCCGGGAACTCTAAGGATACTGCCAGTGACAAACTGGA
GGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGTAGGTACAGAGGGCAGCTACACAGCGATGTGATGCGAATCTCAAAAAGCCTATCGTAG
TCCAGATTGGAGTCTGCAACTCGACTCCATGAAGTAGGAATCGCTAGTAATCGCGGATCA

>gi|816379784|gb|KR023911.1| Arthrobacter sp. SLS1-O0 16S ribosomal RNA gene, partial sequence
TGCAGTCGACGATGATTCCCAGCTTGCTGGGGGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTTCAGGATAAGCCTTGGAAACGGGGTCTAATACTGGATATAGAC
TTTTCCTCGCATGGGGGATGGTTGAAAGATTTATTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACC
GGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTTCGGGTTGTAAA
CCTCTTTCAGTAGGGAACAAGGCCAGCATTTTTGTTGGTTGAGGGTACTTGCAGAAGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGCGCAAGCGTTATCCGGAATTAT
TGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAG
CGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCG
TAAACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGC
ACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGAATCGGAAACGCTTGGAAACAAGTGCCCCACTTTGTGGTCGGTTTACAGGTGGTGCATGG
TTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTGATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAG
GTGAGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGG
ATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCA

>gi|816379785|gb|KR023912.1| Psychrobacter sp. SLS1-O4 16S ribosomal RNA gene, partial sequence
TGCAGTCGAGCGGAACGATGATAGCTTGCTATCAGGCGTCGAGCGGCGGACGGGTGAGTAATACTTAGGAATCTACCTAGTAGTGGGGGATAGCTCGGGGAAACTCGAATTAATACCGCATACG
ACCTACGGGAGAAAGGGGGCAACTTGTTGCTCTCGCTATTAGATGAGCCTAAGTCGGATTAGCTAGATGGTGGGGTAAAGGCCTACCATGGCGACGATCTGTAGCTGGTCTGAGAGGATGATCA
GCCACACCGGGACTGAGACACGGCCCGGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAG
CACTTTAAGCAGTGAAGAAGACTCCGTGGTTAATACCCACGGACGATGACATTAGCTGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCG
GAATTACTGGGCGTAAAGCGAGCGTAGGTGGCTTGATAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTCGATACTGTTAGGCTAGAGTAGGTGAGAGGAAGGTAGAATTCCA
GGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCTTCTGGCATCATACTGACACTGAGGCTCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGTCTACTAGTCGTTGGGTCCCTTGAGGACTTAGTGACGCAGCTAACGCAATAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATATCTAGAATCCTGCAGAGATGCCTTCCTTCGGGAATTAGAATACAGGTGCTG
CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTTCCTTAGTTACCAGCGGGTTAAGCCGGGAACTCTAAGGATACTGCCAGTGACAAACTGGA
GGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGTAGGTACAGAGGGCAGCTACACAGCGATGTGATGCGAATCTCAAAAAGCCTATCGTAG
TCCAGATTGGAGTCTGCAACTCGACTCCATGAAGTAGGAATCGCTAGTAATCGCG

>gi|816379786|gb|KR023913.1| Pseudomonas sp. SCS1-O2 16S ribosomal RNA gene, partial sequence
TGCAGTCGAGCGGTAGAGAGAGCTTGCTTCTCTTGAGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCT
ACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCACACCGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCTACGATCCGTAACTGGTCTGAGAGGATGATCAGT
CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCA
CTTTAAGTTGGGAGGAAGGGCAGTTACCTAATACGTGATTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGA
ATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTG
TGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCTAGAGATAGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCA
TGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGG
AAGGTGGG

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>gi|816379787|gb|KR023914.1| Pseudomonas sp. SCS1-O4 16S ribosomal RNA gene, partial sequence
CATGCAGTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGT
CCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCTACGATCCGTAACTGGTCTGAGAGGATGATC
AGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAA
GCACTTTAAGTTGGGAGGAAGGGCAGTTACCTAATACGTGATTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATC
GGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGGTAGAGGGTGGTGGAATTTC
CTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTGTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGG
GCCCGCACAAGCGGTGGAGCATGTGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCTAGAGATAGATTGGTGCCTTCGGGAACATTGAGACAGGTGCT
GCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGG
AGGAAGGTGGGG

>gi|816379788|gb|KR023915.1| Arthrobacter sp. SCS2-O2 16S ribosomal RNA gene, partial sequence
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ACTGCCGCATGGTGGAAAGTTGAAAGATTTATTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACC
TCTTTCAGTAGGGAACAAGGCCAGTGTTTAGCTGGTTGAGGGTACTTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGG
GCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCGG
TGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTA
AACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGAACTGGAAATACCTGGAAACAGGTGCCCCGCTTGCGGTCGGTTTACAGGTGGTCATGGTTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTG
AGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATT
GGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGC

>gi|816379789|gb|KR023916.1| Arthrobacter sp. SCS2-O6 16S ribosomal RNA gene, partial sequence
TGCAGTCGAACGATGAACCTCGCTTGCGGGGGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTTGGAAACGGGGTCTAATACTGGATATTGACT
TACTGCCGCATGGTGGAAAGTTGAAAGATTTATTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCG
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACC
CTCTTTCAGTAGGGAACAAGGCCAGTGTTTAGCTGGTTGAGGGTACTTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTG
GGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCG
GTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTA
AACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGAACTGGAAATACCTGGAAACAGGTGCCCCGCTTGCGGTCGGTTTACAGGTGGTCATGGTTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTG
AGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATT
GGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCA

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>gi|816379790|gb|KR023917.1| Pseudomonas sp. SCS3-O1 16S ribosomal RNA gene, partial sequence
TGCAGTCGAGCGGCAGCACGGGTACTTGTACCTTGGTGGCGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGAAACGGACGCTAATACCGCATACGT
CCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCACTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATC
AGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAA
GCACTTTAAGTTGGGAGGAAGGGCAGTAAATTAATACTTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATC
GGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACAAGCTAGAGTATGGTAGAGGGTGGTGGAATTTC
CTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGG
GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACATTGAGACAGGTGC
GCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGG
AGGAAGGTG

>gi|816379791|gb|KR023918.1| Pseudomonas sp. SCS3-O3 16S ribosomal RNA gene, partial sequence
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TCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGAT
CAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAA
AGCACTTTAAGTTGGGAGGAAGGGCAGTAAATTAATACTTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAAT
CGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACAAGCTAGAGTATGGTAGAGGGTGGTGGAATTT
CCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAG
TCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGG
GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCTAGAGATAGATTGGTGCCTTCGGGAACATTGAGACAGGTGC
GAGGAAGGTGGGAAAGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCAGAAAACCGATCGT
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>gi|816379792|gb|KR023919.1| Arthrobacter sp. SCS3-O4 16S ribosomal RNA gene, partial sequence
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CACCGTTGACCGCATGGTTTTTGGTGGAAAGATTTTTGGTCTGCAGGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTG
ACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGT
AAACCTCTTTCAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCGTAA
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GCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTG
GGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGC
GGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTCCAGACCGCCGTGGAAACACGGTTTCCCCTTTGGGGCTGGTTCACAGGTGGTGCATGGTTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCGCGTAAAGGCGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
ACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTC
TGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCA

>gi|816379793|gb|KR023920.1| Arthrobacter sp. SCS3-O6 16S ribosomal RNA gene, partial sequence
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TACCTCGCATGGGTTTTGTTGAAAGATTTATTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACC
TCTTTCAGTAGGGAACAAGGCCAGCATTTTTGTTGGTTGAGGGTACTTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTG
GGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCG
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AACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGAACCGGAAATACCTGGAAACAGGTGCCCCGCTTGCGGTCGGTTTACAGGTGGTGCATGGTTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTTATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTG
AGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATT
GGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCA

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>gi|816379794|gb|KR023921.1| Arthrobacter sp. SCS3-O7 16S ribosomal RNA gene, partial sequence
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TTACCTCGCATGGGGTTTTGTTGAAAGATTTATTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCG
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAAC
CTCTTTCAGTAGGGAACAAGGCCAGCATTTTTGTTGGTTGAGGGTACTTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTG
GGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACCCCGGATCTGCGGTGGGTACCGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCG
GTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTA
AACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGAACCGGAAATACCTGGAAACAGGTGCCCCGCTTGCGGTCGGTTTACAGGTGGTGCATGGTTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTTATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGT
GAGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGAT
TGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCA

>gi|816379795|gb|KR023922.1| Arthrobacter sp. SCS3-O8 16S ribosomal RNA gene, partial sequence
TGCAGTCGAACGATGAACCCCGCTTGCGGGGGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTTGGAAACGGGGTCTAATACTGGATATTGACT
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GGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACCCCGGATCTGCGGTGGGTACCGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCG
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>gi|816379796|gb|KR023923.1| Arthrobacter sp. MVS1-O2 16S ribosomal RNA gene, partial sequence
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AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACGTAAGGCGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGAC
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>gi|816379797|gb|KR023924.1| Arthrobacter sp. MVS1-O3 16S ribosomal RNA gene, partial sequence
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>gi|816379798|gb|KR023925.1| Arthrobacter sp. MVS1-O4 16S ribosomal RNA gene, partial sequence
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>gi|816379799|gb|KR023926.1| Arthrobacter sp. MVS1-O5 16S ribosomal RNA gene, partial sequence
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>gi|816379800|gb|KR023927.1| Arthrobacter sp. SCS3-L2O2a 16S ribosomal RNA gene, partial sequence
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A-3a-2 part 7 of 9

>gi|816379801|gb|KR023928.1| Arthrobacter sp. SCS3-L2O2b 16S ribosomal RNA gene, partial sequence
TGCAAGTCGAACGATGATCCCAGCTTGCTGGGGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTGACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATACGACC
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>gi|816379802|gb|KR023929.1| Variovorax sp. SCS3-L2O3 16S ribosomal RNA gene, partial sequence
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>gi|816379803|gb|KR023930.1| Arthrobacter sp. SLS1-P7 16S ribosomal RNA gene, partial sequence
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>gi|816379804|gb|KR023931.1| Arthrobacter sp. SLS1-P8 16S ribosomal RNA gene, partial sequence
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>gi|816379805|gb|KR023932.1| Carnobacterium sp. SLS1-P10 16S ribosomal RNA gene, partial sequence
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>gi|816379806|gb|KR023933.1| Arthrobacter sp. SLS1-P11 16S ribosomal RNA gene, partial sequence
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>gi|816379807|gb|KR023934.1| Arthrobacter sp. SLS1-P13 16S ribosomal RNA gene, partial sequence
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TTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTA
GCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCC
GTAAACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGAATCGGAAACGCTTGGAAACAAGTGCCCCACTTTGTGGTCGGTTTACAGGTGGTGCATG
GTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTGATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAA
GGTGAGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCG
GATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAG

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>gi|816379808|gb|KR023935.1| Arthrobacter sp. SCS2-P1 16S ribosomal RNA gene, partial sequence
TGCAAGTCGACGATGACTTTTGTGCTTGCACAGAATGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCTGACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACTGGATATG
CACCGTAGACCGCATGGTTTTTGGTGGAAAGAATTTTGGTCAGGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGA
CCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCATGCCGCGTGAGGGATGACGGCCTTCGGGTTGTA
AACCTCTTTCAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCGTAAA
GAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGAGGCTCAACTTCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATG
CGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATCTACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGG
GCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCG
GAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTGCTAGATCGCCATAGAAATATGGTTTCCCCTTTGGGGCTGGTTCACAGGTGGTGCATGGTTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTTATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGA
CGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTG

>gi|816379809|gb|KR023936.1| Arthrobacter sp. SCS2-P6 16S ribosomal RNA gene, partial sequence
TGCAGTCGAACGATGAACCTCGCTTGCGGGGGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTTGGAAACGGGGTCTAATACTGGATATTGACT
TACTGCCGCATGGTGGAAAGTTGAAAGATTTATTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCG
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAAC
CTCTTTCAGTAGGGAACAAGGCCAGTGTTTAGCTGGTTGAGGGTACTTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTG
GGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCG
GTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTA
AACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTGCTAGATCGCCATAGAAATACCTGGAAACAGGTGCCCCGCTTGCGGTCGGTTTACAGGTGGTGCATGGTTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTG
AGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATT
GGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAG

>gi|816379810|gb|KR023937.1| Arthrobacter sp. SCS2-P8 16S ribosomal RNA gene, partial sequence
TGCAAGTCGACGATGAACCTCGCTTGCGGGGGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTTGGAAACGGGGTCTAATACTGGATATTGACT
TACTGCCGCATGGTGGAAAGTTGAAAGATTTATTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCG
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAAC
CTCTTTCAGTAGGGAACAAGGCCAGTGTTTAGCTGGTTGAGGGTACTTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTG
GGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCG
GTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTA
AACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTGCTAGATCGCCATAGAAATACCTGGAAACAGGTGCCCCGCTTGCGGTCGGTTTACAGGTGGTGCATGGTTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTG
AGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATT
GGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCA

>gi|816379811|gb|KR023938.1| Arthrobacter sp. SCS2-P14 16S ribosomal RNA gene, partial sequence
TGCAGTCGAACGATGAACCCCGCTTGCGGGGGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTTGGAAACGGGGTCTAATACTGGATATTGACT
TACTGCCGCATGGTGGAAAGTTGAAAGATTTATTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCG
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAAC
CTCTTTCAGTAGGGAACAAGGCCAGTGTTTAGCTGGTTGAGGGTACTTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATT
GGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGC
GTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTA
AACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTGCTAGATCGCCATAGAAATACCTGGAAACAGGTGCCCCGCTTGCGGTCGGTTTACAGGTGGTGCATGGTTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTG
AGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATT
GGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCA

>gi|816379812|gb|KR023939.1| Arthrobacter sp. SCS3-P1 16S ribosomal RNA gene, partial sequence
TGCAAGTCGAACGATGAACCCCGCTTGCGGGGGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTTGGAAACGAGGTCTAATACTGGATATTGAC
TTTACCTCGCATGGGGTTTTGTTGAAAGATTTATTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACC
GGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAA
CCTCTTTCAGTAGGGAACAAGGCCAGCATTTTTGTTGGTTGAGGGTACTTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTAT
TGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAG
CGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCG
TAAACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGC
ACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTGCTAGATCGCCATAGAAATACCTGGAAACAGGTGCCCCGCTTGCGGTCGGTTTACAGGTGGTCATGGTTG
TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTTATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGG
TGAGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGA
TTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCA

>gi|816379813|gb|KR023940.1| Arthrobacter sp. SCS3-P3 16S ribosomal RNA gene, partial sequence
TGCAGTCGACGATGAACCCCGCTTGCGGGGGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTTGGAAACGGGGTCTAATACTGGATATTGACTT
ACTGCCGCATGGTGGAAAGTTGAAAGATTTATTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACC
TCTTTCAGTAGGGAACAAGGCCAGTGTTTAGCTGGTTGAGGGTACTTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGG
GCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCGG
TGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAA
ACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACA
AGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGAACCGGAAATACCTGGAAACAGGTGCCCCGCTTGCGGTCGGTTTACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGA
GGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTG
GGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAG

>gi|816379814|gb|KR023941.1| Arthrobacter sp. SCS3-P8 16S ribosomal RNA gene, partial sequence
TGCAGTCGAACGATGAACCCCGCTTGCGGGGGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTTGGAAACGAGGTCTAATACTGGATATTGACT
TTACCTCGCATGGGGTTTTGTTGAAAGATTTATTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCG
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAAC
CTCTTTCAGTAGGGAACAAGGCCAGCATTTTTGTTGGTTGAGGGTACTTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATT
GGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGC
GGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGT
AAACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCA
CAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGAACCGGAAATACCTGGAAACAGGTGCCCCGCTTGCGGTCGGTTTACAGGTGGTGCATGGTT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTTATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGT
GAGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGAT
TGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCA

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>gi|816379815|gb|KR023942.1| Arthrobacter sp. MVS1-P4 16S ribosomal RNA gene, partial sequence
TGCAGTCGAGCGATGAACTCCAGCTTGCTGGGGGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACTGGATATTGA
CTTTTCACCGCATGGTGGTTGGTTGAAAGATTTATTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGAC
CGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAA
ACCTCTTTCAGTAGGGAACAAGGCCAGCATTTTTGTTGGTTGAGGGTACTTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTA
TTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACTCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTA
GCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCC
GTAAACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGAACTGGAAACACCTGGAAACAGGTGCCCCGCTTGCGGTCGGTTTACAGGTGGTGCATGG
TTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAG
GTGAGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGG
ATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGAT

>gi|816379816|gb|KR023943.1| Arthrobacter sp. SCS1-L1P1 16S ribosomal RNA gene, partial sequence
TGCAAGTCGACGATGACTTTTGTGCTTGCACAGAATGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCTGACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATATG
CACCGTTGACCGCATGGTTTTTGGTGGAAAGATTTTTTGGTCAGGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTG
ACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGT
AAACCTCTTTCAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCGTAA
AGAGCTCGTAGGCGGTTTGTCGCGTCTATCGTGAAAGTCCGAGGCTCAACCTCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAAT
GCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTG
GGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGC
GGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTGCCAGACCGCCGTGGAAACACGGTTTCCCCTTTGGGGCTGGTTCACAGGTGGTGCATGGTTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCGCGTAAAGGCGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACG
ACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTC
TGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGA

Table A-3a-3: Source Identifier of isolated soil communities found underneath seal carcasses in Antarcitca.

