Exploiting the natural products of novel myxobacteria: Phylogenetic and fatty acid perspectives and bioactive compound discovery

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List of Publications

1. **Garcia, R. O.,** D. Krug, and R. Müller. 2009. Discovering natural products from myxobacteria with emphasis on rare producer strains in combination with improved analytical methods, p. 59–91. *In* D. Hopwood (ed), Methods in enzymology: complex enzymes in microbial natural product biosynthesis. vol. 458., part A. Academic Press, Burlington.

2. Krug, D., G. Zurek, B. Schneider, **R. Garcia**, and R. Müller. 2008. Efficient mining of myxobacterial metabolite profiles enabled by liquid chromatography–electrospray ionisation-time-of-flight mass spectrometry and compound-based principal component analysis. Anal. Chim. Acta 624:97–106.

3. **Garcia, R. O.,** H. Reichenbach, M. W. Ring, and R. Müller. 2009. *Phaselicystis flava* gen. nov., sp. nov., an arachidonic acid-containing soil myxobacterium, and the description of *Phaselicystidaceae* fam. nov. Int. J. Syst. Evol. Microbiol. 59:1524–1530.

4. **Garcia, R.,** and R. Müller. *Minicystis rosea*, gen. nov., sp. nov., a pink myxobacterium. Int. J. Syst. Evol. Microbiol. Manuscript to be submitted.

5. **Garcia, R.,** Q. Xiao-Ming, M. Koch, and R. Müller. *Pseudochondromyces catenulatus* gen. nov., sp. nov., nom. rev., a rediscovery of '*Chondromyces catenulatus'* Thaxter, 1904. Int. J. Syst. Evol. Microbiol. Manuscript to be submitted.

6. **Garcia, R.,** M. Stadler, and R. Müller. *Aetherobacter fasciculatus,* sp. nov., *Aetherobacter rufus*, sp. nov., omega-3-rich polyunsaturated fatty acid-producing myxobacteria, and the description of *Aetherobacter* gen. nov. Int. J. Syst. Evol. Microbiol. Manuscript to be submitted.

7. **Garcia, R.,** K. Gerth, M. Stadler, I. J. Dogma Jr., and R. Müller. 2010. Expanded phylogeny of myxobacteria and evidence for cultivation of the 'unculturables'. Mol. Phylogenet. Evol. 57:878–887.

8. **Garcia, R.,** D. Pistorius, M. Stadler, and R. Müller. 2011. Fatty acid-related phylogeny of myxobacteria as an approach to discover polyunsaturated omega-3/6 fatty acids. J. Bacteriol. 193:1930–1942.

Other Publications

1**.** Mohr, K., **R. O. Garcia,** K. Gerth, H. Irschik, and R. Müller. *Sandaracinus amylolyticus* gen. nov., sp. nov., a starch degrading soil myxobacterium, and the description of *Sandaracinaceae*, fam. nov. Int. J. Syst. Evol. Microbiol. In press, DOI:10.1099/ijs.0.033696-0.

2. Gawas, D., **R. O. Garcia,** V. Huch, and R. Müller. 2011. A highly conjugated dihydroxylated C_{28} steroid from a myxobacterium. J. Nat. Prod. 74:1281–1283.

3. Simmons, L., K. Kaufmann, **R. Garcia,** G. Schwär, V. Huch, and R. Müller. Bendigoles D-F, novel anti-inflammatory sterols from the marine sponge-derived *Actinomadura* sp. SBMs009. Bioorgan. Med. Chem. 2011. In press, DOI:10.1016/j.bmc.2011.05.044.

4. Gawas, D., **R. O. Garcia,** J. Hermann, and R. Müller. A family of tyramine glycosides with cytotoxic activity from myxobacterial strain SBNa008. J. Nat. Prod. Manuscript submitted.

List of Patents

1. Synthetic enzymes for the production of Argyrins
Müller, R., S. Wenzel, and **R. Garcia**
2008. European Patent: 08159743.7 – 2405

2. Production of omega-3 fatty acids by myxobacteria
Stadler, M., E., Roemer, R. Müller, **R. Garcia**, D. Pistorius, A. Brachmann.
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Short Lectures / Oral Presentations

1. Pyxidicoccus: A novel source for anti-infectives
34th International Conference on the Biology of the Myxobacteria
Granada, Spain. July 14 -18, 2007

2. Cystobacter as multi- producer of cytotoxic and novel secondary metabolites
VAAM Workshop 'Biology of Bacteria Producing Natural Products'
Nonnweiler, Germany. October 4-6, 2007

3. Search for novel myxobacteria: Possibilities and prospects for novel compounds
VAAM Workshop 'Biology of Bacteria Producing Natural Products'
Technical University, Berlin, Germany. September 28-October 1, 2008

4. Biology of myxobacteria
The Graduate School, University of Santo Tomas
Manila, Philippines. February 2009

5. Myxobacteria as proficient source of novel secondary metabolites
First life science PhD student day
Saarland University, Saarbrücken, Germany. August 21, 2009

6. Comprehensive chemo-phylogeny of myxobacteria based on 16S rDNA and fatty acids
37th international Conference on the Biology of Myxobacteria
European Academy Otzenhausen, Nonnweiler, Germany. September 1, 2010

7. Novel compounds from novel genera of myxobacteria
Australian Society for Microbiology Annual Scientific Meeting & Exhibition
Sydney Convention & Exhibition Centre, Sydney, Australia. July 4-8, 2010

8. *Discovery and biotechnological potential of Aetherobacter gen nov ined. (Myxobacteria) for production of omega-3-polyunsaturated fatty acids (PUFAs) and novel secondary metabolites*
GenoMik-Transfer Statusseminar 2011
Göttingen, Germany. May 12-13, 2011

9. *Novel myxobacteria: Source of new bioactive compounds*
38th International Conference on the Biology of Myxobacteria
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Poster Presentation

Discovery of omega-3 fatty acids in myxobacteria
Australian Society for Microbiology Annual Scientific Meeting & Exhibition.
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Zusammenfassung

Myxobakterien synthetisieren vielfältige und interessante Sekundärmetabolite mit beeindruckenden biologischen Aktivitäten und Wirkmechanismen. Auf der Suche nach neuen bioaktiven Verbindungen wurden Proben aus der ganzen Welt mittels unterschiedlicher Kultivierungs- und Isolierungstechniken erforscht. Einige neue Isolate wurden erfolgreich kultiviert und repräsentieren neue Familien (*Phaselicystidaceae*) und neue Gattungen (*Phaselicystis*, *Aetherobacter*, *Minicystis, Pseudochondromyces*). 16S-rRNA-Gensequenzanalysen ergaben, dass sie die bisher 'unkultivierte' Gruppe von Myxobakterien zu vertreten scheinen.

Bei der chemischen Charakterisierung der neuen Isolate mittels GC-MS-Analyse wurden große Mengen mehrfach ungesättigter Fettsäuren (PUFAs) von hohem kommerziellem Wert nachgewiesen. Acht PUFAs, die verschiedene ω-3 und ω-6 Fettsäuren (FAs) umfassen, wurden erstmals in Myxobakterien identifiziert. Bei FA-Analysen und 16S-rRNA-Gensequenzanalysen erwiesen sich die Myxobakterien abermals als einheitliche Gruppe. Diese Feststellung bereitet nicht nur den Weg für die chemo-phylogenetische Zuordnung der Myxobakterien, sondern hilft ferner dabei potenzielle neue Stämme, die PUFAs produzieren, zu identifizieren.

Die vorliegende Arbeit hebt auch die Entdeckung neuer bioaktiver Verbindungen aus der neuen Art *Aetherobacter rufus* SBSr003^T hervor. Mittels semi-präparativer HPLC-Auftrennungen wurden zwei neuartige bioaktive Verbindungen gewonnen. NMR-Analysen ermöglichen derzeit die Strukturaufklärung dieser Verbindungen. Insgesamt wurde veranschaulicht, dass Myxobakterien als Modelorganismen für viele umfangreiche und vielversprechende Anwendungen dienen können.

Abstract

Myxobacteria synthesise diverse and interesting secondary metabolites with impressive biological activities and modes of action. In an effort to uncover new bioactive compounds, world-wide samples were explored using various cultivation and isolation techniques. Several novel isolates were successfully cultivated representing a new family (*Phaselicystidaceae*) and new genera (*Phaselicystis*, *Aetherobacter*, *Minicystis, Pseudochondromyces*). Based on 16S rRNA gene sequence analysis, they appear to represent the so-far 'uncultivated' group of myxobacteria.

During the chemical characterisation of novel isolates, large quantities of commercially valuable polyunsaturated fatty acids (PUFAs) were detected by GC-MS analysis. Eight PUFAs, comprising different ω-3 and ω-6 fatty acids (FAs), were identified for the first time in myxobacteria. Based on FA and 16S rRNA gene analyses, myxobacteria were again proven to be a coherent group. This finding not only pioneers the chemo-phylogenetic correlation of myxobacteria but also aids in the identification of potentially new PUFA-producing strains.

The study also highlights the discovery of new bioactive compounds in the novel species *Aetherobacter rufus* SBSr003^{T}. Semi-preparative HPLC separations yielded two novel bioactive compounds. Ongoing NMR analysis will enable elucidation of the compounds' structures. Overall, myxobacteria were exemplified as model organisms for many wide and promising applications.

CONTENTS

Chapter I. Introduction

Myxobacteria are one of the most fascinating Gram-negative spore-forming prokaryotes. They exhibit unique and complex life cycles leading to the formation of multicellular fruiting bodies (Shimkets *et al.,* 2006), a structure more commonly attributed to eukaryotic fungi. In over 30 years of work on myxobacteria, roughly 7,000 strains have been isolated, covering most of the described species and genera (Reichenbach, 2005). To date, 53 species of myxobacteria have been validly recognised (Table 1). The number of isolates discovered in the past appears to be a reflection of the efficiency of cultivation methods derived from standard microbial baiting and biomacromolecule degradation. Despite the success of these methods, however, there are still validly described myxobacterial strains which remain uncultivated, exemplified by *Haploangiun* and many species of *Polyangium* (Reichenbach, 2005; Peterson, 1959).

Metagenomic studies based on 16S rRNA gene unveal high similarities of many sequences to clones of uncultured bacteria, suggesting that myxobacteria are far more diversified than previously thought (Jiang *et al.,* 2007). Their presence in deep sea vents (Moyer, *et al.,* 1995), hydrothermal springs (Iizuka *et al.,* 2006), marine samples (Iizuka *et al.,*1998; Iizuka *et al.,* 2003a - 2003b; Li *et al.,* 2002), and fresh water environments have also been documented and explored (Jahn, 1924; Hook *et al.,* 1980). Unknown nutritional behaviour and metabolism are believed to be the major contributing factors to the unsuccessful cultivation of many uncultured myxobacterial strains.

Myxobacteria have gained attention not only for their social and developmental lifestyle (Dworkin, 1996; Hoiczyk *et al.,* 2009; Kearns *et al.,* 2001; Reichenbach, 1984) but also for their ability to produce diverse secondary metabolites and complex mega-biosynthetic enzymes (Weissman & Müller, 2010; Wenzel & Müller, 2009; Kopp *et al.,* 2004; Müller & Gerth, 2006; Reichenbach, 2001). From approximately 7,000 identified myxobacterial strains (Gerth *et al.,* 2003; Reichenbach, 2005), around 100 core structures and 500 derivatives have been structurally elucidated (Bode & Müller, 2008), securing their reputation as one of the premier sources of natural products. Although the

Table 1. List of validly described taxa in myxobacteria.

I. Suborder *Cystobacterineae*
- Family *Myxococcaceae*
  - **Genus *Myxococcus*** (5)
    - *Myxococcus xanthus*
    - *Myxococcus virescens*
    - *Myxococcus fulvus*
    - *Myxococcus stipitatus*
    - *Myxococcus macrosporus*
  - **Genus *Corallococcus*** (2)
    - *Corallococcus coralloides*
    - *Corallococcus exiguus*
  - **Genus *Pyxidicoccus*** (1)
    - *Pyxidicoccus fallax*
  - **Genus *Anaeromyxobacter**** (1)
    - *Anaeromyxobacter dehalogenans*
- Family *Cystobacteraceae*
  - **Genus *Cystobacter*** (10)
    - *Cystobacter badius*
    - *Cystobacter armeniaca*
    - *Cystobacter violaceus*
    - *Cystobacter miniatus*
    - *Cystobacter minus*
    - *Cystobacter gracilis*
    - *Cystobacter velatus*
    - *Cystobacter ferrugineus*
    - *Cystobacter fuscus*
    - *Cystobacter disciformis*
  - **Genus *Archangium*** (1)
    - *Archangium gephyra*
  - **Genus *Stigmatella*** (3)
    - *Stigmatella erecta*
    - *Stigmatella aurantiaca*
    - *Stigmatella hybrida*
  - **Genus *Melittangium*** (3)
    - *Melittangium lichenicola*
    - *Melittangium boletus*
    - *Melittangium alboraceum*
  - **Genus *Hyalangium*** (1)
    - *Hyalangium minutum*
  - **(Genus *Angiococcus*)**
    - *Angiococcus disciformis = C. disciformis*

II. Suborder *Sorangiineae*
- Family *Polyangiaceae*
  - **Genus *Polyangium*** (7)
    - *Polyangium vitellinum*
    - *Polyangium aureum*
    - *Polyangium luteum*
    - *Polyangium sorediatum*
    - *Polyangium spumosum*
    - *Polyangium fumosum*
    - *Polyangium parasiticum*
  - **Genus *Chondromyces*** (6)
    - *Chondromyces crocatus*
    - *Chondromyces apiculatus*
    - *Chondromyces robustus*
    - *Chondromyces catenulatus*
    - *Chondromyces pediculatus*
    - *Chondromyces lanuginosus*
  - **Genus *Sorangium*** (1)
    - *Sorangium cellulosum*
  - **Genus *Byssovorax*** (1)
    - *Byssovorax cruenta*
  - **Genus *Haploangium*** (2)
    - *Haploangium rugiseptum*
    - *Haploangium minus*
  - **Genus *Jahnella*** (1)
    - *Jahnella thaxteri*
- Family *Phaselicystidaceae*
  - **Genus *Phaselicystis*** (1)
    - *Phaselicystis flava*

III. Suborder *Nannocystineae*
- Family *Kofleriaceae*
  - **Genus *Kofleria*** (1)
    - *Kofleria flava*
  - **Genus *Haliangium*** (2)
    - *Haliangium tepidum*
    - *Haliangium ochraceum*
- Family *Nannocystaceae*
  - **Genus *Nannocystis*** (2)
    - *Nannocystis exedens*
    - *Nannocystis pusilla*
  - **Genus *Plesiocystis*** (1)
    - *Plesiocystis pacifica*
  - **Genus *Enhygromyxa*** (1)
    - *Enhygromyxa salina*

Total number of species: 53

* Sanford *et al.,* 2002.

Number of validly described species is shown after the generic name.

number of compounds discovered in myxobacteria exceeds than the number of described species, bioactive compound mining is still far from exhaustion, especially after the discoveries of many novel and rare genera from terrestrial and aquatic habitats (Ojika *et al.,* 2008; Kunze *et al.,* 2006). In the continuous secondary metabolite screening program at the Helmholtz Institute for Pharmaceutical Research (HIPS), which has yielded still more novel isolates (Garcia *et al.,* 2010), it is expected that many more new scaffolds will be isolated and elucidated in the future.

The potential of myxobacteria does not appear limited to antibiotics and cytotoxic compounds; they have also surprisingly been implicated in the production of steroids (Bode *et al.,* 2003; Gawas *et al.,* 2011) and recently, in the production of commercially important polyunsaturated fatty acids (Stadler *et al.,* 2010), hence making them promising model bacteria for many industrial, pharmaceutical, and medicinal applications.

Outline of the Study

The study initially focuses on unearthing new myxobacterial producer strains from global samples using a combination of microbiological, chemical, molecular, and phylogenetic techniques (Garcia *et al.,* 2009a). New approaches for isolation, cultivation, and preservation are meticulously described leading to the discovery of novel isolates. An improved screening regimen for secondary metabolites, based on a combined chemical and biological approach, is also emphasised for the mining of interesting compounds from myxobacteria (Garcia *et al.,* 2009a; Krug *et al.,* 2008).

The second part of the work deals with the characterisation of a new myxobacterial family (Garcia *et al.,* 2009b), and the proposal of four other strains into new species and genera. In addition to morphological and chemo-physiological characterisation, their assignments to novel taxa strengthened by molecular and phylogenetic analyses.

Another major highlight of this study is the classification and taxonomic assignment of the novel isolates and representative type strains through a 16S rRNA-based phylogenetic study (Garcia *et al.,* 2010). This involves sequencing of many type-neotype strains, verification and correction of previously published sequences, and careful analysis of their phylogenetic positions. Sequences of clones previously thought to be "uncultured bacteria" were analysed and their possible positions in the phylogenetic tree determined.

During the course of chemical characterisation of the novel isolates, unusual fatty acid (FA) patterns were detected. Interestingly, diverse polyunsaturated fatty acids belonging to omega-3 and omega-6 families were revealed. Findings from the novel isolates' FAs have led this work to further explore and determine the available myxobacterial representative type-neotype strains, allowing them to be correlated in the phylogenetic tree (Garcia *et al.,* 2011). The analysis was also extended to morphologically-related gliding bacteria belonging to *Herpetosiphon* and *Flexibacter*. The discovery of PUFAs in myxobacteria promises great commercial and biotechnological capability for industrial application (Stadler *et al.,* 2010).

Lastly, the work describes the potential of the novel taxa as sources of new bioactive compounds. *Aetherobacter* represents a novel genus in *Sorangiineae*, which, in this study, was mined for novel secondary metabolites. The work encompasses compound purification, isolation, and bioassay assesments.

Myxobacterial Natural Products

Myxobacteria have gained recognition as intriguing sources of new pharmaceutical drugs (Mulzer, 2009). The identification of diverse and structurally unique compounds has established them as one of the most outstanding secondary metabolite producers amongst the prokaryotes (Reichenbach, 2001; Reichenbach & Höfe, 1993, 1999; Gerth *et al.,* 2003, Bode & Müller, 2006; Weissman & Müller, 2010). Table 2 shows the diversity of compounds amongst myxobacterial taxa. *Sorangium* (48.4%), *Chondromyces* (10.3%) and *Polyangium* (5.2%) account for nearly 64% of the currently

Table 2. Distribution of secondary metabolites amongst myxobacterial taxa.

Taxa	Secondary Metabolites	Total
Suborder *Cystobacterineae*		
Family *Cystobacteraceae*		(31)
Genus *Archangium*	archazolid, argyrin, aurafuron, gephyronic acid, **germacran**, myxovalargin, tubulysin, vioprolide	8
Cystobacter	althiomycin, cyrmenin, cystothiazole, myxalamide, melithiazol, pyrrolnitrin, stigmatellin, vioprolide	8
Melittangium	melithiazol, pyrrolnitrin	2
Stigmatella	aurachin, aurafuron, dawenol, myxalamide, myxothiazol, nitroresorcinol, stigmatellin, stigmolone	8
Hyalangium	-	0
(Angiococcus)	althiomycin, angiolam, myxocheline, myxothiazol, tubulysin	5
Family *Myxococcaceae*		(20)
Myxococcus	althiomycin, cittilin, Dkxanthene, harman, myxalamide, myxocheline, myxochromide, myxopyronin, myxothiazol, myxotyroside, myxovalargin, myxovirescin, phenalamide (stipiamide), pyrrolnitrin, rhizopodin, saframycin Mx1	16
Corallococcus	corallopyronin, **pyrrolnitrin**, myxothiazol, myxovalargin	4
Pyxidicoccus *	-	0
Anaeromyxobacter	-	0
Suborder *Sorangiineae*		
Family *Polyangiaceae*		(48)
Polyangium	phenoxan, thiangazol	2
Chondromyces	apicularen, ajudazol, chondramide, chondrochloren, crocacin, crocapeptin, jerangolid, pedein, thuggacin	9
Sorangium	ambruticin, carolactone, chivosazol, chlorotonil, disorazol, eliamide, epothilone, etnangien, eudesmadien, icumazol, invictolid, jerangolid, leupyrrin, maracen, maracin, pellasoren, pentacaronic acid, phoxalone, **pyrrolnitrin**, ratjadon, ripostatin, socein, sorangicin, sorangiadenosine, sorangiolid, soraphen, soraphinol, spirangien, spirodienal, sulasoren, sulfangolid, tartrolon, thuggacin, trichangion, tuscolid, tuscoron	36
Byssovorax	cruentaren	1
Haploangium	-	0
Jahnella	-	0
Family *Phaselicystidaceae*		0
Phaselicystis	-	0
Suborder *Nannocystineaea*		
Family *Nannocystaceae*		(7)
Nannocystis	geosmin, **germacran**, nannochelin, phenylnannolone	4
Plesiocystis	-	0
Enhygromyxa	-	0
'Paraliomyxa' **	miuraenamide	1
Family *Kofleriaceae*		(1)
Kofleria	-	0
Haliangium	haliangiacin	1

(-) Nothing known so far.
* Since it was initially misclassified as "*Angiococcus*," thus appear to share the same production.
** Ojika *et al.,* 2008; Iizuka *et al.,* 2006. Not validly described yet.
Boldface shows the examples of the overlapping compound amongst suborders.
Total number of compound in the family is enclosed in parenthesis.

identified myxobacterial compounds (Gerth *et al.,* 2003), hence making *Sorangiineae* the most efficient source amongst the suborders. Although this group represents only a quarter (26%) of the total myxobacteria collection at the HZI (formerly the German Centre for Biotechnology - GBF), the compound diversity is overwhelming, predicted to soar even higher in the near future after cultivation of many new strains in this suborder (Garcia *et al.,* 2010). The numbers will also likely increase as a result of efforts to cultivate strains which, at the moment, represent the "unculturable" group (Jiang *et al.,* 2007; Jiang *et al.,* 2010).

Although there are only a limited number of myxobacterial species known to date (Garcia *et al*., 2010; Reichenbach, 2005), the number of compounds identified has reached into the hundreds. The unbalanced ratio between species and compounds discovered in myxobacteria can be attributed to the diversity of metabolites produced by a single strain. Interestingly, the compounds and their chemical derivatives identified in myxobacteria usually belong to different structural classes. The majority of these compounds are polyketides, non-ribosomal peptides, or hybrids thereof (Weissman & Müller, 2010).

Myxobacterial compounds can also be classified on the basis of bioactivity. The bulk of them are antimicrobial; approximately 54% are known antifungals which primarily act on the mitochondrial respiratory chain, specifically targeting complexes I and III (Gerth *et al.,* 2003). Examples of these compounds are thiangazole, phenoxan, myxalamid, myxothiazol, haliangiacin, crocain and miuranamide. Other antifungal compounds isolated from myxobacteria disrupt macromolecule synthesis, inhibit cell membrane synthesis, and target acetyl-CoA carboxylase.

Antibacterial metabolites produced by myxobacteria account for nearly one-third of the total number of compounds isolated from these organisms (Gerth *et al.,* 2003). Modes of action are much wider in scope, spanning a large number of different targets. Althiomycin, angiolam, and myxovalargin are, for example, inhibitors of protein synthesis, while myxoviriscins act on cell wall synthesis. In the case of corallopyronin,

etnangien, myxopyronin, ripostatin, and sorangiacin, the eubacterial RNA polymerase appears to be the target. Intriguingly, etnangien also appears to act upon DNA polymerase. Compounds acting against acid-fast mycobacteria have also been discovered, as exemplified by maracin, maracen, and thuggacin; the latter appears to be a promising candidate for anti-tuberculosis therapy.

Recent reviews on myxobacterial secondary metabolites have identified 32 major classes of cytotoxic compounds (Weissman & Müller, 2010). Most interesting are those structural classes targeting the eukaryotic cytoskeleton, which could be encouraging leads for future development as anti-cancer drugs. Chondramide, chivosazol, and rhizopodin are some of the most interesting cytotoxic natural products specifically affecting actin filament formation, while in tubulysin, disorazol and epothilone, tubulin appear to be the target. The latter compound has been studied thoroughly (Mulzer, 2009) and is being marketed as an anti-cancer drug for breast cancer therapy.

Approach to Novel Strain Discovery

Myxobacteria are widely distributed in nature, representing all geographical and climate zones (Dawid, 2000; Reichenbach, 1999b). In the past decades, the search has covered soil samples, herbivores dung, tree bark, and rotting plant materials, yielding diverse species, representing different genera and suborders (Reichenbach & Dworkin, 1992). Samples collected from living tree bark have also yielded novel species (Peterson, 1959). Some groups are often isolated from their niches on particular substrate, reflecting in their social lifestyle. *Myxococcus virescens* and *Cystobacter fuscus* are, for example, commonly isolated in rabbit dung while *Stigmatella aurantiaca* appears common in rotting wood bark. The novel genus *Jahnella* was also discovered colonising rabbit dung, suggesting its proteolytic-bacteriolytic nutrition behaviour (Reichenbach, 2005). In addition, several studies have shown that they can also be isolated from alkaline peat bogs (Hook *et al.,* 1980). Samples collected in the desert, Antarctica (Gerth & Müller, 2005; Dawid *et al.,* 1988), and even those taken from hot springs have also contained myxobacteria (Iizuka *et al.,* 2006). Environments with dynamic interactions of organisms and stark competition for common resources, as exemplified by the tropical

and subtropical forest, appear to be the most promising source for interesting novel strains.

Myxobacteria often inhabit terrestrial environments, growing under aerobic condition. The discovery of three novel "halophilic" and anaerobic myxobacteria was surprising. *Plesiocystis, Enhygromyxa,* and *Hyalangium*, representing the halophilic genera, were discovered from a marine environment, or marine-associated samples (Iizuka *et al.,* 2003a, Iizuka *et al.,* 2003b; Fudou *et al.,* 2002). Halophilic conditions and an unexplored sampling environment illustrate the extensive possibilities for isolation of new taxa. The oxygen-free condition used in the isolation of *Anaeromyxobacter* had never been attempted before in the isolation of myxobacteria (Sanford *et al.,* 2002) and therefore clearly represent a new isolation technique.

In principle, novel myxobacterial strains could possibly be brought into culture in the future from unexplored sources by using different methods of isolation and baiting techniques. The expectation that the new isolates show unusual characteristics is high, thus necessitating a certain familiarity with current biology and taxonomy in order to differentiate and recognise them. Swarming and fruiting bodies are characteristics commonly observed during the isolation and attention should be devoted to the search for these stages. The inability of novel myxobacteria to produce fruiting bodies appears possible (Jiang *et al.,* 2007), a consideration that must be kept in mind during isolation.

Myxobacterial phylogeny

Myxobacteria are a coherent group belonging to the delta Proteobacteria (Woese *et al.,* 1985; Shimkets & Woese, 1992; Spröer *et al.,* 1999). They are postulated to originate from a common ancestor, a hypothesis that is supported by phenotypic, genotypic, and molecular features (Kaiser *et al.,* 1993). The group (order *Myxococcales*) was phylogenetically divided into suborders *Cystobacterineae, Nannocystineae, and Sorangiineae,* all of which exhibit gliding characteristic of the vegetative cells. Myxobacterial diversity and their phylogenetic position have been documented extensively from both marine and terrestrial domains (Brock Neil *et al.,* 2005; Jiang *et*

al., 2007; Jiang *et al.,* 2010; Moyer *et al.,* 1995; Zhang-Cai *et al.,* 2003; Iizuka *et al.,* 2006). From the 26 previously studied culturable morphospecies (Spröer *et al.,* 1999), the number of identified myxobacteria has increased significantly almost doubling in the last decade (Garcia *et al.,* 2010). The escalating number of strains appears to be associated to advances in sequencing of highly conserved housekeeping genes, so much that their phylogenetic position can be clearly verified to allow identification. In myxobacteria, for example, *lepA, fusA, rpoB, gyrB*, and 16S rRNA genes have been used for genotypic classification (Stackerbrandt *et al.,* 2007). The latter gene appears to be the most widely used and established method, and is therefore commonly chosen for phenetic (genotypic and phenotypic traits) studies (Lang *et al.,* 2008; Lang & Spröer, 2008).

Reports have shown that many clone sequences have high similarity with uncultured bacteria, and were phylogenetically determined to cluster with myxobacteria (Jiang *et al.,* 2007; Jiang *et al.,* 2010). These clones may possibly represent the viable but not culturable (VBNC) organisms. Although myxobacteria are often distinguished by such common characteristics as the production of fruiting bodies under starvation conditions, a previous study has shown evidence that non-fruiting groups also exist and, appear much larger than the known fruiting taxa (Jiang *et al.,* 2007). In addition, the study also revealed that the non-fruiting taxa seem to represent the uncultured myxobacteria. Myxobacterial diversity in marine environments was also documented and determined to be phylogeographically distinct from terrestrial myxobacteria at high levels of classification (Jiang *et al.,* 2010). The occurrence of marine myxobacteria seems un-surprising, as it was previously reported in Pele's Vents, Hawaii (Moyer *et al.,* 1995).

Myxobacterial Fatty Acids: Health Benefits and their Commercial Impact

Although many studies have dealt with fatty acids of myxobacteria, the majority of them have focused on a few select species (Bode *et al.,* 2005). *Myxococcus xanthus* DK1622 and *Stigmatella aurantiaca* DW4/3-1 are the subject of many studies on FAs; however, these were concerned with developmental and biosynthetic aspects (Bode *et al.,* 2006a - 2006b; Dickschat *et al.,* 2005; Ring *et al.,* 2006; Ring *et al.,* 2009). FA analysis has also

been widely used in taxonomic characterisation of *Corallococcus*, *Melittangium,* and other organisms (Lang & Spröer, 2008; Lang & Stackerbrandt, 2009; Monteoliva-Sanchez *et al.,* 1987; Yamanaka *et al.,* 1988; Kaneda *et al.,* 1991). Previous studies in *Sorangium* require a re-evaluation due to old analytical methods and differences in extraction procedures (Fautz *et al.,* 1981). Analysis of FA has also been performed in order to differentiate amongst marine isolates and members of the two genera in *Myxococcaceae* (Iizuka *et al.,* 2003a – 2003b; Schäberle *et al.,* 2010; Lang *et al.,* 2008; Lang & Stackerbrandt, 2009; Stackerbrandt *et al.,* 2007). In the suborder *Sorangiineae*, only the previously described novel family *Phaselicystidaceae* has been explored thoroughly for FAs (Garcia *et al.,* 2009b). Myxobacteria not only synthesise diverse FAs but can also distinguished by characteristic FA types. Some groups are remarkable for the absence of hyxdroxy FAs (Iizuka *et al.,* 2003a; Fudou *et al.,* 2002), while some contain extraordinary amounts of the *iso*-branched (iso-FAs) type (Bode *et al.,* 2006), which, in *Myxococcus xanthus,* appears to be involved in the development cycle leading to sporulation (Ring *et al.,* 2006). In other bacteria (e.g. *Listeria monocytogenes*), branched-chain FAs play a role in the pH stress tolerance of the organism (Giotis *et al.,* 2007).

Myxobacteria were surprisingly observed to be capable of polyunsaturated fatty acid (PUFA) synthesis. Originally, PUFAs were previously thought to be absent in bacteria (Erwin & Bloch, 1964), a hypothesis later disproved through the discovery of some PUFA-producing bacterial strains living under high pressure and low temperature environments (De-Long & Yayanos, 1986; Nichols *et al.,* 1993; Nichols & McMeekin, 2002; Yano *et al.,* 1997). Major microbial sources of PUFAs are *Schizochytrium, Ulkemia, Crytocodinium,* and *Mortierella* (Ward & Singh, 2005). In myxobacteria, the halophilic genus *Plesiocystis* was characterised for the production of long chained $C_{20:4}$ fatty acid (Iizuka *et al.,* 2003a), later found in other genera of marine myxobacteria (Iizuka *et al.,* 2003b; Schäberle *et al.,* 2010). In terrestrial soil-myxobacteria belonging to the genus *Phaselicystis*, huge amounts of $C_{20:4}$ FA have been found and identified as arachidonic acid (Garcia *et al.,* 2009b). Recently, important omega-3 fatty acids have also been discovered in some novel strains of myxobacteria (Stadler *et al.,* 2010).

In general, polyunsaturated fatty acids are important and essential components in eukaryotic cells, conferring fluidity, flexibility and membrane permeability. Eicospentaenoic acid (EPA) has been implicated in cardiovascular health benefits, treatment of brain disorders (Fenton *et al.,* 2000; Peet, 2004), and cancer (Tisdale, 1999), while docosahexaenoic acid (DHA) is associated with eye and brain development in infants, and also supports the cardiovascular system. PUFAs are widely and commercially used in the market, and are in high demand as supplements in many food and dairy products. In infant formula alone, the world wholesale market is estimated to be about $10 billion per annum (Ward & Singh, 2005). The growing awareness of the health benefits of PUFAs, is expected to significantly contribute to the expansion and diversification of market products. Among the major targets for improved commercial development of PUFAs are gamma-linolenic acid (GLA), arachidonic acid (ARA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA).

Discovering Bioactive Compounds in Novel Myxobacteria

In this study, classical biological screening and modern chemical analytical techniques play an important role in the discovery of novel bioactive compounds. Myxobacterial cultures for screening are initially grown in small-scale medium containing adsorber resins. Myxobacterial compounds are normally stable when they bound to the resins, helping in the improvement of yields (Reichenbach, 1999a). Most myxobacteria tolerate the presence of XAD-16 in the culture broth without any adverse effect on growth. Chemical characterisation of the extracts is routinely performed by HPLC coupled to MS, time-of-flight (ToF), high resolution LTQ Orbitrap and tandem MS defined by set of parameters. Extracts are simultaneously tested for antimicrobial and cytotoxicity using a range of microorganisms and cell line panels (Fig. 1). Extracts exhibiting biological activity which cannot be correlated to known masses are further evaluated by semi-preparative HPLC fractionation and re-testing against the sensitive organism to determine the unknown active compound. A previous study exemplified this approach (Garcia *et al.,* 2009a). Target compounds are then marked for large scale fermentation after strain improvement and process optimisation (Gerth *et al.,* 2003) and isolation is

carried out using a diverse array of analytic and advanced chromatographic separation techniques.