Sequence_ID	collected-by	collection-date	country	identified-by	isolate	isolation-source	latitude-longitude	specimen-voucher	strain
SLS1-G2	D.A. Cowan	Jan-2012	Antarctica: Shangri-La	J.H. Kuegler	Shangri-La Seal 1-Glucose 2	seal carcass exposed soil	78.062 S 163.780 E	KIT_IBLT_TeBi:antarcticsealsoil:SLS1-G2	SLS1-G2
SLS1-G3	D.A. Cowan	Jan-2012	Antarctica: Shangri-La	J.H. Kuegler	Shangri-La Seal 1-Glucose 3	seal carcass exposed soil	78.062 S 163.780 E	KIT_IBLT_TeBi:antarcticsealsoil:SLS1-G3	SLS1-G3
SLS1-G4	D.A. Cowan	Jan-2012	Antarctica: Shangri-La	J.H. Kuegler	Shangri-La Seal 1-Glucose 4	seal carcass exposed soil	78.062 S 163.780 E	KIT_IBLT_TeBi:antarcticsealsoil:SLS1-G4	SLS1-G4
SLS1-G5	D.A. Cowan	Jan-2012	Antarctica: Shangri-La	J.H. Kuegler	Shangri-La Seal 1-Glucose 5	seal carcass exposed soil	78.062 S 163.780 E	KIT_IBLT_TeBi:antarcticsealsoil:SLS1-G5	SLS1-G5
SCS1-G8	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 1-Glucose 8	seal carcass exposed soil	78.063 S 163.858 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS1-G8	SCS1-G8
SCS1-G9	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 1-Glucose 9	seal carcass exposed soil	78.063 S 163.858 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS1-G9	SCS1-G9
SCS2-G2	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 2-Glucose 2	seal carcass exposed soil	78.067 S 163.862 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS2-G2	SCS2-G2
SCS3-G3	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 3-Glucose 3	seal carcass exposed soil	78.067 S 163.864 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS3-G3	SCS3-G3
SCS3-G4	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 3-Glucose 4	seal carcass exposed soil	78.067 S 163.864 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS3-G4	SCS3-G4
MVS1-G1	D.A. Cowan	Jan-2012	Antarctica: Miers Valley	J.H. Kuegler	Miers Valley Seal 1-Glucose 1	seal carcass exposed soil	78.097 S 163.756 E	KIT_IBLT_TeBi:antarcticsealsoil:MVS1-G1	MVS1-G1
MVS1-G3	D.A. Cowan	Jan-2012	Antarctica: Miers Valley	J.H. Kuegler	Miers Valley Seal 1-Glucose 3	seal carcass exposed soil	78.097 S 163.756 E	KIT_IBLT_TeBi:antarcticsealsoil:MVS1-G3	MVS1-G3
MVS1-G4	D.A. Cowan	Jan-2012	Antarctica: Miers Valley	J.H. Kuegler	Miers Valley Seal 1-Glucose 4	seal carcass exposed soil	78.097 S 163.756 E	KIT_IBLT_TeBi:antarcticsealsoil:MVS1-G4	MVS1-G4
MVS1-G8	D.A. Cowan	Jan-2012	Antarctica: Miers Valley	J.H. Kuegler	Miers Valley Seal 1-Glucose 8	seal carcass exposed soil	78.097 S 163.756 E	KIT_IBLT_TeBi:antarcticsealsoil:MVS1-G8	MVS1-G8
MVS1-G9	D.A. Cowan	Jan-2012	Antarctica: Miers Valley	J.H. Kuegler	Miers Valley Seal 1-Glucose 9	seal carcass exposed soil	78.097 S 163.756 E	KIT_IBLT_TeBi:antarcticsealsoil:MVS1-G9	MVS1-G9
SCS1-N3	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 1-Nutrient 3	seal carcass exposed soil	78.063 S 163.858 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS1-N3	SCS1-N3
SCS1-N5	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 1-Nutrient 5	seal carcass exposed soil	78.063 S 163.858 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS1-N5	SCS1-N5
SCS2-N2	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 2-Nutrient 2	seal carcass exposed soil	78.067 S 163.862 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS2-N2	SCS2-N2
SCS2-N3	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 2-Nutrient 3	seal carcass exposed soil	78.067 S 163.862 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS2-N3	SCS2-N3
SCS3-N2	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 3-Nutrient 2	seal carcass exposed soil	78.067 S 163.864 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS3-N2	SCS3-N2
SCS3-N3	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 3-Nutrient 3	seal carcass exposed soil	78.067 S 163.864 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS3-N3	SCS3-N3
SCS3-N5	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 3-Nutrient 5	seal carcass exposed soil	78.067 S 163.864 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS3-N5	SCS3-N5
SCS3-N7	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 3-Nutrient 7	seal carcass exposed soil	78.067 S 163.864 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS3-N7	SCS3-N7
MVS1-N6	D.A. Cowan	Jan-2012	Antarctica: Miers Valley	J.H. Kuegler	Miers Valley Seal 1-Nutrient 6	seal carcass exposed soil	78.097 S 163.756 E	KIT_IBLT_TeBi:antarcticsealsoil:MVS1-N6	MVS1-N6
SLS1-O0	D.A. Cowan	Jan-2012	Antarctica: Shangri-La	J.H. Kuegler	Shangri-La Seal 1-Oil 0	seal carcass exposed soil	78.062 S 163.780 E	KIT_IBLT_TeBi:antarcticsealsoil:SLS1-O0	SLS1-O0
SLS1-O4	D.A. Cowan	Jan-2012	Antarctica: Shangri-La	J.H. Kuegler	Shangri-La Seal 1-Oil 4	seal carcass exposed soil	78.062 S 163.780 E	KIT_IBLT_TeBi:antarcticsealsoil:SLS1-O4	SLS1-O4
SCS1-O2	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 1-Oil 2	seal carcass exposed soil	78.063 S 163.858 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS1-O2	SCS1-O2
SCS1-O4	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 1-Oil 4	seal carcass exposed soil	78.063 S 163.858 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS1-O4	SCS1-O4
SCS2-O2	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 2-Oil 2	seal carcass exposed soil	78.067 S 163.862 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS2-O2	SCS2-O2
SCS2-O6	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 2-Oil 6	seal carcass exposed soil	78.067 S 163.862 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS2-O6	SCS2-O6
SCS3-O1	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 3-Oil 1	seal carcass exposed soil	78.067 S 163.864 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS3-O1	SCS3-O1
SCS3-O3	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 3-Oil 3	seal carcass exposed soil	78.067 S 163.864 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS3-O3	SCS3-O3
SCS3-O4	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 3-Oil 4	seal carcass exposed soil	78.067 S 163.864 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS3-O4	SCS3-O4
SCS3-O6	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 3-Oil 6	seal carcass exposed soil	78.067 S 163.864 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS3-O6	SCS3-O6
SCS3-O7	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 3-Oil 7	seal carcass exposed soil	78.067 S 163.864 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS3-O7	SCS3-O7
SCS3-O8	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 3-Oil 8	seal carcass exposed soil	78.067 S 163.864 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS3-O8	SCS3-O8
MVS1-O2	D.A. Cowan	Jan-2012	Antarctica: Miers Valley	J.H. Kuegler	Miers Valley Seal 1-Oil 2	seal carcass exposed soil	78.097 S 163.756 E	KIT_IBLT_TeBi:antarcticsealsoil:MVS1-O2	MVS1-O2
MVS1-O3	D.A. Cowan	Jan-2012	Antarctica: Miers Valley	J.H. Kuegler	Miers Valley Seal 1-Oil 3	seal carcass exposed soil	78.097 S 163.756 E	KIT_IBLT_TeBi:antarcticsealsoil:MVS1-O3	MVS1-O3
MVS1-O4	D.A. Cowan	Jan-2012	Antarctica: Miers Valley	J.H. Kuegler	Miers Valley Seal 1-Oil 4	seal carcass exposed soil	78.097 S 163.756 E	KIT_IBLT_TeBi:antarcticsealsoil:MVS1-O4	MVS1-O4
MVS1-O5	D.A. Cowan	Jan-2012	Antarctica: Miers Valley	J.H. Kuegler	Miers Valley Seal 1-Oil 5	seal carcass exposed soil	78.097 S 163.756 E	KIT_IBLT_TeBi:antarcticsealsoil:MVS1-O5	MVS1-O5
SCS3-L2O2a	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 3-Oil liquid2 2a	seal carcass exposed soil	78.067 S 163.864 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS3-L2O2a	SCS3-L2O2a
SCS3-L2O2b	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 3-Oil liquid2 2b	seal carcass exposed soil	78.067 S 163.864 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS3-L2O2b	SCS3-L2O2b
SCS3-L2O3	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 3-Oil liquid2 3	seal carcass exposed soil	78.067 S 163.864 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS3-L2O3	SCS3-L2O3
SLS1-P7	D.A. Cowan	Jan-2012	Antarctica: Shangri-La	J.H. Kuegler	Shangri-La Seal 1-Paraffin 7	seal carcass exposed soil	78.062 S 163.780 E	KIT_IBLT_TeBi:antarcticsealsoil:SLS1-P7	SLS1-P7
SLS1-P8	D.A. Cowan	Jan-2012	Antarctica: Shangri-La	J.H. Kuegler	Shangri-La Seal 1-Paraffin 8	seal carcass exposed soil	78.062 S 163.780 E	KIT_IBLT_TeBi:antarcticsealsoil:SLS1-P8	SLS1-P8
SLS1-P10	D.A. Cowan	Jan-2012	Antarctica: Shangri-La	J.H. Kuegler	Shangri-La Seal 1-Paraffin 10	seal carcass exposed soil	78.062 S 163.780 E	KIT_IBLT_TeBi:antarcticsealsoil:SLS1-P10	SLS1-P10
SLS1-P11	D.A. Cowan	Jan-2012	Antarctica: Shangri-La	J.H. Kuegler	Shangri-La Seal 1-Paraffin 11	seal carcass exposed soil	78.062 S 163.780 E	KIT_IBLT_TeBi:antarcticsealsoil:SLS1-P11	SLS1-P11
SLS1-P13	D.A. Cowan	Jan-2012	Antarctica: Shangri-La	J.H. Kuegler	Shangri-La Seal 1-Paraffin 13	seal carcass exposed soil	78.062 S 163.780 E	KIT_IBLT_TeBi:antarcticsealsoil:SLS1-P13	SLS1-P13
SCS2-P1	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 2-Paraffin 1	seal carcass exposed soil	78.067 S 163.862 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS2-P1	SCS2-P1
SCS2-P6	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 2-Paraffin 6	seal carcass exposed soil	78.067 S 163.862 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS2-P6	SCS2-P6
SCS2-P8	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 2-Paraffin 8	seal carcass exposed soil	78.067 S 163.862 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS2-P8	SCS2-P8
SCS2-P14	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 2-Paraffin 14	seal carcass exposed soil	78.067 S 163.862 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS2-P14	SCS2-P14
SCS3-P1	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 3-Paraffin 1	seal carcass exposed soil	78.067 S 163.864 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS3-P1	SCS3-P1
SCS3-P3	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 3-Paraffin 3	seal carcass exposed soil	78.067 S 163.864 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS3-P3	SCS3-P3
SCS3-P8	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 3-Paraffin 8	seal carcass exposed soil	78.067 S 163.864 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS3-P8	SCS3-P8
MVS1-P4	D.A. Cowan	Jan-2012	Antarctica: Miers Valley	J.H. Kuegler	Miers Valley Seal 1-Paraffin 4	seal carcass exposed soil	78.097 S 163.756 E	KIT_IBLT_TeBi:antarcticsealsoil:MVS1-P4	MVS1-P4
SCS1-L1P1	D.A. Cowan	Jan-2012	Antarctica: Seal Cliff	J.H. Kuegler	Seal Cliff Seal 1-Paraffin liq.1 1	seal carcass exposed soil	78.063 S 163.858 E	KIT_IBLT_TeBi:antarcticsealsoil:SCS1-L1P1	SCS1-L1P1