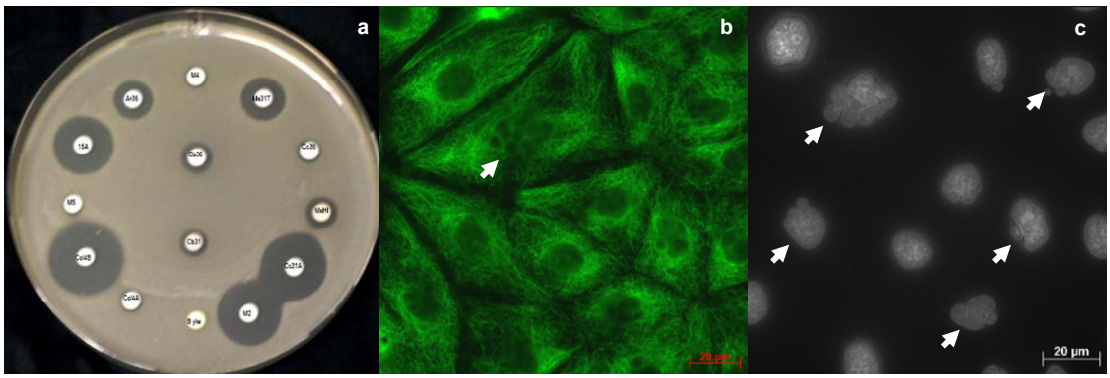

Figure 1. Example of biological screening of myxobacterial crude extracts. (a) Antibacterial test against Gram-positive *Rhodococcus opacus*, (b-c) Cytotoxicity test using potoroo kidney cells (Ptk2) showing nuclear fragmentation (arrows).

The novel isolate *Aetherobacter rufus* SBSr003^{T} vividly illustrates the expectation to isolate and identify new metabolites in myxobacteria. After initial identification of possible bioactive compounds, screening and isolation process aimed at unearthing the novel compounds are described here. Although several more strains were isolated representing novel genera and perhaps even a new family, the study is limited only to this strain; however, there are future plans to further explore the potential of other novel isolates for new and interesting compounds. The discovery of novel myxobacterial compounds in this study appears to be undoubtedly and directly associated with the discovery of new isolates representing new taxa.

Chapter II. Publications

Discovering Natural Products from Myxobacteria with Emphasis on Rare Producer Strains in Combination with Improved Analytical Methods

Ronald O. Garcia, Daniel Krug, and Rolf Müller (2009)

In: D. Hopwood (ed), Methods in Enzymology: Complex Enzymes in Microbial Natural Product Biosynthesis. vol. 458., part A. p. 59–91. Academic Press, Burlington.

This article is available online at:

http://dx.doi.org/10.1016/S0076-6879(09)04803-4

Efficient mining of myxobacterial metabolite profiles enabled by liquid chromatography–electrospray ionisation-time-of-flight mass spectrometry and compound-based principal component analysis

Daniel Krug, Gabriela Zurek, Birgit Schneider, Ronald Garcia, Rolf Müller (2008)

Analytica Chimica Acta **624:** 97–106.

This article is available online at:

http://dx.doi.org/10.1016/j.aca.2008.06.036

Phaselicystis flava, gen. nov., sp. nov., an arachidonic acid-containing soil myxobacterium, and the description of *Phaselicystidaceae*, fam. nov.

Ronald O. Garcia, Hans Reichenbach, Michael W. Ring, and Rolf Müller (2009)

International Journal of Systematic and Evolutionary Microbiology **59:**1524–1530.

Minicystis rosea, gen. nov., sp. nov., a pink myxobacterium

Ronald Garcia and Rolf Müller

Running Title: *Minicystis*: a pink myxobacterium

Subject Category: New Taxa - Proteobacteria

Abstract

A bacterial strain designated as SBNa008^T was isolated from a soil sample collected in the Philippines. It exhibits the general characteristics associated with myxobacteria, such as swarming of Gram-negative rod-shaped vegetative cells, fruiting body formation, and bacteriolytic activity. The strain is mesophilic, chemoheterotrophic, and aerobic. The major fatty acids (FAs) are *iso*−$C_{15:0}$, $C_{17:1\ 2\text{-}OH}$ and $C_{20:4\ \omega6,\ 9,12,15,\ all\ cis}$ (AA-arachidonic acid). The polyunsaturated omega-3 eicosapentaenoic acid (EPA) was also found. The G + C content of the genomic DNA is 67.3 mol %. 16S rRNA gene sequence reveals 95 – 96 % similarity to clones of uncultured bacteria, and 94 - 95% to members of *Sorangiineae*. The clustering of the novel isolate to the "unculturables" and its novel branch in the phylogenetic tree suggest that SBNa008^T represents a novel genus and species, proposed here as *Minicystis rosea*. The type strain for *Minicystis rosea* is SBNa008^T (= DSM 24000^T, = NCCB 100349^T).

Abbreviations:
FA(s): Fatty acid(s)
PUFA: Polyunsaturated fatty acid
AA: Arachidonic fatty acids
EPA: Eicosapentaenoic acid

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Minicystis rosea* SBNa008^T is GU249616.

Reactions to API 32 GN kit are available as supplementary table with the on-line version of this paper.

INTRODUCTION

The strain was isolated in December 2007 from Philippine soil sample containing plant material taken from Landsweiler-Reden collection, Germany. It was recognised as a myxobacterium through its swarming and fruiting body characteristics on agar baited with live *Escherichia coli*. Cells glide coherently on agar in a loose swarming pattern at the edge of the colony, and via several transfers of this material to a new plate, the novel strain was purified and isolated.

Based on fruiting body observations, SBNa008^T was initially classified as a member of the *Nannocystineae* suborder. Among the five recognised genera of this suborder, the closest resemblance to its tiny ovoid sporangiole was observed with *Nannocystis*. This feature, along with some other remarkable characteristics, was partially described in the comprehensive phylogeny of myxobacteria (Garcia *et al.,* 2010). Although the novel bacterium shows many similarities with *Nannocystis*, vegetative cell morphology suggested that SBNa008^T might be a *Sorangiineae*-type myxobacterium, leading us to further characterise the isolate.

METHODS

Isolation and cultivation. Based on swarming of long slender rod-shaped cells and fruiting body formation on water agar, *Minicystis rosea* was identified as a myxobacterium. The strain was purified and isolated by cutting the farthest swarm edge and transferring it repeatedly onto a lean water agar. During screening of our global soil sample collection, we did not encounter this unusually thin and almost transparent colony, which is barely recognisable under the microscope. The organism was routinely cultivated and maintained in buffered VY/2 medium (Garcia *et al.,* 2009a) and stored at -80 °C.

Microscopy and morphological examination. Swarming colonies and fruiting bodies were observed under an Olympus SH−ILLB stereoscopic microscope and photographed using an Axiocam MRC (Zeiss) camera. Vegetative cell morphology and myxospores

were studied using phase-contrast microscopy (Axio-Star, Carl Zeiss). All growth stages were observed on solid agar medium, namely water, buffered VY/2, and cVY/2 (VY/2 containing filtered autoclaved baker's yeast). Vegetative cells were also observed in clear MD1G medium (Garcia *et al.,* 2010), but with a reduced concentration of $MgSO_4 \cdot 7H_2O$ (0.05 %).

Microbial predation test. Overnight cultures of *Escherichia coli, Psedumonas stutzeri* (Gram-negative), *Micrococcus luteus* (Gram-positive), and 36-h-old culture *Hansenula anomala* (yeast) were used as bait in water agar medium. Growth of the myxobacterium and bacterial lysis, as indicated by clearing of the streaked bait and swarm spreading, were determined after 5-7 days of incubation at 30 °C.

Physiological and biochemical tests. Reaction of vegetative cells to Gram- and Congo red stain was determined accordingly. Staining by the latter was performed according to McCurdy (1969). The catalase test was performed with a drop of 3.0 % H_2O_2 on vegetative cells. Cellulose degradation was performed using buffered water, VY/2, and cVY/2 agar overlaid with both sterile filter paper (2.0 x 1.0 cm) and a drop of cellulose powder solution on separate section of the agar. The chitin degradation assay was also performed in the same clear media (except VY/2 agar), but with only a drop of chitin powder (Sigma) solution. All set-ups were incubated at 30 °C for 1 to 2 weeks.

Growth determination on xylan, skim milk agar (SKM), and milk casein was previously described (Garcia *et al.,* 2009b). Biochemical tests were performed using bioMérieux API 32 GN kit according to manufacturer's instructions, but with slight medium modifications to support the nutritional requirements of the myxobacteria (0.05 % ammonium sulphate, 0.1 % monosodium phosphate, 0.05 % $CaCl_2 \cdot 2H_2O$, 0.05 % $MgSO_4 \cdot 7H_2O$). The set-up was incubated at 30 °C for 5-days, and maintained in moist environment.

Growth response to pH, temperature, oxygen, and antibiotics. Growth response to different levels of temperature and antibiotic resistance was tested in buffered VY/2 agar

(Garcia *et al.,* 2009a) supplemented with vitamin solutions (Shimkets *et al.* 2006). For pH tolerance, VY/2 medium was adjusted accordingly. Vegetative cell inocula came from 3-d old culture grown in TG1 medium (0.3 % Bacto Tryptone, 0.3 % Glucose, 0.025 % $CaCl_2 \cdot 2H_2O$, 0.05 % $MgSO_4 \cdot 7H_2O$, pH adjusted to 7.0 with KOH before autoclaving). Cell inoculum was adjusted to 1.0 McFarland (bioMérieux) and spotted (10 µL) on the agar. Oxygen response was also tested in TG1 medium (10 mL) and incubated under stationary conditions. Incubation for all tests was performed for 4 – 7 days at 30 ºC, except for the temperature response tests at 18 ºC, 22 ºC (room temperature), and 37 ºC.

Growth response to nitrogen and sugar sources. Nitrogenous and sugar compounds used in this study were previously described (Garcia *et al.,* 2009a) and supplemented here into water agar.

Fatty acid and G + C content analyses. Cell pellet from actively growing culture was obtained from MD1G medium shaken at 160 rpm, 30 °C. Cellular fatty acid extraction from samples was performed in duplicates using the fatty acid methyl esters (FAME) method and analysed by GC−MS (Garcia *et al.,* 2011). The mol percent DNA G + C content of the novel bacterium was determined by HPLC after nuclease P1 digestion of the genomic DNA (Shimelis & Giese, 2006; Li *et al.,* 2003).

16S rRNA gene sequencing and phylogenetic analyses. Genomic DNA extraction from actively growing culture and amplification of the 16S rRNA gene were prepared accordingly (Garcia *et al.,* 2009b). Other myxobacterial 16S rRNA gene sequences used in this study, mostly representing the type strains in suborder *Sorangiineae*, were obtained from GenBank. Sequence alignments were performed using the rapid multiple sequence alignment based on fast Fourier transform (MAFFT) v.6.814b (Katoh *et al.,* 2002). Distance matrices between sequences were calculated using the Jukes-Cantor model (Jukes & Cantor, 1969). Phylogenetic tree was constructed using the maximum likelihood method (PHYML v2.4.5) (Guindon & Gascuel, 2003), and a bootstrap of 1000 replicates was calculated (Felsenstein, 1985). Phylogenetic relationship was also

confirmed using the neighbour-joining method (Saitou & Nei, 1987). All these programs are packed in the Geneious Pro 5.0.2 software (Drummond *et al.,* 2010).

RESULTS AND DISCUSSION

Swarm. The film-like colony is barely visible on agar medium (e.g. VY/2, cVY/2 and water agar). Culture agar plates needed to be tilted at an angle for it to be seen. A light pink colony was observed on VY/2 agar after 1-2 weeks of incubation in a bright environment. Unlike other myxobacteria, no radial veins or ripples were found in the colony. The cells swarm in a circular pattern but with unstructured or loose colony edges (Fig. 1a). In some cases, small flare-like swarms are produced at the colony border, reminiscent of some members of *Myxococcaceae*. In contrast to many *Sorangiineae*, SBNa008^T barely penetrates deep into agar and sometimes exhibits only shallow depressions. Furthermore, the novel isolate differs from *Nannocystis* through the absence of deep agar holes and excavations.

Vegetative cells. The vegetative cells are phase-dark, long (1.2 x 4.0 – 8.0 μm), slender rods with blunted ends (Fig. 1b), typical of members of the suborder *Sorangiineae* and *Kofleriaceae.* In liquid medium (e.g. MD1G), vegetative cell clumps appear light peach to rose pink.

Fruiting body. Fruiting bodies are composed of tiny spherical to ovoid sporangioles (4.0 – 12.0 μm diameter), arranged solitarily or as clusters (Fig. 1c). Dense clusters commonly developed on agar surfaces, especially close to the centre of the colony. In water agar containing a streak of live *E. coli*, a thin layer of sporangioles matted on lysed bait. Although *Nannocystis* was also able to form small fruiting bodies, large sporangioles (40.0 x 110.0 μm) were seen (Reichenbach, 2005). *Nannocystis pusilla* is unique for its uniformly tiny sporangioles (8.0 – 15.0 μm). To date, it appears that *M. rosea* may be one of the smallest sporangiole-bearing myxobacteria, which may be the reason it has been undetectable in the past. The novel bacterium was also remarkably different from *Nannocystis* in its ability to form fruiting bodies on the surface of solid medium; the latter

commonly developed deep into corroded agar (Reichenbach, 2005). Unlike *Polyangium, Cystobacter,* and *Kofleria*, which have tightly packed fruiting bodies, the novel isolate exhibited more loosely arranged sporangioles. Its absence of sorus enclosing the bunch of sporangioles distinguishes them from *Sorangium*. The tiny fruiting body of SBNa008^T which is often barely detectable might in some instances be mistaken for encysted amoebae.

Myxospores. The myxospores of SBNa008^T are phase-dark, slender fat rods measuring 1.2 x 2.0 – 3.0 μm, with dark granules at the poles (Fig. 1d). These features are typical for *Sorangiineae*. Although *Nannocystis* appears to be closest morphological relative to SBNa008^T, their myxopores and vegetative cells differ. *Nannocystis* is distinct for its almost rounded myxospores enclosed in small sporangioles, and produces short rod-shaped vegetative cells (sometimes almost cuboidal) without clearly evident dark granule formation at its poles.

Physiological and biochemical characteristics

Staining characteristics and temperature tolerance. The novel strain was Gram- and Congo-red negative. The latter stain confirms that SBNa008^T does not belong to the suborder *Cystobacterineae*. Optimal growth was observed at 30 °C, while minimal growth was observed at 18 °C and at 37 °C. The latter incubation showed the most evident agar depression among the temperatures tested. No growth was observed at above 37 °C. Colonies appeared transparent to lightly pink coloured on agar, a trait that appears common in many myxobacteria when incubated in a bright environment. In some myxobacterial genera, carotenoids appear to be responsible for the pigments (Jansen *et al.,* 1995; Reichenbach & Kleinig, 1971), although it may also be attributed to some secondary metabolites (Ohlendorf *et al.,* 2008; Trowitzsch-Kienast *et al.,* 1993)

pH and oxygen tolerance. SBNa008^T exhibited wide pH tolerance from 5.0 – 8.5. Optimal growth, represented by colony diameter, was found between pH 7.0 – 8.0 in

VY/2 agar. No growth was observed at pH 4.0 and beyond 9.0. Growth was film-like on the sides of the test tube, suggesting aerotolerant or facultative behaviour.

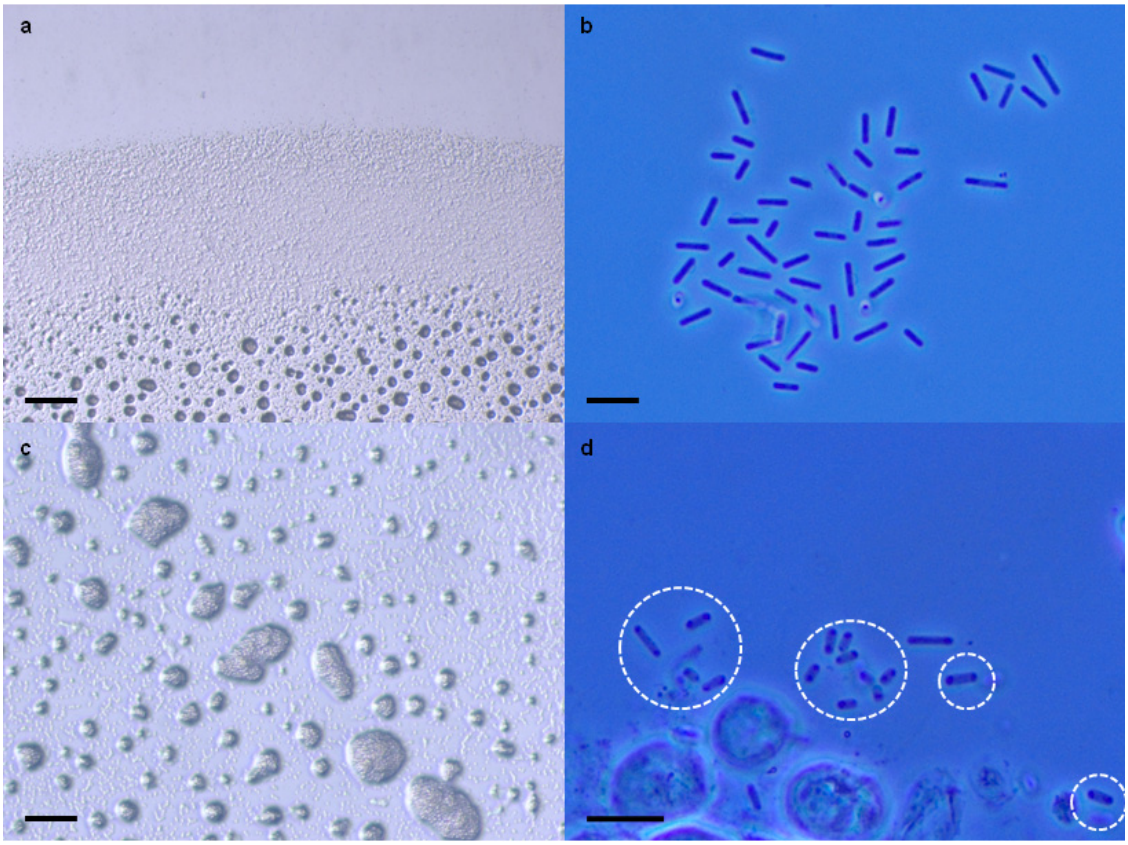

Figure 1. Growth stages of *Minicystis rosea* SBNa008^{T}. (a) Swarm colony on agar surface, (b) Phase-dark vegetative cells, (c) Clusters of tiny fruiting bodies and cellular aggregations, (d) Myxospores released from sporangioles (encircled). Photos taken under dissecting- (a, c) and phase-contrast- (b, d) microscope. Bar, 3.75 mm (a), 1.0 mm (c), 10.0 µm (b, d).

Degradation of biomacromolecules, reaction to different biochemicals, and predatory ability. Filter paper, cellulose, and chitin powder solution were not degraded, indicating non-cellulolytic and non-chitinoytic behaviour. Xylan was also not degraded. Unlike *Nannocystis,* the novel isolate only exhibits slight depressions on solid medium, often found close to the centre of the colony, which suggests weak agar degradation. No growth was also observed in skim milk agar (SKM). Supplementary table S1 shows the results of different biochemical tests.

The predatory lytic behaviour of the novel isolate to live microbial bait was only observed with Gram-negative bacteria, such as *Escherichia coli* DH10B. At later stages of growth, cell aggregation and fruiting bodies developed on the lysed bait. *Pseudomonas stutzeri, Micrococcus luteus,* and *Hansenula anomala* were not cleared, implying its selectivity to lyse and out-comfit them.

Sugar sources. SBNa008^T grew well in the presence of soluble starch, fructose, saccharose, molasses, and lactose. Poor growth was observed in the same medium supplemented with maltose, D-glucose, xylose, sorbitol, D-galactose, arabinose, and mannose. In agar containing 0.35 % soluble starch, no clear halo was produced in the colony after flooding with iodine solution, suggesting its non-hydrolysis.

Nitrogen and peptone sources. Swarming cells were observed in the presence of potassium nitrate and aspartic acid. Better growth was exhibited on the latter substrate, but with more scattered migrating cells. No evident colony swarming was observed upon incorporation of glutamic acid, urea, and potassium nitrate into the medium.

Best swarming, as reflected by the colony diameter, was seen in medium supplemented with peptone and neopeptone. Poor growth was observed in casitone, casamino acids, peptone, tryptone, and phytone.

Antibiotic resistance. *Minicystis rosea* was resistant to apramycin, gentamycin, neomycin, and spectinomycin. Poor swarming was observed in tobramycin, while no growth was found on ampicillin, carbenicillin, kanamycin, oxytetracycline, streptomycin, tetracycline, and rifampicin, indicating its sensitivity.

Fatty acid characteristics. Major fatty acids of *M. rosea* were iso-$C_{15:0}$, $C_{17:1\ 2OH}$, and $C_{20:4\omega6}$ (arachidonic acid) (Table 1)**.** Low amount of omega-3 FA $C_{20:5\omega3}$ (EPA) and trace amounts of omega-6 FA $C_{18:3\omega6}$ (GLA - γ linolenic acid) were also detected. Polyunsaturated fatty acids appear to be rare in prokaryotes (Nichols *et al.,* 1999; Nichols & McMeekin, 2002), but myxobacteria are quite well-equipped to synthesize them. To

date, the production of EPA has been correlated to only a few genera of myxobacteria (Garcia *et al.,* 2011; Stadler *et al.,* 2010), whereas AA is far more common among the *Sorangiineae* and *Nannocystineae* suborders (Garcia *et al.,* 2011).

The presence of $C_{17:1\ 2OH}$ FA supports the clustering of the novel isolate to *Sorangiineae*, simultaneously disqualifying it as a member of *Nannocystineae*. The latter suborder is hallmarked for the absence of hydroxy FA (Garcia *et al.,* 2011). A predominance of straight-chain FA over the branched-chain type further supports the affiliation of SBNa008^T to *Sorangiineae*.

Table 1. Fatty acid characteristic of *Minicystis rosea* SBNa008^T.

Fatty acids (FAs)	**Percent***
Straight-chain	
$C_{13:0}$	0.16
$C_{14:0}$	0.08
$C_{15:0}$	0.56
$C_{16:0}$	1.98
$C_{17:0}$	1.76
$C_{18:0}$	2.23
$C_{17:1\ 2\text{-}OH}$	**17.27**
$C_{16:0\ OAG}$	0.43
$C_{18:3\omega6}$ (GLA)	0.19
$C_{20:4\omega6}$ (AA)	**14.18**
$C_{20:5\omega3}$ (EPA)	4.18
unknown FA	6.63
Branched-chain	
iso-$C_{14:0}$	0.11
iso-$C_{15:0}$	**27.28**
iso-$C_{16:0}$	0.94
iso-$C_{17:0}$	3.17
iso-$C_{17:0\ 2\text{-}OH}$	0.08
iso-$C_{15:0\ DMA}$	**13.61**
iso-$C_{15:0\ OAG}$	5.14

* Major fatty acids (> 10 %) are marked in boldface.

16S rRNA gene and phylogenetic analysis. 16S rRNA gene sequence of the novel bacterium shows 95% similarity to *Byssovorax cruenta* (AJ833647) and 94% to *Phaselicystis flava* (EU545827), *Chondromyces lanuginosus* (FJ176774), and *Sorangium cellulosum* (AM746676). 95-96% similarity to clones of uncultured environmental bacteria (EU662572, EU104167, AM490752) was also observed. The clustering of *M. rosea* to these clones in the phylogenetic tree suggests that it represents the "uncultured" taxon of myxobacteria. This finding is unsurprising after the discovery of several other novel myxobacterial isolates also showing close similarity to them (Garcia *et al.,* 2009a; Garcia *et al.,* 2010).

Phylogenetic analysis reveals the alliance of SBNa008^T to *Sorangiineae*, forming a monophyletic cluster closely related to the "unculturables" (Fig. 2). A previous study, covering more than hundred strains, in the expanded phylogeny of myxobacteria supports its phylogenetic positioning (Garcia *et al.,* 2010).

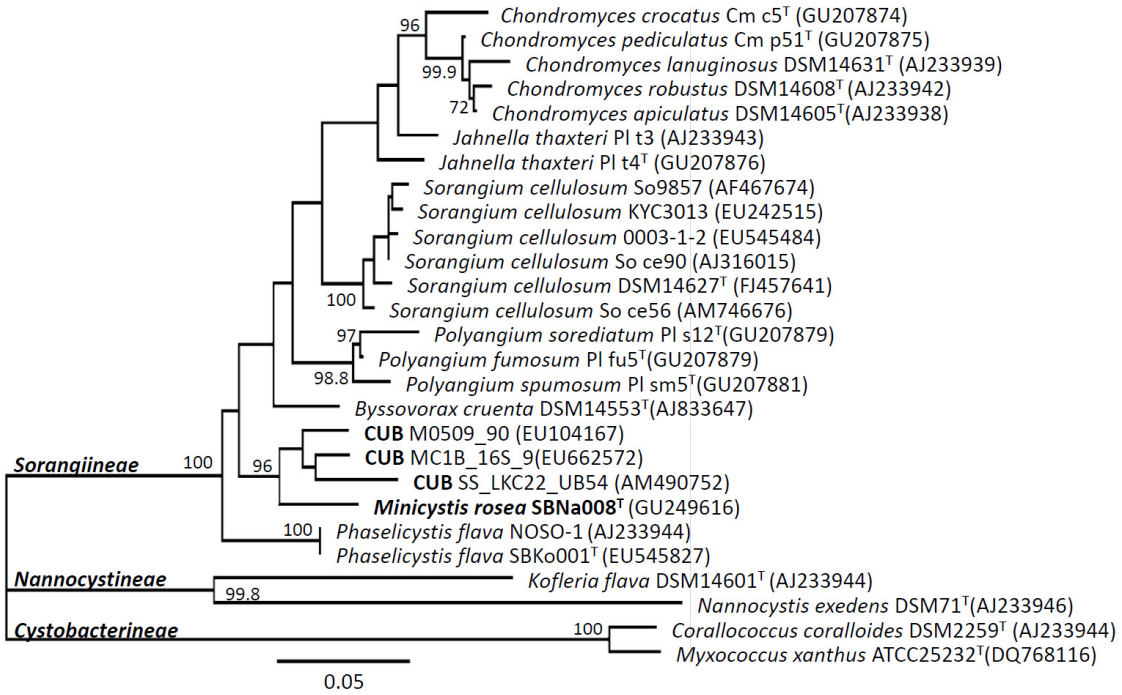

Figure 2. Phylogenetic position of *Minicystis rosea* SBNa008^T and its clustering with clones of unculturable bacteria (CUB). The tree was constructed based on 16S rRNA gene sequences using the maximum likelihood method (PHYML). The numbers at branch points indicate the percentage bootstrap support based on 1000 resamplings. Values greater than 60 % are shown. Bar, 0.05 substitution per nucleotide position.

Description of *Minicystis, gen. nov.,* Garcia and Müller

Minicystis: Mi.ni.cys'tis. L. comp. minor -us, less, smaller inferior; Gr. fem. n. kustis (Latin transliteration cystis), the bladder, a bag; N.L. fem. n. *Minicystis*, intended to mean that the sporangiole size is smaller than those of *Nannocystis*.

Soil myxobacterium. *Vegetative cells* are long and cylindrical rods with blunt ends; movement occurs by gliding on agar surface. *Swarm* is thin, transparent, exhibits a non-distinct radial vein pattern, and is non-adsorbent to Congo red. Colony edges with loose migrating cells; agar partially depressed. *Myxospores* are non-refractive, phase-dark, cylindrical slender rods shorter than vegetative cells, and enclosed in sporangial wall. *Fruiting bodies* appear as small, ovoid sporangioles. Bacteriolytic type, does not degrade cellulose or chitin. Phylogenetic analysis based on 16S rRNA gene sequence shows clustering with *Sorangiineae.* Type species: *Minicystis rosea.*

Description of *Minicystis rosea*, *sp. nov*., Garcia and Müller

rosea: ro'se.a. L. fem. adj. rosea, rose-coloured, rosy.

Exhibit all characteristics of its genus. *Vegetative cells* are fat rods, 1.0−1.5 x 3.5−10.5 μm in size and phase-dark. *Swarms* are composed of scattered loose cells, sometimes with flame-like extensions at the colony edge, and produced shallow agar depressions. *Fruiting bodies* are composed of tiny sporangioles (20.0 – 49.0 x 25.0 – 56.0 μm), typically as monolayered clusters (32.0 – 86.0 x 52.0 – 193.0 μm). *Myxospores* are non-refractive, phase-dark, stout and short rods (1.0 – 1.2 x 3.2 – 4.0 μm) with rounded ends, enclosed in sporangial wall. Bacteriolytic nutritional type. Mesophilic, aerotolerant or facultative anaerobe. Cellulose and chitin not degraded. Good growth in saccharose, fructose, mannose, and arabinose. Resistant to apramycin, gentamycin, neomycin, spectinomycin and tobramycin. Sensitive to ampicillin, carbenicillin, kanamycin, oxytetracycline, tetracycline, streptomycin, and rifampicin. Major cellular fatty acid components are *iso*−$C_{15:0}$, $C_{17:1\ 2-OH}$ and arachidonic acid. Mol percent G + C content is 67.3.

The type strain is SBNa008^T (= DSM 24000^T = NCCB 100349^T), isolated in December 2007 from Philippine soil sample taken from Landsweiler-Reden collection, Germany.

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Supplementary Table S1. Biochemical characteristics of *Minicystis rosea* SBNa008^T obtained from API ID32GN kit.

Substrates	Reaction *
L-rhamnose	+
N-acetyl-glucosamine	+
D-rhibose	+
Inositol	+
D-saccharose	+
itaconic acid	+
suberic acid	+
sodium malonate	+
lactic acid	+
L-alanine	+
potassium 5-ketogluconate	+
Glycogen	-
3-hydroxybenzoic acid	-
L-serine	+
D-mannitol	+
D-glucose	+
Salicin	-
D-melibiose	-
L-fucose	+
D-sorbitol	+
L-arabinose	+
propionic acid	+
capric acid	-
valeric acid	+
trisodium citrate	+
L-histidine	+
potassium 2-ketogluconate	+
3-hydroxy butyric acid	+
4-hydroxy benzoic acid	+
L-proline	-

***** Positive sign indicates growth, negative indicates no growth

Pseudochondromyces catenulatus gen. nov., sp. nov., nom. rev., a rediscovery of '*Chondromyces catenulatus'* Thaxter 1904

Ronald Garcia, Qian Xiao-Ming, Marcus Koch, Rolf Müller

Running Title: Rediscovery of *Chondromyces catenulatus*

Subject Category: New Taxa-Proteobacteria

Abstract

A bacterial strain designated as SBCm007^T was isolated from decaying dried wood material collected in the Wuyishan Nature Reserve, Fujian Province, China. The organism was unique in its elegant hanging chain of sporangioles arising from a slender stalk, and at the same time for producing sporangioles deep in the agar, suggesting dimorphic fruiting body morphology. The sporangioles contain short rod myxospores and, after inoculation onto agar, burrowing swarms composed of Gram-negative rod-shaped vegetative cells were produced. The strain also exhibits both bacteriolytic- and cellulolytic-type of nutrition. SBCm007^T is classified as mesophilic, aerobic to microaerophilic, chemoheterotrophic, and with narrow resistance to various antibiotics. Major fatty acids are straight chain $C_{16:1\omega7c}$, $C_{18:1\omega9c,}$ and $C_{16:0}$. The 16S rRNA gene sequence shows closest similarity to *Sorangium cellulosum* strains (96 %), *Chondromyces apiculatus,* and *C. lanuginosus* (each 95 %). Morphological characteristics suggest that SBCm007^T represents the myxobacterium described by Thaxter (1904), and documented photographically by McNeil and Skerman (1972) as the uncultured *Chondromyces catenulatus*. However, phylogenetic and chemo-physiologic analyses strongly support its classification as *Pseudochondromyces catenulatus* gen. nov., sp. nov.

Abbreviations:
FA: Fatty acid
FAME: Fatty acid methyl esters

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of SBCm007^T is GU249617.

Further images of various growth phases of strain SBCm007^T and biomacromolecule degradation are available as supplementary material with the online version of this paper.

INTRODUCTION

Chondromyces is a rod-shaped Gram-negative myxobacterium, often mistaken as fungus for its miniature tree-like fruiting body. This genus has one of the largest fruiting bodies in myxobacteria visible to the naked eye, and has a microscopically striking stalk and sporangiole clusters. Its morphological complexity varies among species, which are identifiable by their unique shape and sporangiole arrangement, such as the turnip- and bell-shaped appearances of *Chondromyces apiculatus* and *C. pediculatus,* respectively.

To date, six species of *Chondromyces* are validly described (Reichenbach, 2005), though only five are included in the approved list of bacterial names with standing nomenclature (http://www.bacterio.cict.fr/alintro.html#c). These species represent the neotype strains in the open collection (Reichenbach, 2006), except for *C. catenulatus*.

Thaxter (1904) first discovered *C. catenulatus* from a rotten poplar log near Hannover in New Hampshire, U.S.A. He described the fruiting body as showing a remarkable resemblance with the higher imperfect fungi. *C. catenulatus* is peculiar for its chain arrangement of the sporangioles. Unfortunately, the species was only obtained once despite all attempts to cultivate in various media. At present, Thaxter's type specimen TC 4517^T is housed in the Farlow Herbarium, Harvard University, Cambridge, MA, U.S.A. (Reichenbach, 2005) but unfortunately cannot be grown. The absence of available live type specimen in the culture collection renders this study to describe completely the novel bacterium.

The second occurrence of *C. catenulatus* was reported after almost eight decades in Southeast Queensland, Australia (McNeil & Skerman, 1972). Scanning electron photomicrographs of the fruiting body taken from the natural wood substrate shows the characteristic chain sporangiole arrangement. However, no details were given to further describe the bacterium. Since then, *C. catenulatus* has not been found again for almost 40 years. In both reported occasions, it was never successfully cultivated, and the species description relied on Thaxter's observation in crude culture, yet it was still included in the approved list of bacterial names due to its inclusion in the 8th edition of Bergey's Manual of Determinative Bacteriology (Skerman *et al.,* 1980).