3b Biosurfactant producing prokaryotes inhabiting raised bog peat

A-3b-1

Table A-3b-1: Complete list of isolated peat-bog strains and NCBI accession numbers for identified strains

#	strain	NCBI accession no.
1	**111A**	-
2	**111B**	-
3	**111C**	-
4	**111D**	-
5	***Janthinobacterium* sp. 112A**	KP642150
6	**112B**	-
7	***Janthinobacterium* sp. 112C**	KP642151
8	**112D**	-
9	**113A**	-
10	**113B**	-
11	***Pseudomonas* sp. 114**	-
12	***Serratia* sp. 210**	KP642152
13	***Rouxiella* sp 213**	KP642153
14	***Serratia* sp. 214**	KP642154
15	***Serratia* sp. 221**	KP642155
16	***Serratia* sp. 222**	KP642156
17	***Rouxiella* sp. 223**	KP642157
18	**225**	-
19	***Pseudomonas* sp. 230**	KP642158
20	***Citrobacter* sp. 322**	KP642160
21	***Rouxiella* sp. 323 DSM 100043**	KP642161
22	***Serratia* sp. 324**	KP642162
23	***Serratia* sp. 411**	KP642163
24	***Rouxiella* sp. 421**	KP642164
25	***Pseudomonas* sp. 423**	KP642165
26	**321**	-

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Table A-3b-2: 16S ribosomal RNA gene partial sequence of peat communities isolated from a raised bog in the northern Black Forest. Identifier numbers: gi=NCBI PropSet; gb=NCBI GenBank

>gi|816379842|gb|KP642150.1| Janthinobacterium sp. 112A 16S ribosomal RNA gene, partial sequence
CGGAGCTTGCTCTGGTGGCGAGTGGCGAACGGGTGAGTAATATATCGGAACGTACCCTGGAGTGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATACGATCTAAGGATGAAAGTGGGGG
ATCGCAAGACCTCATGCTCGTGGAGCGGCCGATATCTGATTAGCTAGTTGGTAGGGTAAAAGCCTACCAAGGCATCGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGAC
ACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAA
ACGGTGAGAGCTAATATCTcTTGCTAATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC
GTGCGCAGGCGGTTTTGTAAGTCTGATGTGAAATCCCCGGGCTCAACCTGGGAATTGCATTGGAGACTGCAAGGCTAGAATCTGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCG
TAGATATGTGGAGGAACACCGATGGCGAAGGCAGCCCCCTGGGTCAAGATTGACGCTCATGCACGAAAGCGTGGGGAGCAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCTA
CTAGTTGTCGGGTCTTAATTGACTTGGTAACGCAGCTAACGCGTGAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGAT
GTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGGATGGAATCCCGGAGAGATTTGGGAGTGCTCGAAAGAGAACCATTACACAGGTGCTGCATGGCTGTCGTCAGCTCG
TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCTACGAAAGGGCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCT
CATGGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTACATACAGAGCGCCGCCAACCCGCGAGGGGGAGCTAATCGCAGAAAGTGTATCGTAGTCCGGATTGTAGTCTGCAACTCGACT
GCA

>gi|816379843|gb|KP642151.1| Janthinobacterium sp. 112C 16S ribosomal RNA gene, partial sequence
GGCAGCACGGAGCTTGCTCTGGTGGCGAGTGGCGAACGGGTGAGTAATATATCGGAACGTACCCTGGAGTGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATACGATCTAAGGATGAAA
GTGGGGGATCGCAAGACCTCATGCTCGTGGAGCGGCCGATATCTGATTAGCTAGTTGGTAGGGTAAAAGCCTACCAAGGCATCGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAA
CTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAG
GGAAGAAACGGTGAGGGCTAATATCTTTTGCTAATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGC
GTAAAGCGTGCGCAGGCGGTTTTGTAAGTCTGATGTGAAATCCCCGGGCTCAACCTGGGAATTGCATTGGAGACTGCAAGGCTAGAATCTGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTG
AAATGCGTAGATATGTGGAGGAACACCGATGGCGAAGGCAGCCCCCTGGGTCAAGATTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAAC
GATGTCTACTAGTTGTCGGGTCTTAATTGACTTGGTAACGCAGCTAACGCGTGAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGG
TGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGGATGGAATCCCGGAGAGATTTGGGAGTGCTCGAAAGAGAACCATTACACAGGTGCTGCATGGCTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCTACGAAAGGGCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGT
CAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTACATACAGAGCGCCGCCAACCCGCGAGGGGGAGCTAATCGCAGAAAGTGTATCGTAGTCCGGATTGTAGTCTGCA
ACTCGACTGCATGAAGTNGGAATCGCT

>gi|816379844|gb|KP642152.1| Serratia sp. 210 16S ribosomal RNA gene, partial sequence
GAGCGGTAGCACAGGAGAGCTTGCTCTCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCT
TCGGACCAAAGTGGGGGACCTTCGGGCCTCACGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC
CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCA
CTTTCAGCGAGGAGGAAGGGTAGTGTTAATAGCACATTGCATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGA
ATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGCTTAACGTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGG
TGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCTGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCACAGAATTCGGCAGAGATGCCTTAGTGCCTTCGGGAACTGTGAGACAGGTGCTGCA
TGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCACGTAATGGTGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGG
AAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAAAGAGAAGCGAACTCGCGAGAGCCAGCGGACCTCATAAAGTACGTCGTAGTC
CGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCGGAATC

>gi|816379845|gb|KP642153.1| Rouxiella sp. 213 16S ribosomal RNA gene, partial sequence
GCAGTCGAGCGGTAGCACGGGAGAGCTTGCTCTCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATG
ATGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCACGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATG
ACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTAGGGTTGT
AAAGCACTTTCAGCGAGGAGGAAGGGTAGTGTTAATAACTGCAACGATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTA
ATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGAGCTTAACTTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAAT
TCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT
AGTCCACGCTGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACG
GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATTCGCTAGAGATAGCTTAGTGCCTTCGGGAACTCTGAGACAGG
GCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCACGTAATGGTGGGAACTCAAAGGAGACTGCCGGTGATAAAC
CGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGAACTCGCGAGAGCAAGCGGACCTCATAAAGTATGTC
GTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGC

>gi|816379845|gb|KP642153.1| Rouxiella sp. 213 16S ribosomal RNA gene, partial sequence
TGCaAGTCGAGCGGTAGCACAGGAGAgCTTGCTCTCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCA
TGACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCACGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGA
TGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTAGGGTT
GTAAAGCACTTTCAGCGAGGAGGAAGGGTAGTGTGTTAATAGCACATTGCATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGT
TAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGAGCTTAACTTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGA
ATTCCAGGTGTAgCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCaGGTGCGAAAGCGTGGGGAGCAAACAGGATtaGATACCCTG
GTAGTCCACGCTGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATTCGCTAGAGATAGCTTAGTGCCTTCGGGAACTCTGAGACAG
GTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGAGTAATGTgCtGGGAACTCAAAGGAGACTGCCGGTGAT
AAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGAACTCGCGAGAGCAAGCGGACCTCATAAAGTA

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>gi|816379847|gb|KP642155.1| Serratia sp. 221 16S ribosomal RNA gene, partial sequence
GTCGAGCGGTAGCACAGAGAGAGCTTGCTCTCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGGATAACTACTGGAAACGGTAGCTAAATACCGCATAA
CGTCTACGGACCAAAGTGGGGGACCTTCGGGCCTCACGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGA
CCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAA
AAGCACTTTCAGCGAGGAGGAAGGGTTCAGTGTTAATAGCACTGTTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAA
TCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTTAACGTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAATT
CCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGG
GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATTCGCTAGAGATAGCTTAGTGCCTTCGGGAACTCTGAGACAGGTG
CTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGATTCGGTCGGGAACTCAAAGGAGACTGCCGGTGATAAACCG
GAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTA

>gi|816379848|gb|KP642156.1| Serratia sp. 222 16S ribosomal RNA gene, partial sequence
GTCGAGCGGTAGCaCAGGAGAGCTTGCTCTCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTTAAACCGCATAACG
TCTACGGACCAAAGTGGGGGACCTTCGGGCCTCACGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGA
AGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTAGGGTTGTAAA
GCACTTTCAGCGAGGAGGAAGGGTTCAGTGTTAATAGCACTGTTCATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATC
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>gi|816379849|gb|KP642157.1| Serratia sp. 223 16S ribosomal RNA gene, partial sequence
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>gi|816379850|gb|KP642158.1| Pseudomonas sp. 230 16S ribosomal RNA gene, partial sequence
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>gi|816379853|gb|KP642161.1| Rouxiella sp. 323 16S ribosomal RNA gene, partial sequence
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>gi|816379854|gb|KP642162.1| Serratia sp. 324 16S ribosomal RNA gene, partial sequence
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>gi|816379855|gb|KP642163.1| Serratia sp. 411 16S ribosomal RNA gene, partial sequence
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A-3b-2 part 3 of 3

>gi|816379856|gb|KP642164.1| Rouxiella sp. 421 16S ribosomal RNA gene, partial sequence
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>gi|816379857|gb|KP642165.1| Pseudomonas sp. 423 16S ribosomal RNA gene, partial sequence
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3c Glycolipids produced by *Rouxiella* sp. DSM 100043 and isolation of the biosurfactants via foam-fractionation

A-3c-1

Table A-3c-1: Masses present in purified fractions of foam extracts of *Rouxiella* sp. DSM 100043. Masses observed during ESI-ToF mass spectrometry of lithium chloride supplemented samples in positive mode; assigned lithium $[M_d^+Li^+]^+$ and sodium $[M_d^+Na^+]^+$ adduct ions led to its resulting neutral mass $[M_{neutral}]$. The strongest relative peak intensity and it's adduct ion type is indicated.

Subfraction	$[M_d + Li^+]^+$	$[M_d + Na^+]^+$	$[M_{neutral}]$	strongest relative peak intensity [%]
63-65 A	289.24	305.22	282.23	100 (Li^+)
	256.23	272.21	249.22	56 (Li^+)
	400.26	416.24	393.25	32 (Li^+)
	374.24	390.23	367.23	20 (Li^+)
63-65 B	212.19	228.16 / 230.20	205.17 / 207.21	100 (Li^+) / 70 (Na^+)
	238.21 / 240.22	254.18 / 256.21	231.19 / 233.20	67 (Li^+) / 54 (Na^+)
	307.22	323.19	300.20	52 (Li^+)
	271.24	287.22	264.22	33 (Li^+)
63-65 C	288.28 / 286.27	304.27 / 302.26	281.28 / 279.27	100 (Li^+) / 40 (Li^+)
	374.24	390.22	367.23	54 (Li^+)
	400.26	416.24	393.25	41 (Li^+)
63-65 D	288.28 / 286.27	304.26 / 302.26	281.28 / 279.27	100 (Li^+) / 41 (Li^+)
	213.17	229.14	206.15	56 (Li^+)
	383.23	399.20	376.21	39 (Na^+)
	357.21	373.18	350.19	35 (Na^+)
63-65 E	220.09	204.11	197.10	100 (Li^+)
	307.21 / 309.22	323.18 / 325.19	300.19 / 302.20	75 (Na^+) / 23 (Na^+)
	271.24	287.20	264.22	75 (Na^+)

A-3c-2

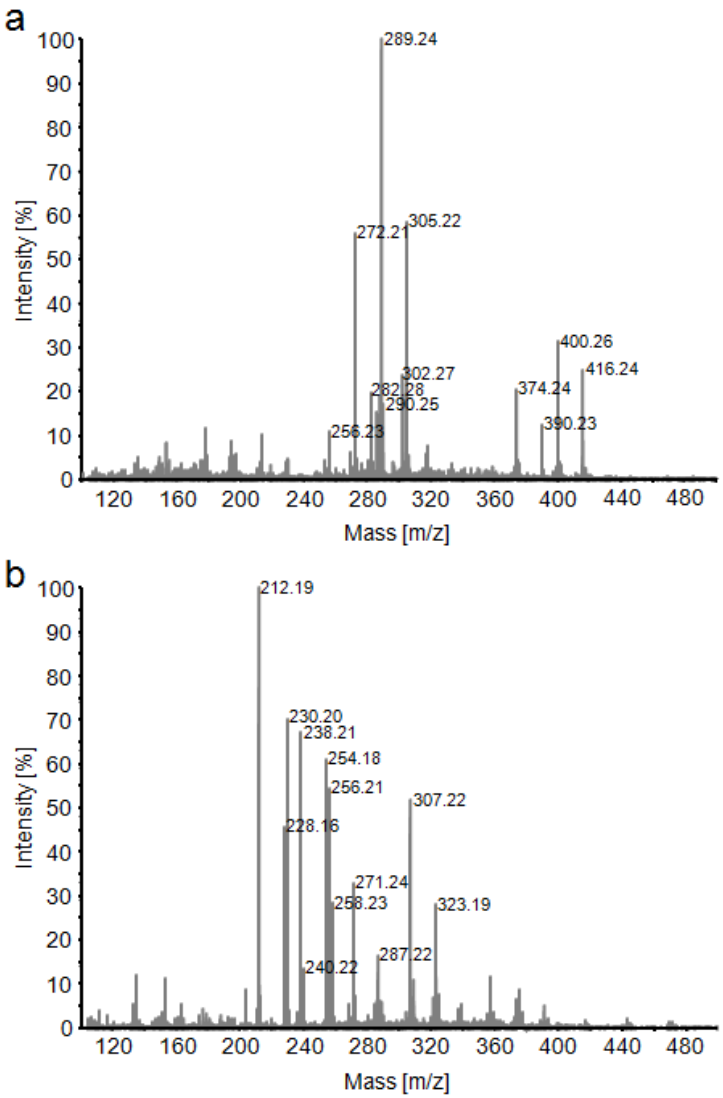

Figure A-3c-2: ESI-ToF mass spectrometry plot of purified foam extracts of *Rouxiella* sp. DSM 100043. Lithium chloride supplemented subfractions (a) 63-65 A and (b) 63-65 B measured in positive mode. Both lithium $[M_d{}^+Li^+]^+$ and sodium $[M_d{}^+Na^+]^+$ adduct ions are present