The intensive search for myxobacteria in the past decades has yielded more than 7000 isolated strains (Reichenbach, 2006). Another group has spent 18 years on worldwide isolation of myxobacteria, covering 64 countries and representing all geographical locations (Dawid, 2000). Despite their efforts, *C. catenulatus* was never encountered and is thus thought to be rare (Reichenbach 2005). We describe here the isolation and characterisation of strain SBCm007^{T}. Evidence for its transfer from *Chondromyces* to a proposed novel genus (*Pseudochondromyces*) is hereby presented.

METHODS

Myxobacterial isolation, purification, and cultivation. Strain SBCm007^{T} was isolated in August 2008 from a piece of decaying wood collected along the riverbank in Wuyishan Nature Reserve. The sample was air dried after collection and stored at room temperature. Enrichment was prepared from mineral salts agar (Shimkets *et al.,* 2006) overlaid with two 7.5 x 15mm filter paper strips, and inoculated with 1 – 2 small pieces of decaying wood sample. Purification and culture maintenance was performed in VY/2 buffered yeast agar (Garcia *et al.,* 2009a).

Morphological observations. Growth stage morphology observations were according to the described methods (Garcia *et al.,* 2009b). To further evaluate the fine structure of the fruiting body and its developmental stages without drying, an environmental scanning electron microscope (ESEM, FEI Quanta 400 FEG) was used in wet conditions (ESEM

mode). A small part of the agar containing the fruiting body was excised and mounted on an aluminum stub. The sample was then cooled down to 276 K and imaged with secondary electrons (GSED detector) at 800 Pa, and with an accelerating voltage of 10 kV. At these conditions samples were stable for several hours.

Physiological tests. Reactions of vegetative cells to Gram and Congo Red stains were determined; staining with the latter was according to McCurdy (1969). Catalase test was performed with 3.0% H_2O_2. Cellulose degradation was performed using buffered VY/2 agar overlaid with filter paper (2 x 1cm), and water agar containing drop of powdered cellulose solution. Degradation assay for chitin was done using CT6−, CT7− agar (Reichenbach, 2006). Agar degradation was tested by inoculation of the actively growing vegetative cells on VY/2 and water agar medium containing 1.0 – 2.0 % Bacto agar.

Microbial predation tests. Overnight cultures of *Escherichia coli, Pseudomonas stutzeri, Micrococcus luteus, Bacillus subtilis*, and 36-h-old yeasts *Hansenula anomala* and *Saccharomyces cerevisiae* were spot-inoculated (approximately 10 mm in diameter) on buffered water agar. These microbial baits were air-dried before inoculated with SBCm0007^T at the edge of the plate. Cultures were then incubated at 30ºC for one week and checked daily for clearing of the baits, indicating lytic activity.

Growth responses to temperature, pH, antibiotic sensitivity, and effect of sugars and nitrogen compounds. Tests for growth responses at different levels of temperature, and antibiotic resistance were performed in buffered VY/2 agar. For pH tolerance tests, VY/2 medium was adjusted accordingly. Vegetative cell inocula came from liquid versions of the same medium taken from an overnight culture. Tests for nitrogen and sugar utilisation were previously described based on CM agar (Garcia *et al.,* 2009a). Incubation for all tests was performed for 4 – 7 days at 30ºC, except for temperature response tests at 18ºC and 37ºC. Also tested were milk casein and skim milk agar (Garcia *et al.,* 2009a).

Fatty acid and G + C content analyses. Cell pellet from actively growing culture was obtained in VY/2 medium, which consequently turned clear after a day of shaking

incubation (160 rpm, 30 °C). Cellular fatty acid extraction from duplicate samples was performed using the fatty acid methyl esters (FAME) method, and analysed by GC−MS (Garcia *et al.,* 2011). The mol percent DNA G + C content of the novel bacterium was determined by HPLC after nuclease P1 digestion of genomic DNA (Li *et al.,* 2003; Shimelis & Giese, 2006).

16S rRNA gene sequencing and phylogenetic analyses. Genomic DNA extraction from actively growing culture and amplification of the 16S rRNA gene was done as described (Garcia *et al.,* 2009b). Other myxobacterial 16S rRNA gene sequences used in this study, mostly representing the type strains in the suborder *Sorangiineae*, were obtained from GenBank. Sequence alignments were performed using Clustal W, version 2.0 (Larkin *et al.,* 2007). Distance matrices between sequences were calculated using the K80 model (Kimura, 1980). A phylogenetic tree was constructed using the PHYML v2.4.5 maximum likelihood method (Guindon & Gascuel, 2003), and bootstrap of 1000 replicates was calculated (Felsenstein, 1985). Phylogenetic relationships were also confirmed using the neighbour-joining (Saitou & Nei, 1987) and MrBayes (Huelsenbeck & Ronquist, 2001) methods. These programs are packed in Geneious Pro 5.0.2 software (Drummond *et al.,* 2010).

RESULTS AND DISCUSSION

Isolation and purification. After almost two weeks of incubation, the strain was observed to form tree-like fruiting bodies arising from decaying bark inoculum in mineral salt agar. The bacterium had also colonised onto the surrounding filter paper, as marked by the appearances of fruiting bodies and aggregates of cells. Macroscopically, SBCm007^T fruiting bodies are visible to the naked eye as orange spots on substrate, and exhibiting typical *Chondromyces* appearance. By plucking the sporangiole hanging at the tip of the chain using a fine sterile glass needle and inoculating them onto filter paper overlaid on buffered yeast medium, SBCm007^T was brought into cultivation. After 3 – 4 days, swarms in the agar were produced, and subsequent transfers from the colony edge allowed the bacterium to be isolated for the first time after more than a century.

In two occurrences of *C. catenulatus* (Thaxter, 1904; McNeil & Skerman, 1972), including SBCm007^T, both occurred on wood, which appears to be their common ecological niche. Based on its first discovery in Hanover, New Hampshire, (geographically bordering the Vermont river), and present isolation taken from riverbank in Wuyishan, it appears that *C. catenulatus* is associated with a high water content or moist environment. This characteristic correlates with its ability to grow well into soft (< 1%) agar and in liquid broth medium.

Morphological and cultural characterisations

Swarm. In buffered VY/2 or water agar, pseudoplasmodial swarms with characteristic band-shaped end were produced deep in the agar (Fig. 1a). These were typically pale to orange in colour. The same pattern was also observed in nutrient free medium (buffered water agar). Deep and long agar corrosion was architecturally engraved by the migrating cells (Supplementary Fig. S1a). These characteristics are reminiscent of *Polyangium* swarming (Supplementary Fig. S1b).

A long orange fan-shaped pseudoplasmodium swarm was observed on filter paper overlaid on water, and VY/2 agar (Garcia *et al.,* 2010). This pattern fits to the description of *Chondromyces catenulatus* (Thaxter, 1904), and finds a counterpart with *Byssovorax cruenta* which also produced this type of swarming on agar but not on filter paper (Reichenbach *et al.,* 2006). The presence of wrinkled parchment-like slime trails on filter paper differentiates the latter from *P. catenulatus*. After the colony of SBCm007^T reaches the end of the paper, or when it comes in contact with agar, the bacterium penetrates into the medium, developing band-shaped swarming.

A light peach coloured diffusing pigment around the colony was produced in mineral salts agar, becoming even more intense upon supplementation with maltose. No fluorescence was observed in and around the colony at both short and long UV wavelengths.

Vegetative cells. Vegetative cells were slender fat rods (1·2 − 1·4 x 5·0 − 15·0 μm) with blunted ends, and phase-dark (Fig. 1b). Members of the suborder *Sorangiineae* and most of the *Nannocystineae* have these characteristics. In old culture, several refractile rounded vesicles (< 10) were observed inside the cell, perhaps fat globules. Motility was by gliding in solid substrate.

Myxospores. The myxospores are slightly optically refractile fat rods, measuring half the size of the vegetative cells (3 – 7 μm x 1.2 – 1.3 μm), with blunted ends (Fig. 1c), and containing dark spots at the ends. The average length of the spores ranged from 4 – 6 μm. They are dormant and desiccation-resistant.

Fruiting body. The multicellular organisation of the SBCm007^T fruiting body includes a stalk and cluster of chain of sporangioles (Fig. 1d). These tree-like features appear common only to *Stigmatella* in *Cystobacterineae* and *Chondromyces* in *Sorangiineae*. Its difference to *Stigmatella* is mirrored morphologically in the vegetative cells, swarm, and myxospores. *Stigmatella* exhibits fusiform vegetative cells with pointed ends, radial swarming with flame-like edges, and irregular curved rod myxospores. The novel isolate is strikingly different to other *Chondromyces* species by its chain arrangement of sporangioles, which so far has only been observed in *C. catenulatus*. Overall, the SBCm007^T fruiting body shows closest morphological similarity to *Chondromyces* .

P. catenulatus fruiting body is composed of three to five oval sporangioles in a chain, with four (125 – 175 μm) being the most common. Two sporangioles are often observed in smaller fruiting bodies. The number of sporangioles described by Thaxter (1904) was more than double (10 or 12) of that found for SBCm007^T. This may depend on the number of cells that cooperatively aggregate during morphogenesis, and is perhaps influenced by the nutrient source present in the sample material. As noted before, *C. catenulatus* was described based on its appearance on a piece of wood, which seems to provide good growth conditions for supporting longer sporangial chains. The fruiting body is usually composed of 50 or more chains of sporangioles and appears as an inverted broom. The bifurcation of the sporangiole chain, as described by Thaxter,

(1904), is somewhat rare, and only noticeable in long chains. However, this developmental phenomenon appears to be a consequence of chains merging which occurs during sporangiole elongation, and usually results in large fruiting bodies.

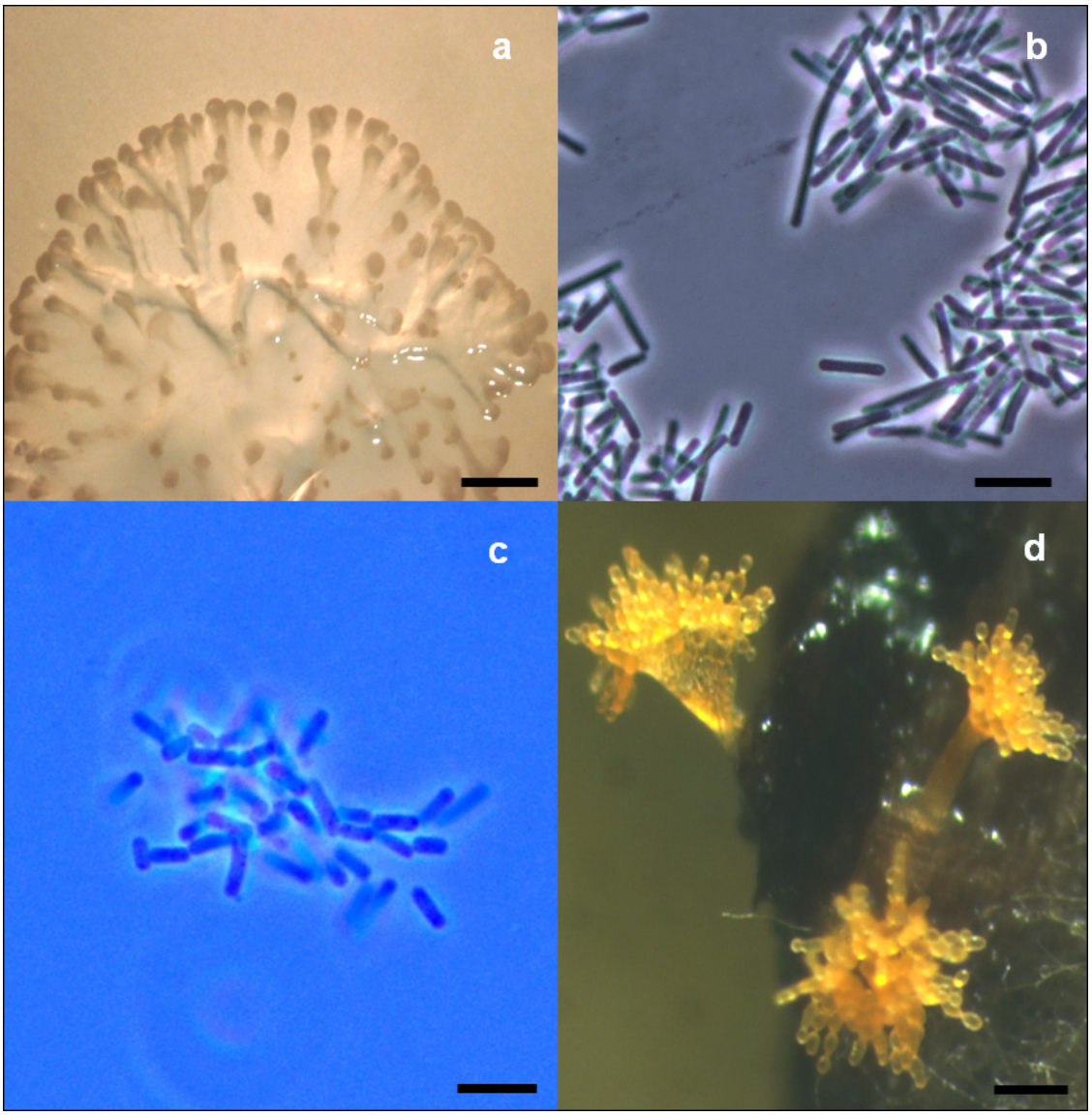

Figure 1. Growth morphology stages of *Pseudochondromyces catenulatus* SBCm007^{T}. (a) Swarm colony in agar, (b) Vegetative cells, (c) Myxospore stage, (d) Fruiting bodies on crude culture (wood). Dissecting photomicrographs (a, d), phase-contrast images (b-c). Bar. 250 µm (a), 10 µm (b, d).

Sporangioles show a pale yellow or peach colour during early stage of fructification and become yellow-orange at maturity. Its shape varies from oval to spherical, depending on its location in the chain. Often, the smallest and spherical sporangioles (22.5 x 25 μm) are located at the end or tip of the chain, while the largest appear closest to the stalk. The size of sporangioles (20 – 50 x 18 μm) is in agreement with Thaxter (1904). Figure 2 and 3 show these developmental stages on agar surface. A discovery of spherical masses with an enclosing wall in the agar is suggestive of sporangiole (Supplementary Fig. S2). They are commonly arranged solitarily or in clusters, containing slightly refractile rod-shaped cells with blunted ends. Their desiccation resistance and germination ability leading to swarm formation was indicative of myxospores.

A slime thread-like structure, or 'isthmus' (5.0– 7.5 x 37.5μm), connects to the stalk and between sporangioles in a chain (Supplementary Fig. S3). Sometimes they fused at the stalk end to produce a flower-like arrangement of the sporangioles. We did not find a chain connected by an alternating series of sporangiole and isthmuses, as those illustrated by Thaxter (1904) in his schematic diagram of *C. catenulatus* (Plate 26, No. 4). Occasionally, a white spine attached to the hanging-end sporangiole can be found.

A stalk supporting the chain of sporangioles was not only observed in crude culture, but nevertheless could be found axenically (Supplementary Fig. S4). In some instances, several of them may develop over time in a specific site to form clusters of fruiting bodies. This condition seems to be illustrated well in Figure 2 of Thaxter's Plate 26 (Thaxter 1904). At an early stage, the stalk appears to be white and later becomes yellowish-brown. The stalk conforms to Thaxter's description of cytosphores, broad at the base and narrow at the tip.

Atypical fruiting bodies of some other species, for example *Chondromyces crocatus,* may also exhibit chain arrangement of sporangioles (Reichenbach, 2005); however they are shorter and composed only of a few chains devoid of isthmus. The novel isolate can also be differentiated significantly in other features, such as swarm, chemo-physiology, and genetic characteristics.

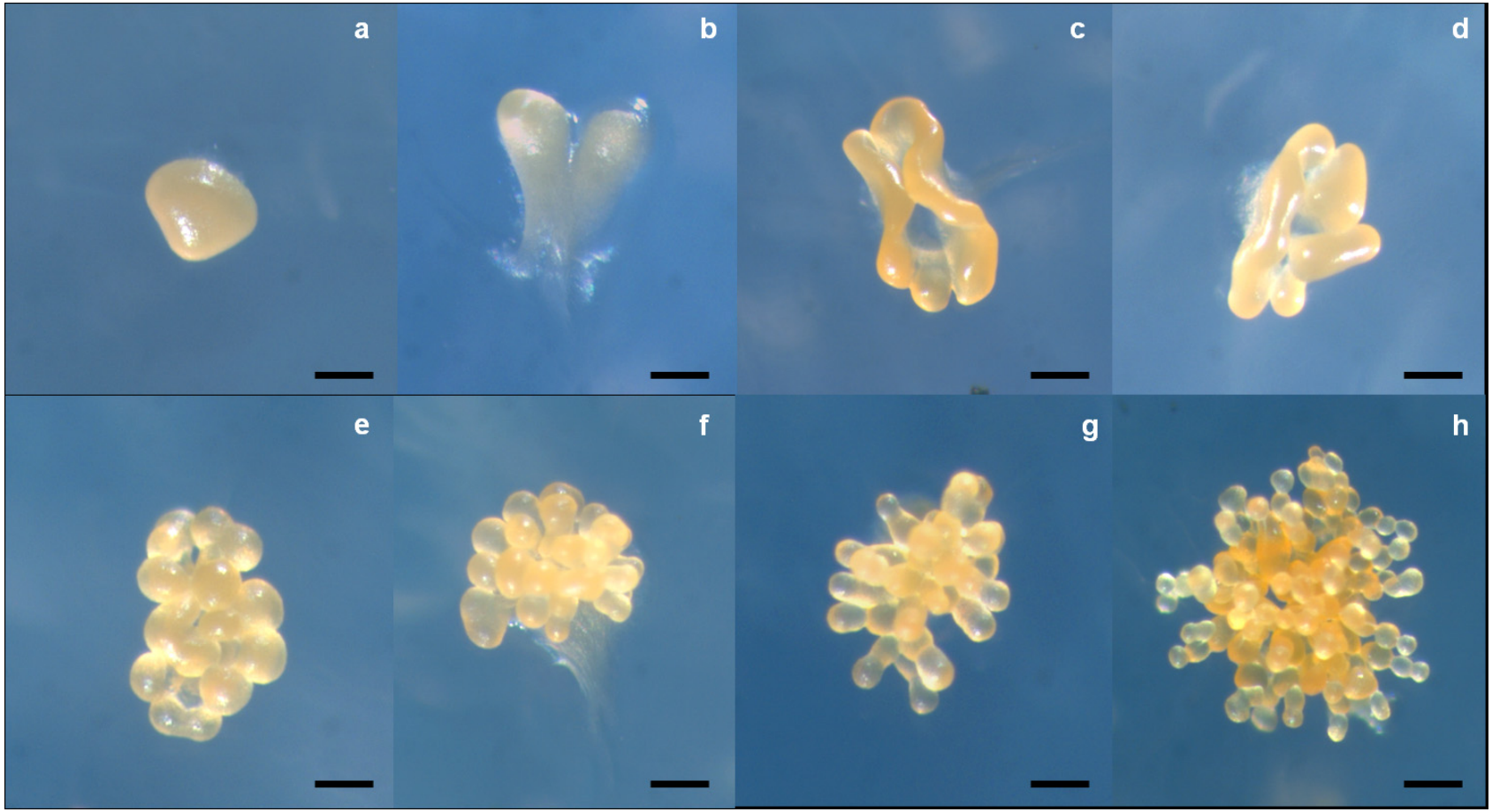

Figure 2. Fruiting body developmental stages of SBCm007^{T} on agar medium. (a) mound formation of vegetative cells, (b-d) differentiation to ridges of cells and upliftment by stalk development. (e-f) transformation to globular knobs. (g) knob cell elongation and cleavage (h) differentiation to chain of sporangioles. Bar, 100 μm.

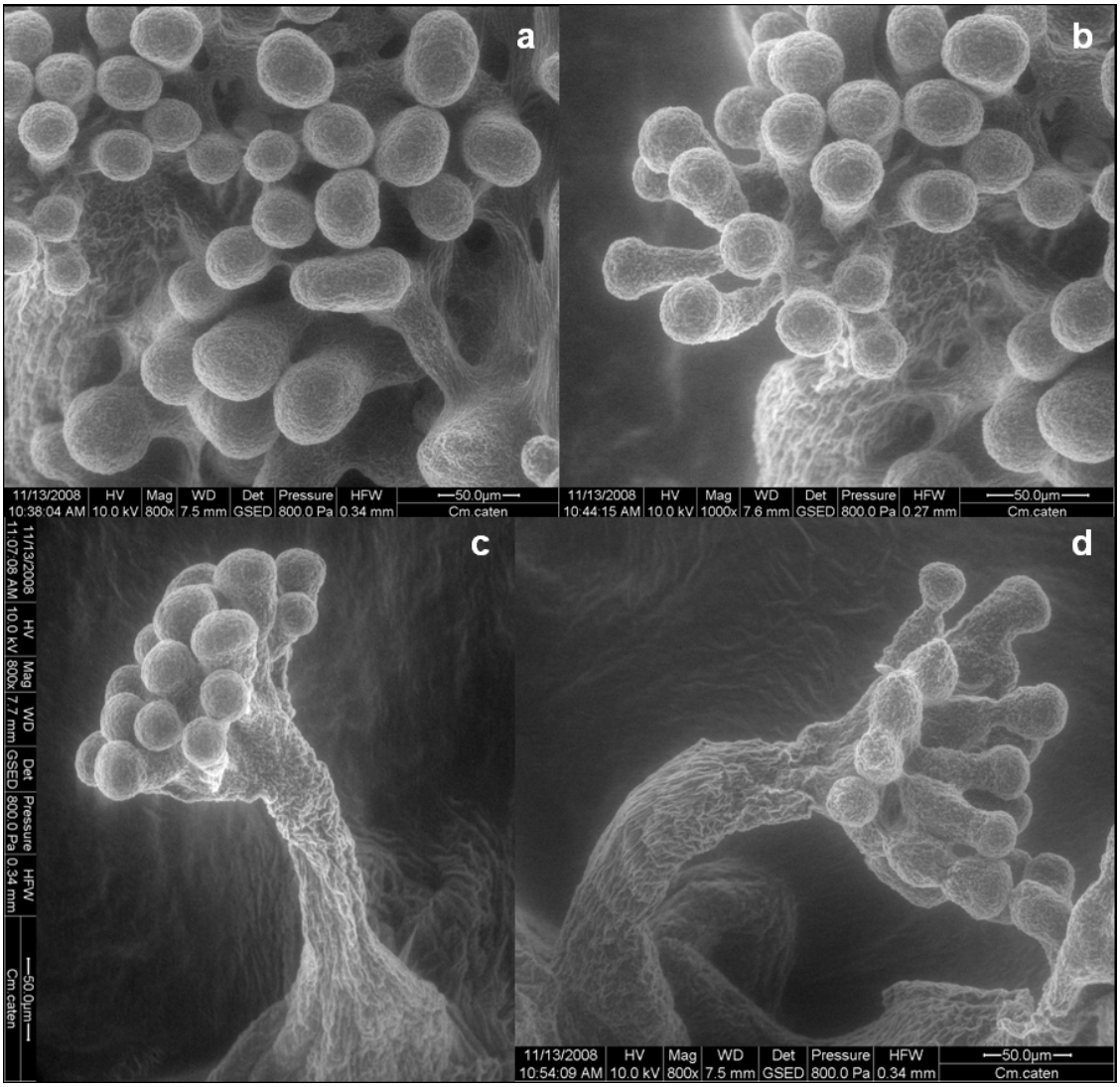

Figure 3. ESEM photomicrographs of *P. catenulatus* fruiting body development (a) Humped cellular aggregations. (b) Cellular mass elevation (c) Differentiation into developing sporangioles and stalk. (d). Chain of sporangiole development.

Physiological characteristics

Staining and lytic property. The vegetative cells are Gram-negative and catalase-positive. The actively migrating swarm cells were Congo-red negative, as is typical among suborder *Sorangiineae*.

Temperature and pH responses. On buffered VY/2 agar, optimal growth was observed at 30 °C. Minimal growth could also be observed at room temperature (23 °C) and at

33 °C. No growth could be seen at 18 °C and 37 °C. SBCm007^T exhibited pH tolerance of 5.0 – 8.0. Optimal growth was found at pH 7.0 in VY/2 agar. No growth was observed at pH 4.0 and beyond 8.0.

Degradation of biomacromolecules and predatory ability. Chitin and xylan were not degraded. No growth was observed in SKM medium. Agar was deeply corroded, splited and liquefied (Supplementary Fig. S5); the effect was especially obvious at an agar concentration of less than 1.5 %. In our years of experience with myxobacteria, we have not found a strain with such strong agarolytic characteristic as SBCm007^T. A closer similarity, but to a lesser degree, was found among members of the *Polyangium*, which upon incubation form soft and watery agar.

Lysis of live microbial bait was observed in Gram-negative *Escherichia coli* and *Pseudomonas stutzeri.* Gram-positive *Bacillus subtilis* was also lysed, but not *Micrococcus luteus,* or yeast *(Saccharomyces cerevisiae*, *Hansenula anomala).* The predatory behaviour of SBCm007^T is perhaps attributed to its lytic enzymes and bioactive compounds, which myxobacteria have become famous for.

Filter paper was slowly degraded (Supplementary Fig. S6a). To date, this is the third group of myxobacteria discovered after *Sorangium* and *Byssovorax*, capable of cellulose breakdown (Supplementary Fig. S6b-c).The novel isolate pulls apart the cellulose fiber strands and eventually degrades them (Supplementary Fig. S6d). Filter paper strips become smaller, leaving imprints of border edges on the agar (Supplementary Fig. S6e). The strain was initially suspected to be a cellulose degrader when it grew well on a lean medium (Water agar) with only filter paper as the carbon source. The degradation process usually takes more than 3 weeks of incubation on filter paper overlaid on agar. In *Sorangium,* we observed strong and complete cellulose degradation, with fiber strands filled with chains or clusters of fruiting bodies (Supplementary Fig. S6b). In *Byssovorax*, cellulose fibers are degraded by the migrating swarm colony, leaving a hole in the centre of the paper (Supplementary Fig. S6c).

Sugar utilisation. In medium supplemented with sugar, SBCm007^T grew well in the presence of starch, fructose, saccharose, molasses and lactose. Poor growth was observed upon supplementation with maltose, D-glucose, xylose, sorbitol, D-galactose, arabinose, and mannose. In the presence of soluble starch, a clear halo was produced after flooding with iodine solution, suggesting hydrolysis.

Nitrogen and peptone sources. Swarming cells were observed in the presence of potassium nitrate and aspartic acid. Better growth was exhibited in the latter substrate, showing more scattered migrating cells in the agar. However, no evidence of swarming could be observed after the incorporation of glutamic acid, urea, and potassium nitrate.

The most pronounced swarming, as reflected by the colony diameter, was seen in medium supplemented with Marcor peptone and neopeptone. Poor growth was observed in casitone, casamino acids, Bacto peptone, tryptone, and phytone.

Antibiotic resistance. SBCm007^T was found to be sensitive to carbenicillin, tetracycline, spectinomycin, tobramycin, gentamycin, oxytetracycline, rifamficin, apramycin, ampicillin, and streptomycin. Growth was observed in neomycin, kanamycin, and hygromycin, indicating its resistance.

Fatty acid and DNA G + C analyses.

The predominance of straight chain fatty acids (90.10 %) (Table1), and presence of the biomarker hydroxy FA $C_{17:1\ 2OH}$ supports SBCm007^{T}affiliation to the *Sorangiineae* suborder. The major fatty acids were $C_{16:1\omega7c}$ (29.79 %), $C_{18:1\omega9c}$ (29.24 %), and $C_{16:0}$ (13.28 %). The amount of $C_{16:1\omega7c}$ shows similarity with *Polyangium, Chondromyces*, and *Jahnella*, but differs from the first two genera by the unusual elevated amount of $C_{18:1\omega9c}$. In addition, SBCm007^T also differs in the total amount of branched-chain fatty acids, amounting to almost 10 %. The absence of the polyunsaturated fatty acid $C_{18:2\omega6,9c}$ and branched-chain hydroxy fatty acid further supports the dissimilarity of SBCm007^T to *Chondromyces* and *Polyangium*. The detection of anteiso $C_{17:0}$ FA (5.15 %) appears to be not only exclusive to marine myxobacteria, but also seems distributed to some terrestrial-

dwelling isolates (Garcia *et al.,* 2011). The DNA G + C content of the novel isolate is 65.6 mol %.

Table 1. Percent cellular fatty acid composition of SBCm007^T.

Fatty acid	%
Saturated straight-chain	
$C_{14:0}$	0.43
$C_{15:0}$	0.19
$C_{16:0}$	13.28
$C_{17:0}$	1.21
$C_{18:0}$	5.45
Unsaturated straight-chain	
$C_{14:1\ \omega2c}$	0.74
$C_{14:1\omega5c}$	0.72
$C_{15:1\omega5c}$	0.08
$C_{16:1\omega7c}$	29.79
$C_{16:1\omega9c}$	4.72
$C_{17:1\omega7c}$	0.74
$C_{18:1\omega9c}$	29.24
Hydroxy straight-chain	
$C_{17:1\ 2\text{-}OH}$	1.51
O-Alkylglycerols (OAG)	
$C_{15:0}$	0.07
$C_{16:0}$	1.93
Branched-chain	
iso-$C_{15:0}$	3.61
iso-$C_{16:0}$	0.34
anteiso-$C_{17:0}$	5.15
Branched-chain OAG	
iso-$C_{15:0}$	0.13
Branched-chain DMA	
iso-$C_{15:0}$	0.67

Phylogenetic analysis. SBCm007^T shows closest 16S rRNA gene sequence identity (95 %) to a clone of an uncultured bacterium (AF280858), *Sorangium cellulosum* strains (95 %), and with other undescribed *Sorangiineae* isolates (96-97 %, GenBank Accession number GU249611-GU249613). Its similarity to type strains of *Chondromyces* was determined between 94.2 % – 95.2 %, whereas there was 96 % – 97.3 % identity with *Polyangium*. In addition, its close identity with the latter genus supports its clustering in the phylogenetic tree (Fig. 4). The phylogenetic divergence of SBCm007^T to the

Chondromyces cluster was unexpected, as it exhibited typical *Chondromyces* fruiting body formation. SBCm007^T represents a novel taxon closely related to *Polyangium*, and is proposed herewith to occupy a novel genus and species. In addition, the topology clearly shows the divergence of the novel isolate to the type strain of *Sorangium* (95.3 %) and *Byssovorax* (95.2 %). Its position in the phylogenetic tree was also supported by a previous study (Garcia *et al.,* 2010).

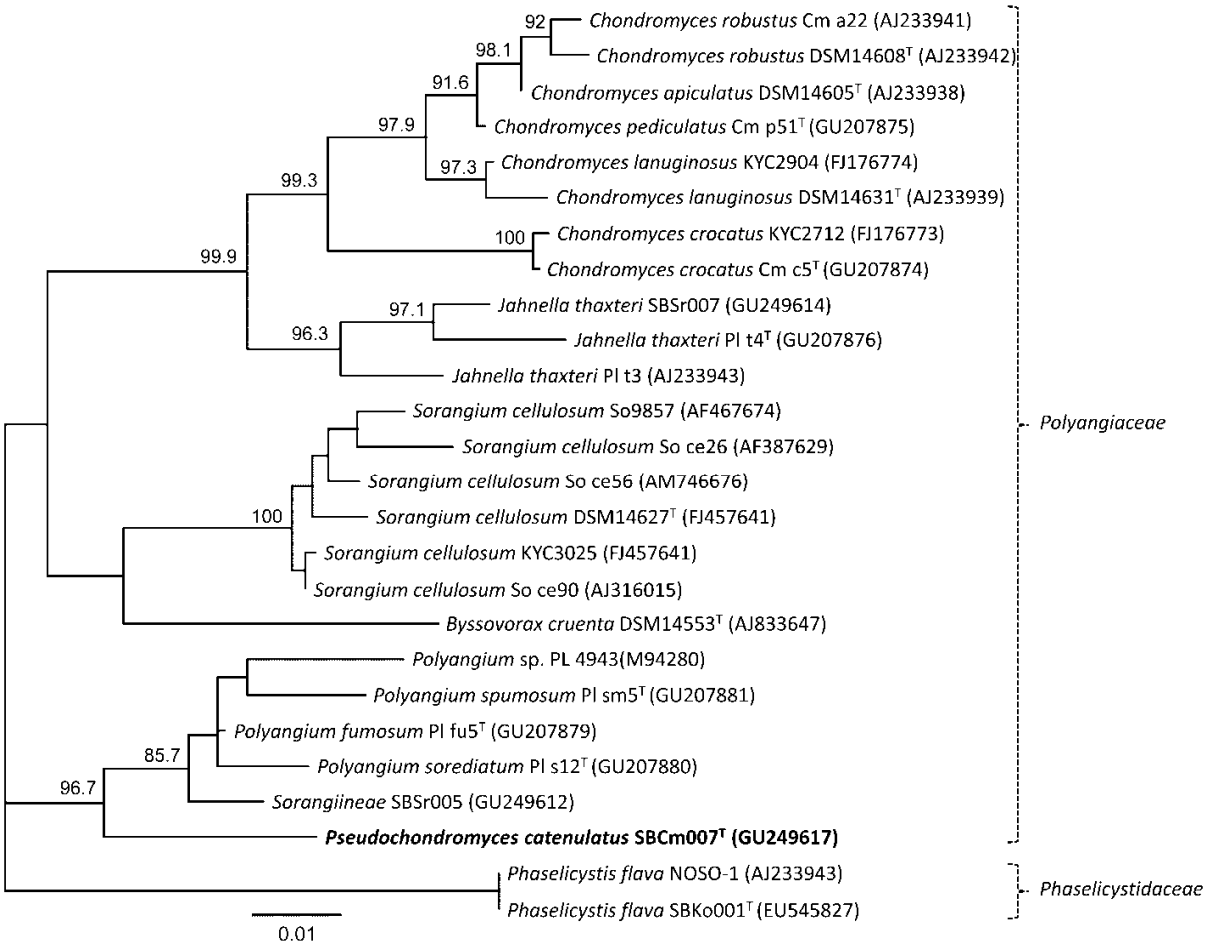

Figure 4. Phylogenetic position of *Pseudochondromyces catenulatus* SBCm007^T in suborder *Sorangiineae*, constructed using the maximum likelihood program (PHYML). The numbers at branchpoints show the level of bootstrap support based on 1000 resamplings. Only values greater than 60 are shown. Bar, 0.01 substitution per nucleotide position.