A-3c-3

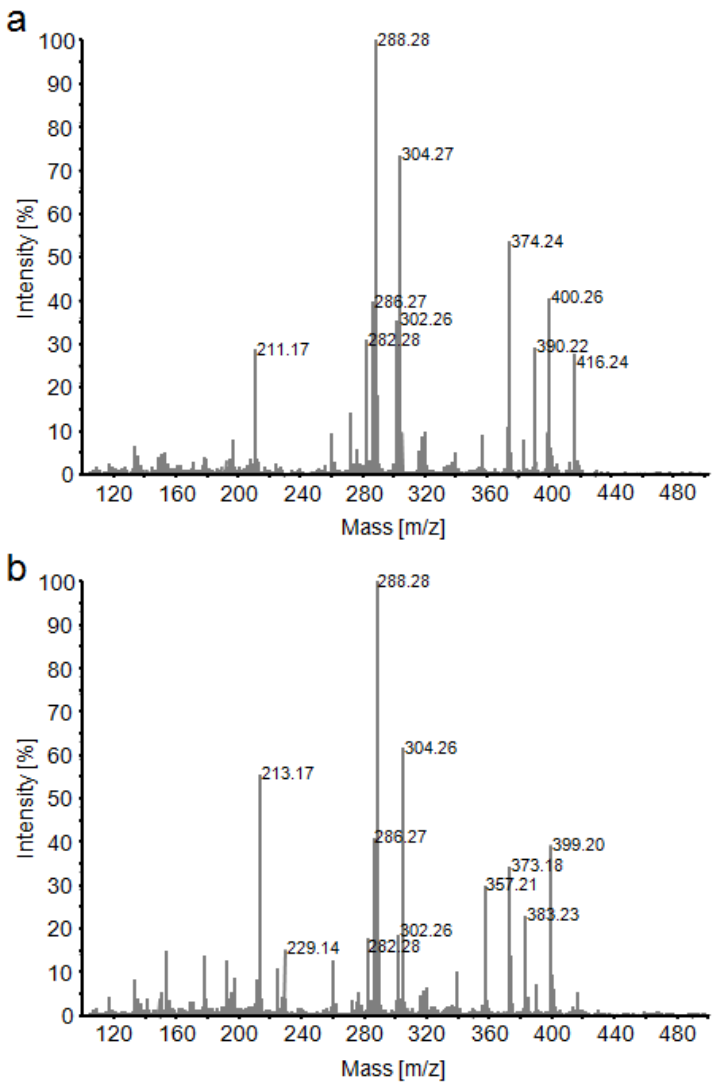

Figure A-3c-3: ESI-ToF mass spectrometry plot of purified foam extracts of *Rouxiella* sp. DSM 100043. Lithium chloride supplemented subfractions (a) 63-65 C and (b) 63-65 D measured in positive mode. Both lithium $[M_d{}^+Li^+]^+$ and sodium $[M_d{}^+Na^+]^+$ adduct ions are present

A-3c-4

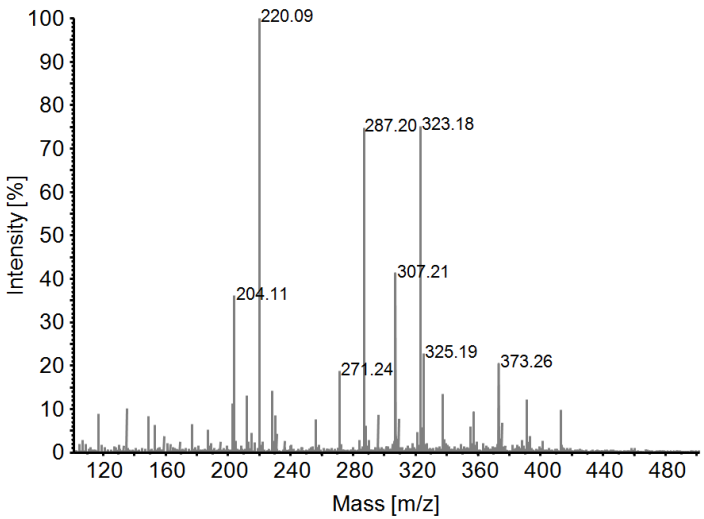

Figure A-3c-4: ESI-ToF mass spectrometry plot of purified foam extracts of *Rouxiella* sp. DSM 100043. Lithium chloride supplemented subfraction 63-65 E measured in positive mode. Both lithium $[M_d^+Li^+]^+$ and sodium $[M_d^+Na^+]^+$ adduct ions are present

A-3c-5

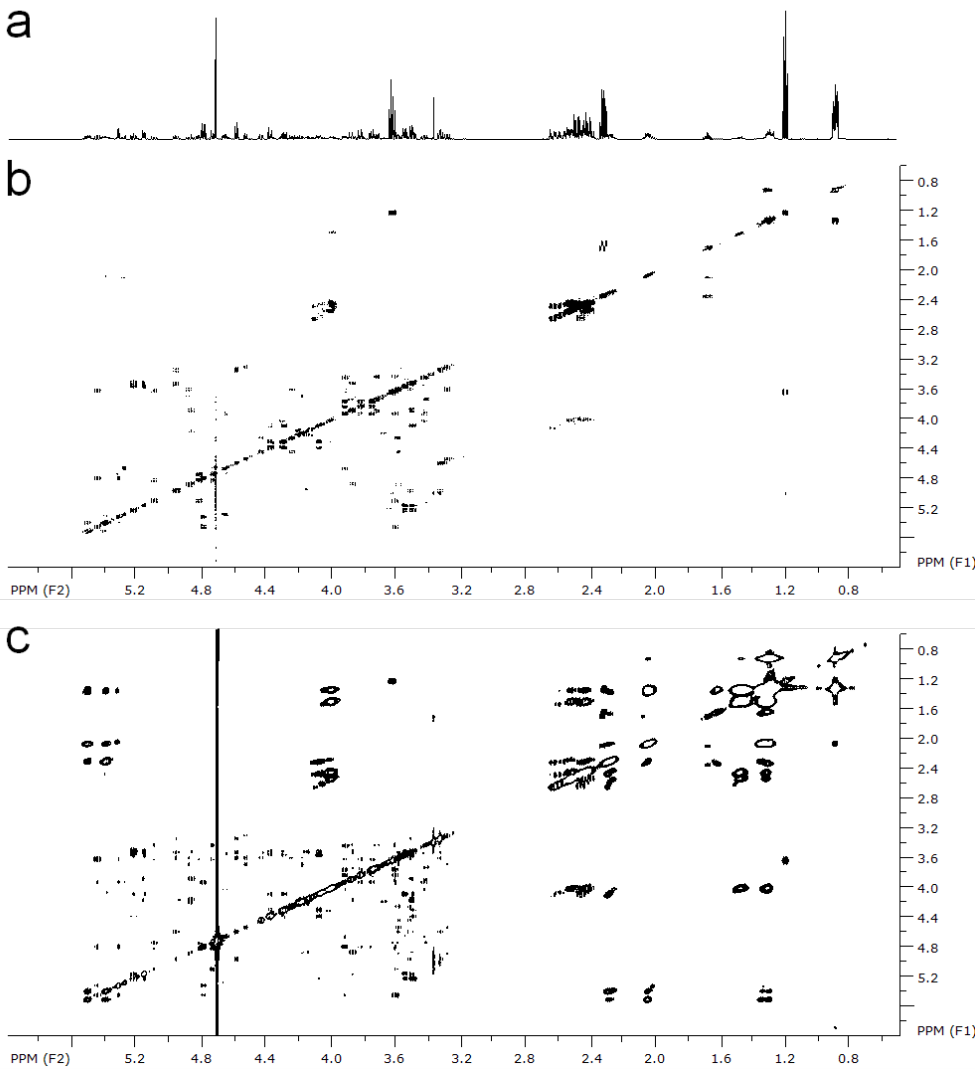

Figure A-3c-5: Full NMR spectra of Rouxiella sp. DSM 100043 glycolipids present in fractions 64-65. (a) ^{1}H, (b) $^{1}H/^{1}H$ COSY and (c) $^{1}H/^{1}H$ TOCSY spectra recorded from fractions 64-65 after removal of residual fatty acids

A-3c-6

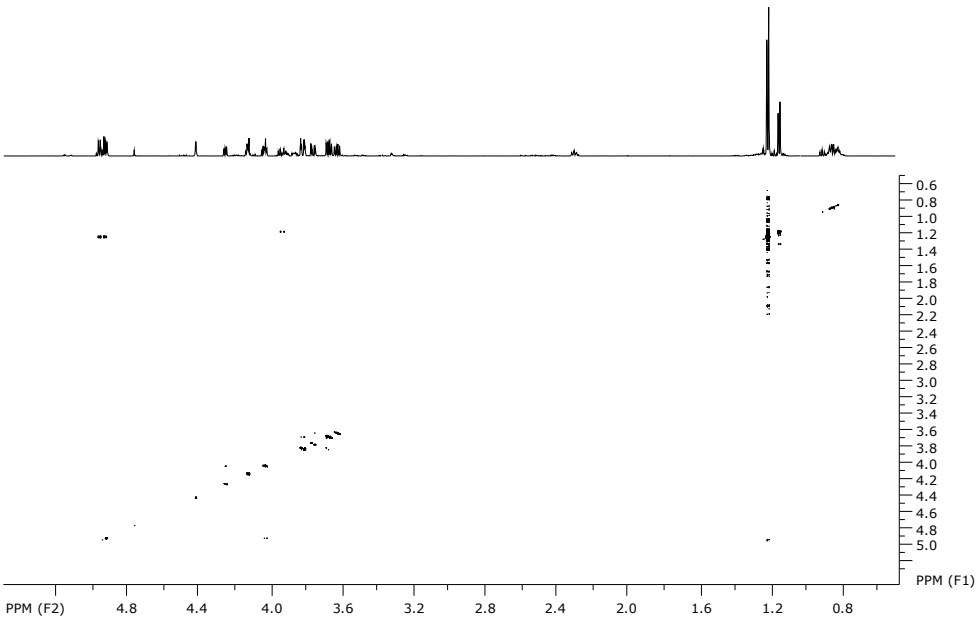

Figure A-3c-6: Full two dimensional $^{1}H/^{1}H$ correlated NMR spectra (COSY) of subfraction 63-67 E containing two talose moieties

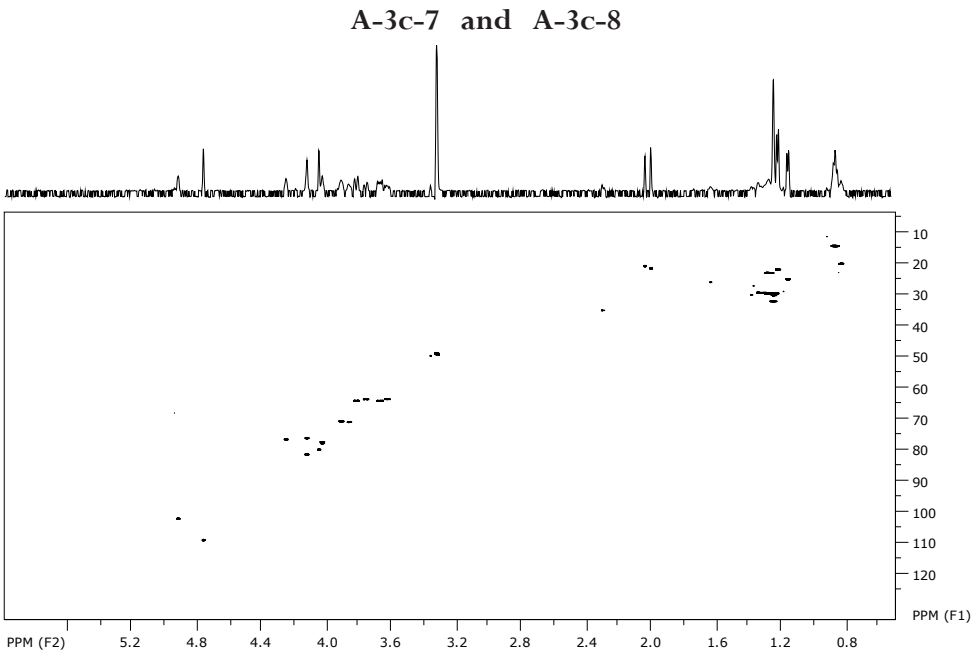

Figure A-3c-7: Two dimensional $^{1}H/^{13}C$ heteronuclear single quantum correlation spectroscopy (HSQC) NMR of subfraction 63-65 E containing two talose moieties

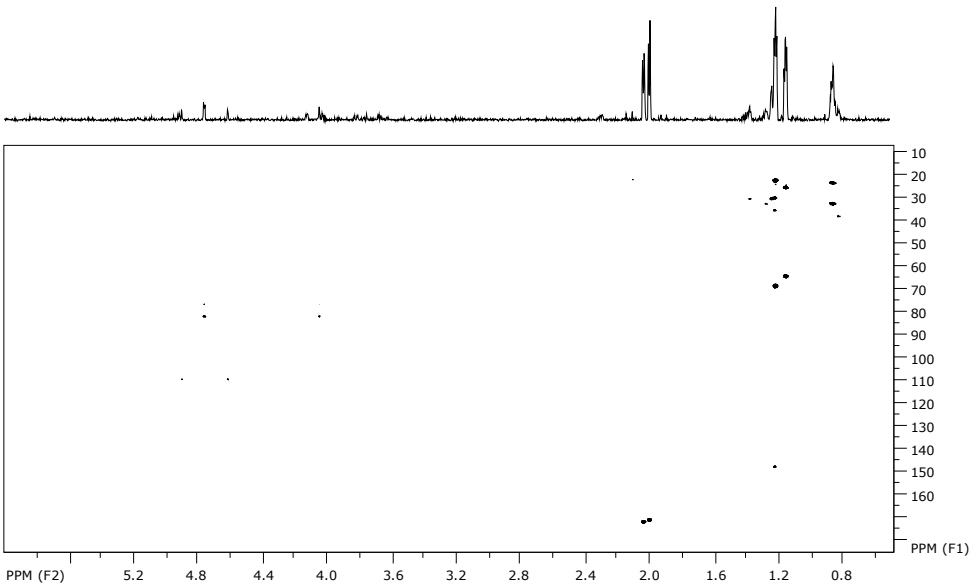

Figure A-3c-8: Two dimensional $^{1}H/^{13}C$ heteronuclear multiple bond correlation spectroscopy (HMBC) NMR of subfraction 63-65 E containing two talose moieties

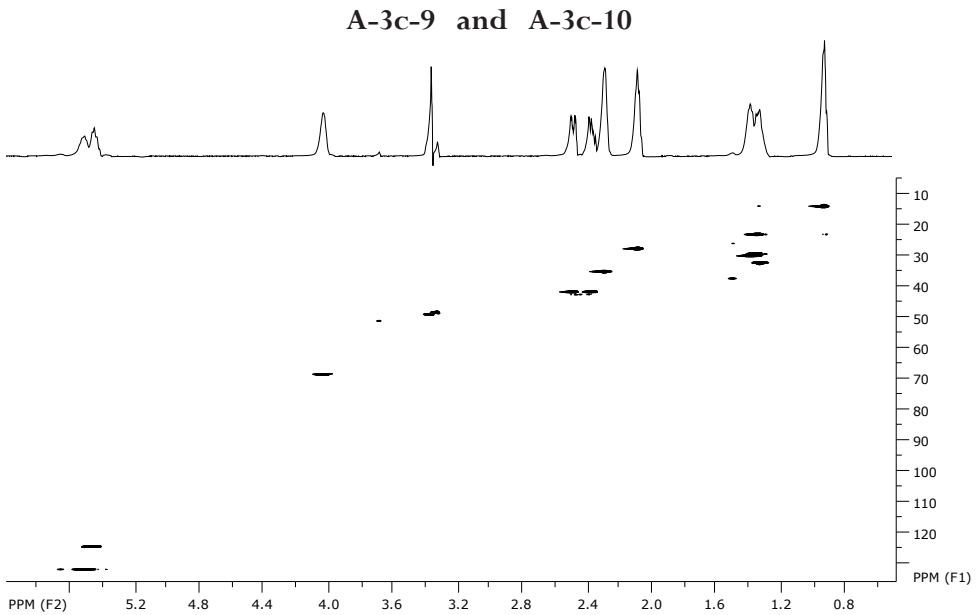

Figure A-3c-9: Two dimensional $^{1}H/^{13}C$ heteronuclear single quantum correlation spectroscopy (HSQC) NMR of samples 60-61 containing 3' hydroxyl lauroleic acid