Description of *Pseudochondromyces* gen. nov.
Garcia, Xiao-Ming and Müller

Pseudochondromyces Pseu. do. chon. dro. my'ces. Gr. adj. *pseudês* false; N.L. masc. n. *Chondromyces* a myxobacterial genus name, *Chondromyces*; N.L. masc. n. *Pseudochondromyces* the false *Chondromyces*.

Vegetative cells are long cylindrical rods with rounded ends. Colony moves by gliding pseudoplasmodial bands, often penetrating to the agar. Swarm stains negative in Congo-red. Myxospores resemble vegetative cells but are slightly refractile, smaller, and enclosed in sporangioles. Fruiting body is composed of sporangioles. Degrade bacteria, yeast, and cellulose. The type species is *Pseudochondromyces catenulatus*.

Description of *Pseudochondromyces catenulatus*. sp. nov., nom. rev. (*ex* Thaxter 1904). Garcia, Xiao-Ming and Müller

Pseudochondromyces catenulatus ca. te. nu' la. tus. L. dim.n. catenula, a small chain; L. masc. suff. -atus, suffix used in adjectives meaning provided with; N.L. masc. adj. *catenulatus*, with small chains (of sporangioles).

Show all the characteristics of the genus. Vegetative cells are long slender fat rods, 1.2–1.4 x 5.0–15.0 μm, and phase dark. Swarms are pale to deep orange, pseudoplasmodial-type with band-shape end, burrowing deep into the medium. Fruiting body is composed of a stalk and chains (2 – 5, common 3 – 4) of oval to spherical sporangioles connected by thin string of slime. Solitary to cluster of sporangioles without stalk are also produced in the agar. Myxospores are cylindrical rods (3 – 7 μm x 1.2 – 1.3 μm) with rounded ends, partially refractile, and enclosed in sporangiole. Agar is degraded and liquefied. Bacteria and yeast are lysed. Cellulose is also degraded. Exhibits resistance to neomycin, kanamycin, and hygromycin. Sensitive to carbenicillin, tetracycline, spectinomycin, tobramycin, gentamycin, oxytetracycline, rifamficin, apramycin, ampicillin, and

streptomycin. Major cellular fatty acid components are straight chain $C_{16:1\omega7c}$, $C_{18:1\omega9c}$, and $C_{16:0}$. It has DNA G + C content of 65.6 mol %.

The type strain is SBCm007^T (= DSM 24112^T, = NCCB 100348^T), isolated from a piece of decaying wood collected in China.

ACKNOWLEDGEMENTS

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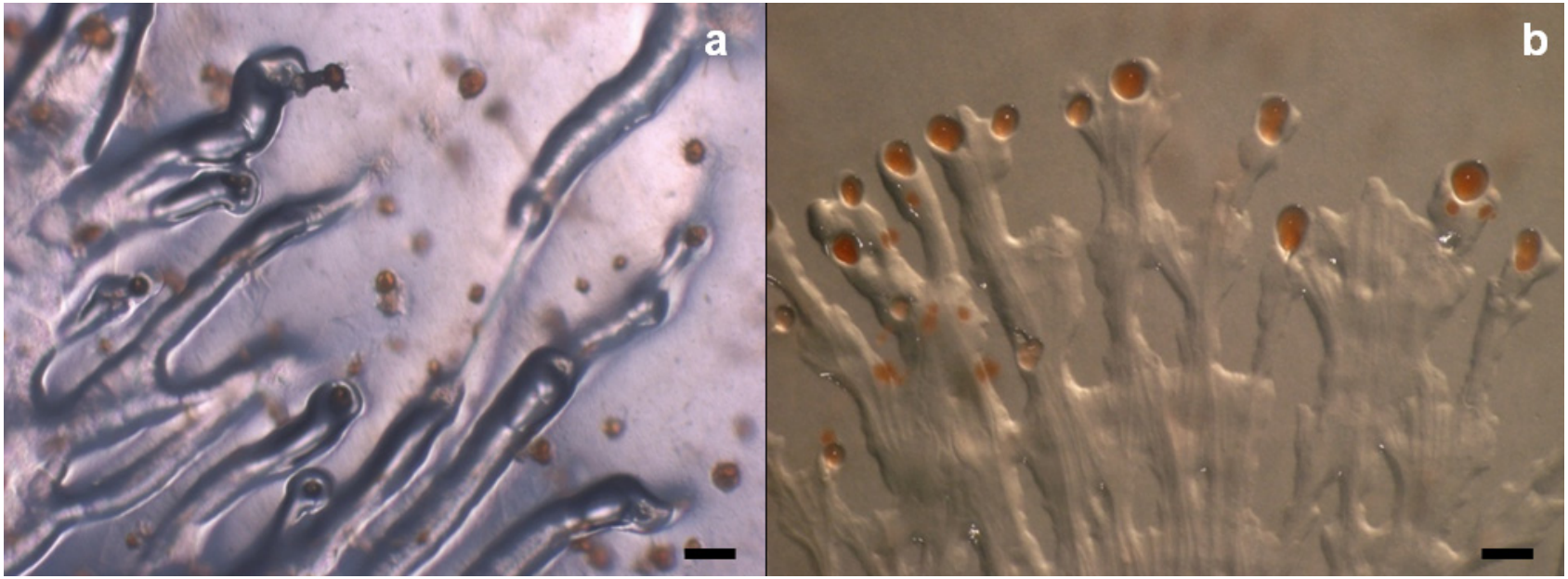
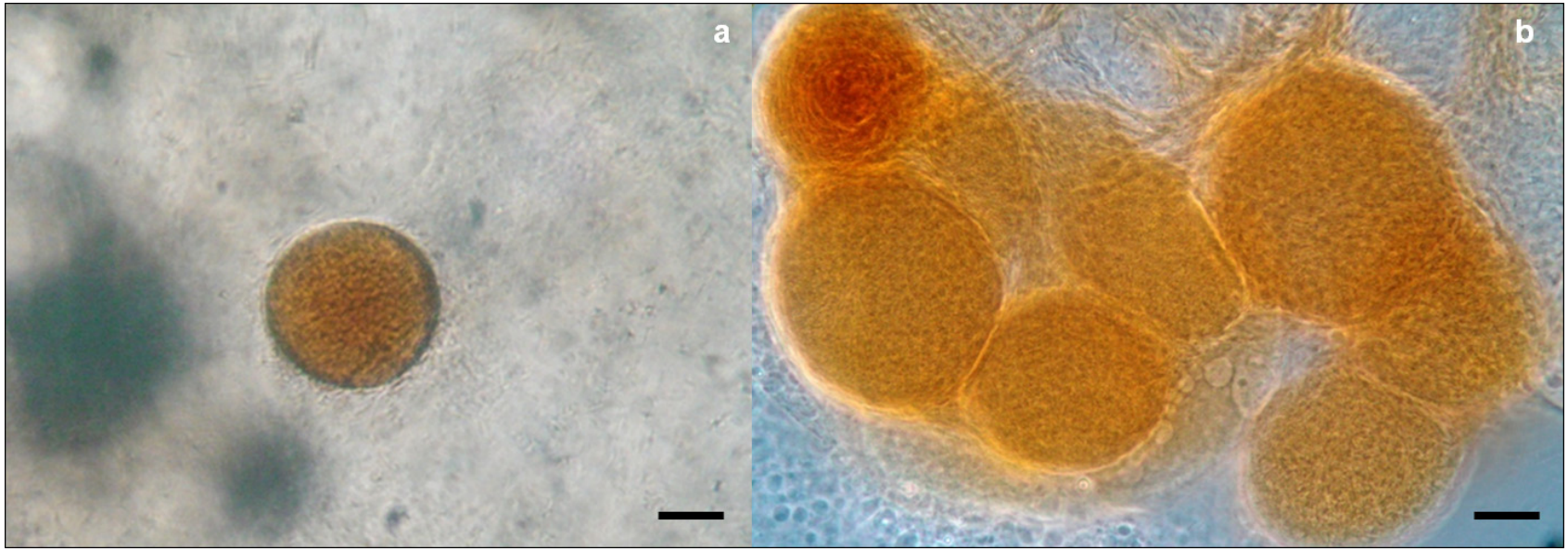
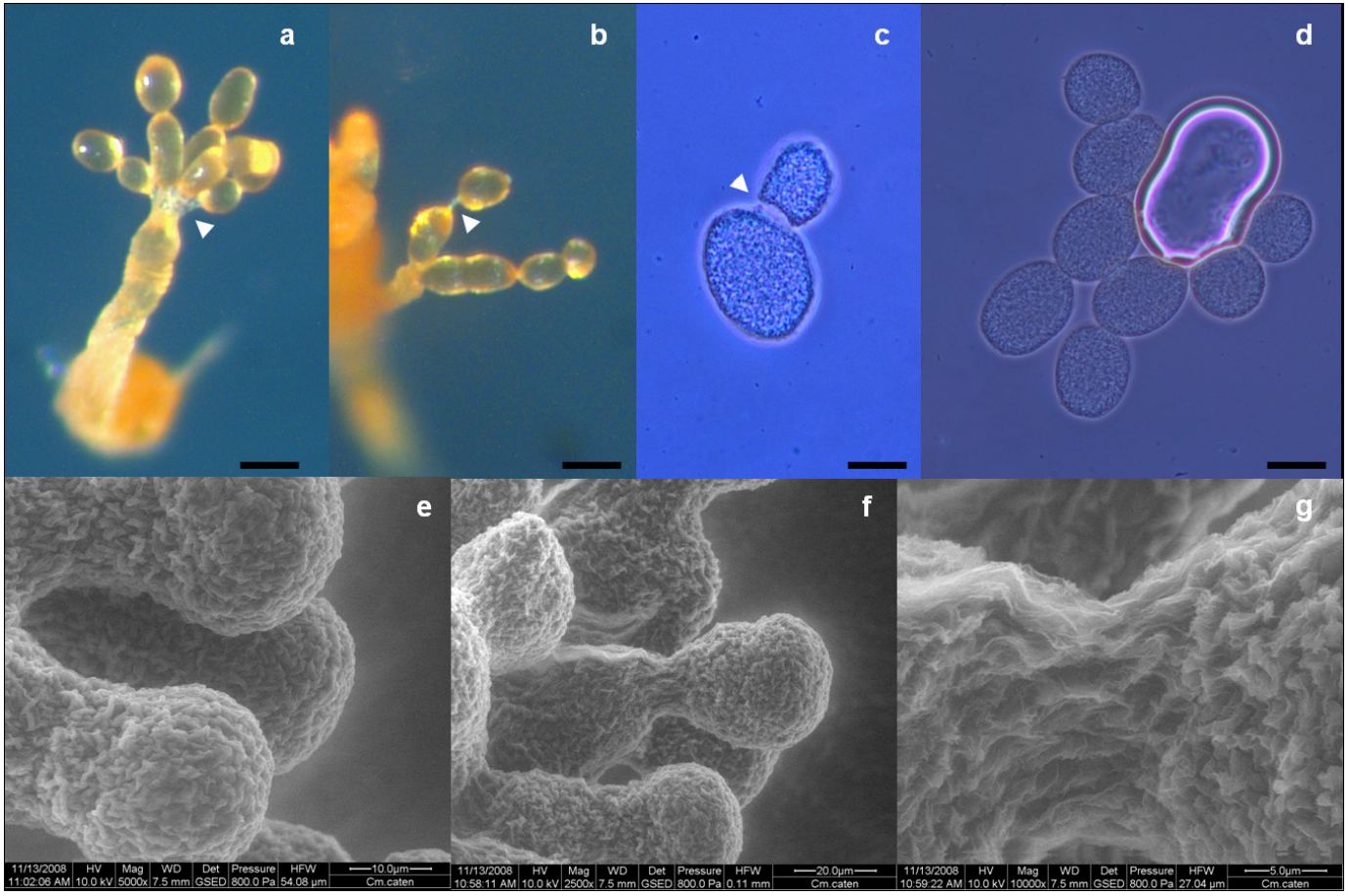


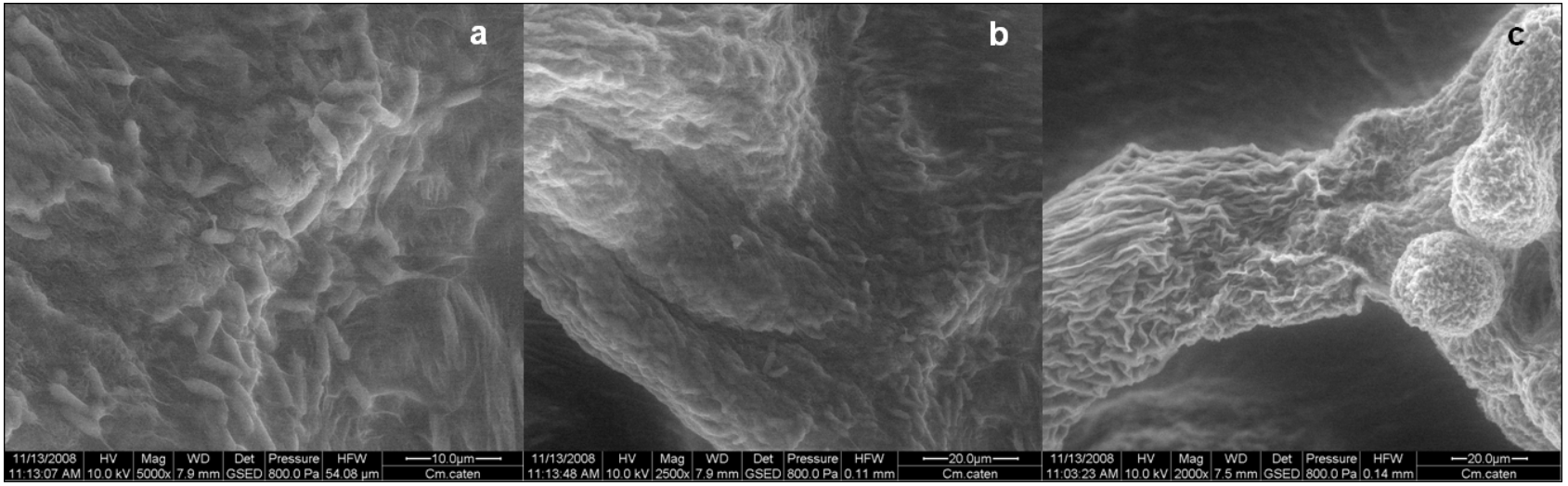
Supplementary Figure S1. Dissecting photomicrographs of swarm colonies on agar. (a) *Polyangium sorediatum* Pl s12^{T}, (b) *Pseudochondromyces catenulatus* SBCm007^{T} Bar, 90 µm.



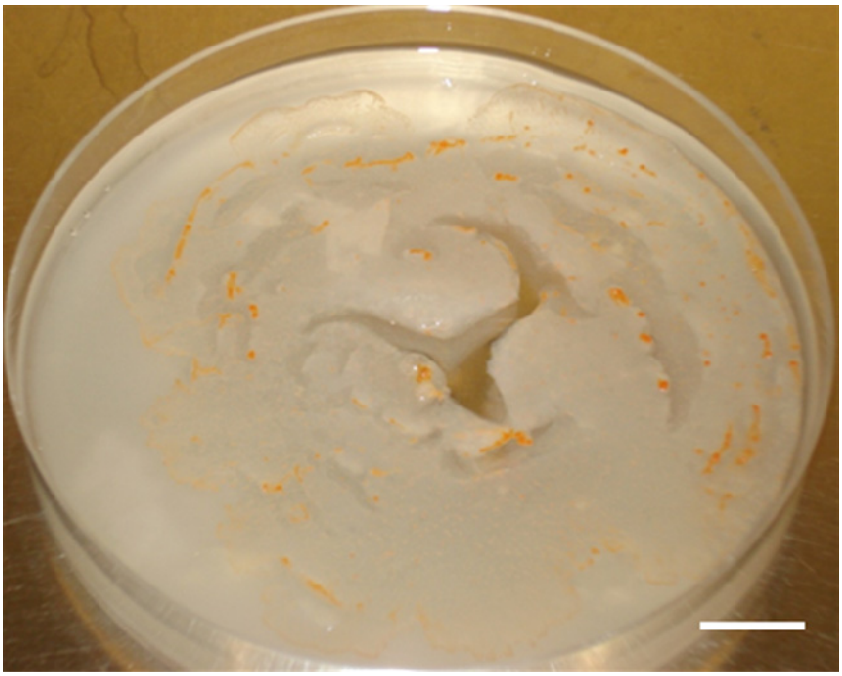
Supplementary Figure S2. Fruiting body of SBCm007^{T} in agar (a) Dissecting photomicrograph of solitary sporangiole. (b) Slide mount of sporangiole cluster, phase-contrast. Bar. 15 µm.