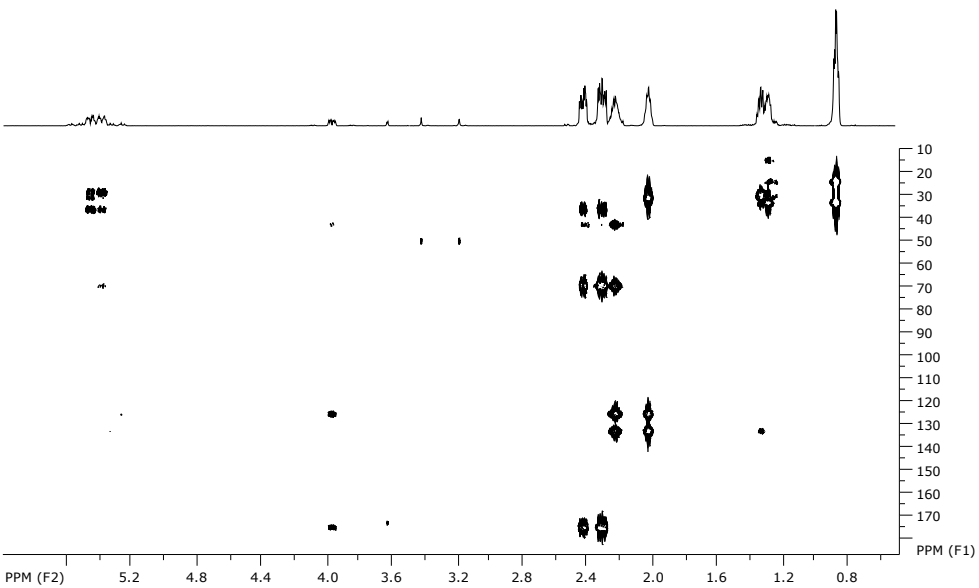

Figure A-3c-10: Two dimensional $^{1}H/^{13}C$ heteronuclear multiple bond correlation spectroscopy (HMBC) NMR of samples 60-61 containing 3' hydroxyl lauroleic acid

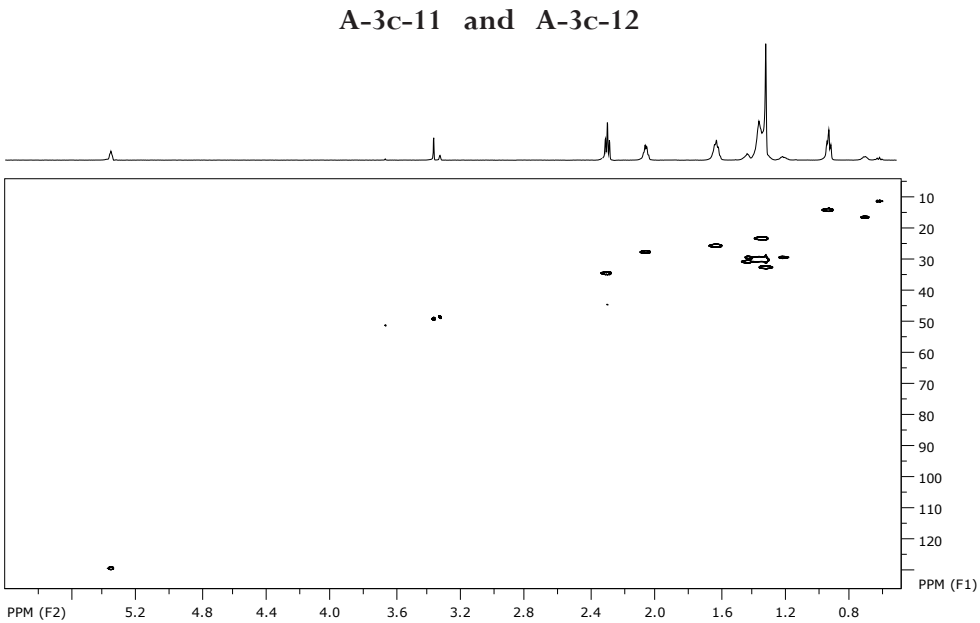

Figure A-3c-11: Two dimensional $^{1}H/^{13}C$ heteronuclear single quantum correlation spectroscopy (HSQC) NMR of samples 67-69 containing myristic and myristoleic acid

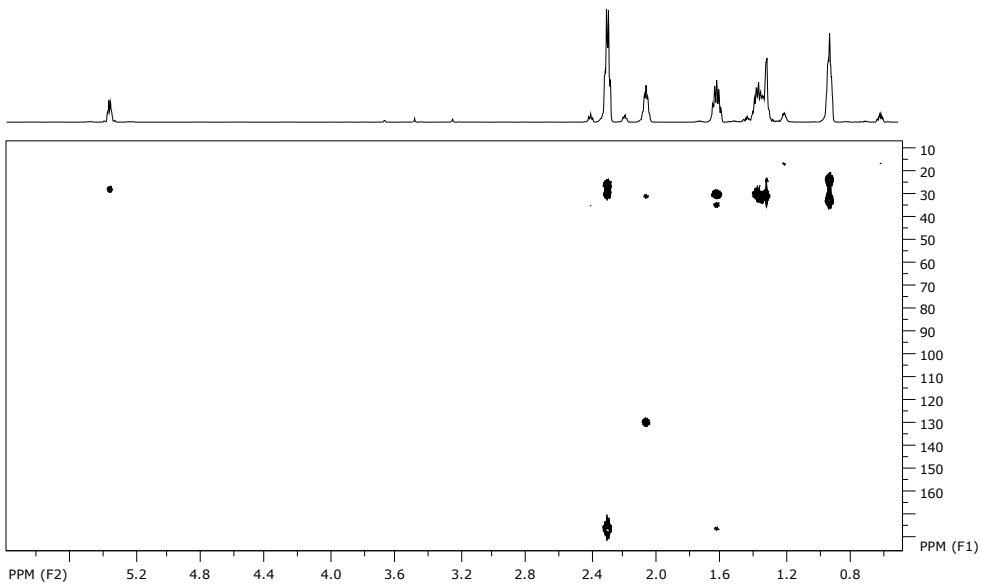

Figure A-3c-12: Two dimensional $^{1}H/^{13}C$ heteronuclear multiple bond correlation spectroscopy (HMBC) NMR of samples 67-69 containing myristic and myristoleic acid

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Academic background

2003	Abitur (A-level; University entrance degree) in Königswinter (GER) with stay abroad (08/00–02/01) in Ottawa (CAN)
2004-2006	Undergraduate studies (Vordiplom) of Biology (technical orientation) University of Stuttgart (GER)
2006-2009	Diplom-Biotechnologe / Diplôme d'Ingénieur en Biotechnologie Ecole Supérieure de Biotechnologie Strasbourg three national study program at Strasbourg engineering school of Biotechnology in cooperation with EUCOR upper-Rhine Universities: Karlsruhe Institute of Technology (GER) University of Basel (CH) Albert-Ludwigs University Freiburg (GER) University of Strasbourg (F)
2008-2009	Master of Science "Drug Engineering and Biotechnology" University of Strasbourg (F)
2011-2015	Doctoral thesis in Technical Biology, Institute of Process Engineering in Life Sciences, Faculty of Chemical- and Biological Engineering, Karlsruhe Institute of Technology (GER): „Screening, Production and characterization of extracellular microbial surfactants"

Working experience and internships

2003 Internship	AGFA-Photo AG, Leverkusen (GER) Mechanical and electrical apprentice, film and photographic paper production
2004 Internship	stUaK Cologne Environmental Agency, Bonn, (GER) Biological and chemical analysis; classification/surveillance of streaming water
2005-2006 Student assistance	University of Stuttgart (GER), Department of Zoology (Prof. Görz) Tutor for the course zoology
2007 Research internship	ETH Zürich (CH), Eawag aquatic research (Prof. Egli) Detection of pathogens by immunomagnetic separation and flow cytometry
2008 Research internship	University of Kyoto (JP), Graduate School of Medicine (Prof. Ito) Stem-and induced pluripotent cell differentiation and implantation into inner ear
2009 Diploma thesis	University of Queensland (AUS), Institute for Molecular Bioscience (Prof. Hankamer) Inverted repeat transgenes for down regulation of photosystems II major light harvesting complexes 1 and 2 in *Chlamydomonas reinhardtii*
2011-2015 Research associate	Karlsruhe Institute of Technology (GER), Process Engineering in Life Sciences, Section II: Technical Biology (Prof. Syldatk) Tutor for microbiology and enzyme technology, supervision of student thesis
2012 Research trainings	University of the Western Cape (RSA), IMBM (Prof. Tuffin) Cape Peninsula University of Technology (RSA), (Dr. LeRoes-Hill) Biosurfactant screening of extremophiles and actinobacteria; metagenomics

Publications

Müller MM, Kügler JH, Henkel M, Gerlitzki M, Hörmann B, Pöhnlein M, Syldatk C, Hausmann R (2012). „Rhamnolipids—Next generation surfactants?“ *J. Biotech.* 162:366-380

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Kügler JH, Le Roes-Hill M, Syldatk C, Hausmann R (2015). Surfactants tailored by the class *Actinobacteria*. *Front. Microbiol.* 6:00212

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