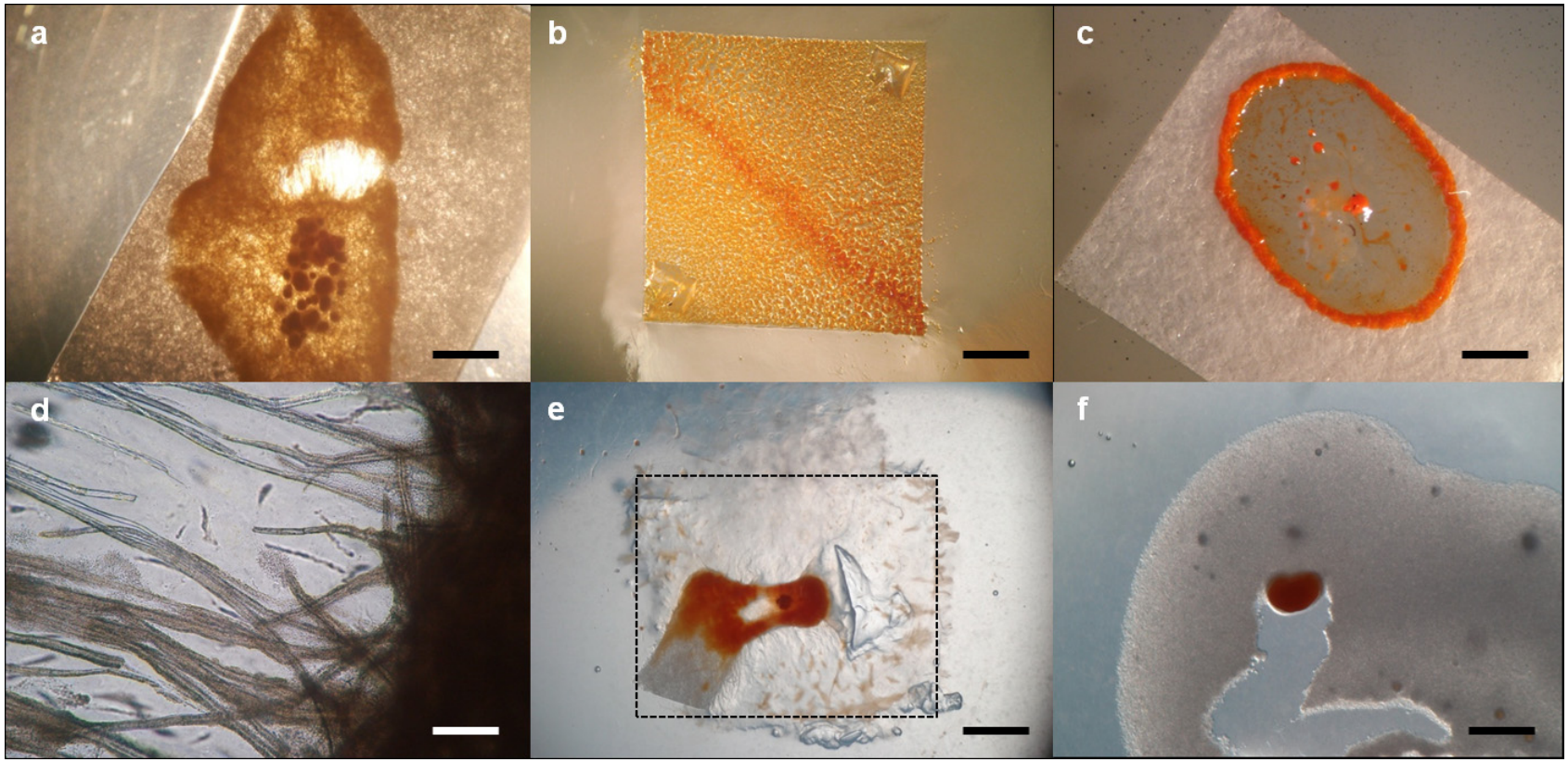
Supplementary Figure S3. Chain of sporangioles. (a) Dissecting photomicrograph of glassy slime connecting the stalk and chain of sporangioles (arrow). (b-c) String connection between sporangioles (arrow). (d) Long chain of sporangioles. (e) end section of developing sporangial chain, (f-g) cleavage site between sporangioles. Photomicrographs taken under dissecting (a-b), phase-contrast (c-d), and ESEM (e-f) microscope. Bar, 50 µm (a-b), 20 µm (c), 40 µm (d).



Supplementary Figure S4. ESEM photomicrographs of SBCm007^T stalk. (a) Rod-shaped cells on the base of the stalk. (b) base of the stalk showing their union. (c) stalk close to the sporangiole chain.



Supplementary Figure S5. Agar degradation and liquifaction of *Pseudochondromyces catenulatus* SBCm007^{T} on yeast medium (VY/2). Bar, 10 mm.



Supplementary Figure S6. Cellulose degradation pattern in myxobacteria. (a) *Pseudochondromyces catenulatus* SBCm007^{T}. (b) *Sorangium cellulosum.* (c) *Byssovorax cruenta.* (d-f) Characteristic pattern in *P. catenulatus*. (d) Cellulose fibre breaks, (e) Filter paper degradation with agar retraction. Dotted square shows the original size of the paper. (f) Clearing of cellulose powder spot on agar by the gliding cell clump. Bar, 1.5mm (a), 4.0mm (b), 2.0mm (c), 500µm (d), 4.5mm (e), 300µm (f).

Aetherobacter fasciculatus sp. nov., *Aetherobacter rufus* sp. nov., omega-3-rich polyunsaturated fatty acid-producing myxobacteria, and the description of *Aetherobacter* gen. nov.

Ronald Garcia, Marc Stadler, and Rolf Müller

Running Title: *Aetherobacter*, omega-3-producing myxobacteria

Subject Category: New Taxa – Proteobacteria

Abstract

Bacterial strains designated as SBSr002^T and SBSr003^T were isolated in 2007 from an Indonesian soil sample containing pieces of roots and other decaying plant material taken from the Landsweiler-Reden collection, Germany. The organisms were recognised for their burrowing swarm in the agar and cluster of sporangioles containing short rod myxospores. Vegetative cells are Gram-negative rods. The novel isolates exhibit a bacteriolytic-type of nutrition. The strains are mesophilic, aerobic to microaerophilic, chemoheterotrophic, and show resistance to various antibiotics. Major fatty acids are straight chain iso-$C_{15:0}$, and $C_{22:6}$ (*n*-3) (docosahexaenoic acid, DHA). The G + C content of their genomic DNA is 68 – 69 mol %. The 16S rRNA gene sequence shows closest similarity (96-98%) to clones of uncultured bacteria (GenBank accession number: FJ479473, FN421522), *Byssovorax cruenta*, and *Sorangium cellulosum* strains.

Abbreviations

PUFA: Polyunsaturated fatty acid

EPA: Eicosapentaenoic acid

DHA: Docosahexaenoic acid

AA: Arachidonic acid

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the novel strains are GU249609 and GU249610.

INTRODUCTION

Myxobacteria have gained recognition in the past for their distinctive biology, developmental cycle, and production of novel and interesting secondary metabolites (Weissman & Müller, 2010; Wenzel & Müller, 2009). Myxobacterial cells glide and, under starvation conditions, develop into resting cells which are safely packed in the fruiting body. The production of secondary metabolites by myxobacteria appears to be a defense mechanism of the gliding cells to protect themselves from other microorganisms in the surrounding environment. In this study, we present different elements of exciting application found in novel myxobacteria during chemical characterisation. Surprising was the discovery of omega-3 polyunsaturated fatty acid (PUFA) in the novel isolates, although this is not the first report of PUFAs in myxobacteria (Garcia *et al.,* 2009b). Due to their health benefits for humans, PUFAs are commercially and medically important fatty acids (Horrocks & Yeo, 1999). Many issues have been raised concerning the current market source (fish oil), thus necessitating the ongoing search for a more secure and sustainable alternative source (Ward & Singh, 2005). In this study, we highlight the unique characteristic of the novel myxobacterial isolates to produce high percentages of omega-3 fatty acids and describe their unusual phenotypic features along with phylogenetic and molecular data, leading to the proposal of new taxa.

Methods

Isolation and Cultivation. Strain SBSr003^T was isolated using the filter paper-baiting method on mineral salt agar, while SBSr002^T was isolated from water agar baited with *Escherichia coli*. (Shimkets *et al.,* 2006). By cutting the colony edge and repeated transfers onto new medium (water agar), the strains were isolated. They are routinely cultivated and maintained in buffered yeast agar (VY/2) medium (Garcia *et al.,* 2009a).

Microscopy and Morphological Examination. Swarming colonies and fruiting body morphology observations were described previously (Garcia *et al.,* 2009b) Vegetative cells and myxospores were studied using phase-contrast microscope (Axiostar, Carl Zeiss). All growth stages were observed on water agar, MDIG agar (0.3 % Casitone, 0.35 % Glucose, 0.05 % $CaCl_2 \cdot 2H_2O$, 0.2 % $MgSO_4 \cdot 7H_2O$, pH 7.2 adjusted with KOH), and buffered yeast agar.

Microbial predation test. Overnight culture of *Escherichia coli, Hansenula anomala, Psedumonas stutzeri, Micrococcus luteus* were used as bait in water agar medium. Growth of

myxobacterium and microbial lysis, as indicated by clearing of the streaked bait, was determined after 5-7 days of incubation at 30°C.

Physiological and biochemical tests. Reactions of vegetative cells to Gram stain was analysed according to the standard microbiological method, whereas Congo Red staining of the swarm colony was done according to McCurdy (1969). Catalase test was performed using 3.0% H_2O_2. Cellulose degradation was performed in water and VY/2 agar overlaid with filter paper (2.0 x 1.0 cm) and a drop of cellulose powder on the other section of the agar. Degradation assay for chitin was also performed in the same media but only with a drop of chitin powder (Sigma) solution. Growth determination on xylan and skim milk agar was previously described (Garcia *et al.,* 2009b). All incubations were performed at 30°C for 1 to 2 weeks.

Growth responses to pH, temperature, sugar and nitrogen sources, and antibiotics. Growth responses to different levels of temperature, and antibiotic resistance were tested in buffered VY/2 agar. For pH tolerance, VY/2 agar was adjusted accordingly. In parallel, the latter test and the temperature response tests were also studied in broth MD1G and CG (same as MD1G, but without salts) medium, shaken at 160 rpm, 30 °C. Tests for different sugar sources were also performed in the same media but glucose was substituted with the sugar being tested. Tests for different peptone and inorganic nitrogen sources were also performed in MD1G medium, but substituted with the corresponding substrate being tested. All tests were incubated for 8 days at 30ºC, except for the temperature response tests, which were also incubated at 18ºC, 22ºC (room temperature), 35°C, and 37ºC.

Fatty acid and G + C content analyses. Cell pellet from actively growing culture was obtained from MD1G medium shaken at 160 rpm, 30°C. Cellular fatty acid extraction from triplicate samples taken from 5-d-old culture was performed using the fatty acid methyl esters (FAME) method and analysed by GC−MS (Garcia *et al.,* 2011). The mol percent DNA G + C content of the novel bacteria was determined according to standard HPLC methods (Shimelis & Giese, 2006; Li *et al.,* 2003).

16S rRNA gene sequencing and phylogenetic analyses. Isolation of genomic DNA for 16S rRNA gene amplification was previously described (Garcia *et al.,* 2009b) and prepared from actively growing culture taken from MD1G medium. The other 16S rRNA gene sequences

used in this study were obtained from GenBank. Alignment of the sequences was performed using the rapid multiple sequence alignment based on fast Fourier transform (MAFFT), v.6.814b (Katoh *et al.,* 2002), and distance matrices were calculated using the Jukes-Cantor model (Jukes & Cantor, 1969). The phylogenetic tree was then constructed using the maximum likelihood method (PHYML v2.4.5) (Guindon & Gascuel, 2003), with bootstrap calculated for 1000 replicates (Felsenstein, 1985). The phylogenetic tree was also confirmed using the neighbour-joining method (Saitou & Nei, 1987). All programs are packed in the Geneious Pro 5.0.2 software suite (Drummond *et al.,* 2010).

RESULTS AND DISCUSSION

Morphological and cultural characteristics

Vegetative cells. The vegetative cells in slide mounts were long and slender phase-dark rods measuring 1.1−1.2 x 3.0−7.0 μm (Fig. 1a-1b). Poles of the cell were rounded, representing *Sorangiineae* like morphology. Cells were motile by gliding on the surface as well as under the agar. Vegetative cell pellets taken from MD1G medium were whitish to yellowish-orange.

Swarm. On lean water agar medium, the strains grew as thin and transparent colonies, while a yellowish colour could be observed on MD1G medium. In buffered yeast agar, both strains produced characteristic deep agar swarming patterns, with high cell density along their colony edges (Fig. 1c-1d). Migrating cells move coherently while, at the same time, clearing the yeast cells and leaving the centre of the colony clear. Shallow colony depressions were found on surface of the medium. The two species can be distinguished through their swarm colour. *A. rufus* SBSr003^T exhibits a white to cream-coloured colony, whereas the colonies of *A. fasciculatus* SBSr002^T appear yellowish-orange. Another remarkable feature of these bacteria in yeast agar is that swarming cells convert into fruiting bodies once they reach the edges of the Petri dish. The alternative fate is death of the swarming cells, thus rendering them unrecognisable.

Fruiting body. In *A. fasciculatus* SBSr002^T, the fruiting bodies appear as yellowish-orange to golden in colour (Fig. 1e), often found under the agar as larger sori (30.0 x 50.0 μm) composed of 5-20 tiny sporangioles (10.4 x 11.4 μm). The novel isolates produce much smaller sporangioles and sori in comparison to *Byssovorax* and *Sorangium*. The ability of the

latter to develop fruiting bodies within cellulose fibre strands contributes to the significant difference to *Aetherobacter*. We also have never encountered black sporangioles, as in some strains of *Sorangium*, in our novel bacterial isolates. Their difference to *Byssovorax* is clearly evident in the size and colour of sporangioles, as they are bigger (60–180 μm) and more intensely red in colour.

In VY/2 and water agar, SBSr003^T fruiting bodies appeared red to vermilion in colour, developing as mounds (120 x 140 μm) or as very long rolls (340 x 400 μm - 1900 x 2900 μm), visible to the naked eye (Fig. 1f). The fruiting body was composed of tiny oval to spherical sporangioles (6.0 – 12.0 μm), compactly arranged in small sori (14.0 x 15.0 μm - 16 x 26 μm), and development starts as white humps of cellular aggregation.

Myxospores. Crushed sporangioles released tightly packed and slightly optically refractive rod cells, presumably myxospores. These were short and nearly as wide as the vegetative cells, with rounded ends, and desiccation-resistant (Fig. 1g-1h). In *A. fasciculatus*, myxopores appear much larger (1.0 – 1.2 μm x 3.2 – 4.0 μm) than in *A. rufus* (1.0 – 2.0 μm).

Physiological characteristics

Staining and lytic properties. Vegetative cells are Gram-negative and catalase-positive, while the swarm colony was unstained with Congo red, suggesting it is not a *Cystobacterineae*. In water agar baited with live *E. coli*, the swarm spreads thinly and, upon contact with bait, wave-like or rolls of swarm are produced. The baited bacterium was completely degraded after 2-3 days of incubation, depending on the inoculum diameter. Fruiting bodies typically developed after lysis of bait. The novel isolates differ from the related genera *Sorangium* and *Byssovorax* by their inability to degrade cellulose on Cel−3 agar and filter paper in ST21 agar. Chitin powder on CT−7 agar was not degraded by the advancing swarms. Partial agar degradation was commonly observed in most agar media used for cultivation. In soft yeast agar medium (< 1% agar), the novel isolates swarm quickly, with migrating cells typically localised under the agar.

Growth responses to temperature and pH. In yeast agar, the fastest spreading colonies and largest swarm diameters were seen at 30ºC. Both novel isolates can tolerate growth at 18ºC on the same agar but no growth occurred at 37ºC. The two strains differ in their temperature

optima. SBSr003^T can tolerate shaking cultivation at 22°C, whereas SBSr002^T cannot. Although both strains exhibit optimal growth at 28°C – 30°C, *A. rufus* tolerates a wider temperature range (22 °C – 30 °C) than *A. fasciculatus*.

Both strains can also grow in yeast agar in a pH of range 5 – 8, with the largest swarm diameter observed at pH 7.0. The same observations were also determined in broth media, but clearly more evident through optimal growth at pH 6.5 – 7.2, as indicated by dense biomass. No evidence of growth for both strains was found below a pH of 4.0 or above a pH of 9.0.

Sugar Source. The two strains also differ in their sugar requirements. Strain SBSr003^T tolerated all sugars tested (glucose, fructose, galactose, lactose, saccharose, starch, raffinose, mannose, and maltose), displaying almost the same amount growth; in contrast, SBSr002^T cannot tolerate starch and grows poorly in mannose. This is in agreement with the supplementation experiment performed in agar, as measured by the swarm diameter.

Peptone and Inorganic Nitrogen Sources. In SBSr003^T, casitone produced better growth than peptone, neopeptone, or tryptone. Poor growth was observed in phytone, while no evidence of growth was observed in casamino acids when they were supplemented. SBSr002^T shows almost the same growth reaction to different peptone sources. Casitone, peptone, neopeptone all yield high cell densities, whereas moderate yields were seen with tryptone, and poor yields were observed in phytone and casamino acids.

Inorganic nitrogen sources, including glutamic acid, aspartic acid, ammonium sulfate, and potassium nitrate, result in good growth of SBSr003^T, while poor growth was observed in urea. In SBSr002^T, good growth was exhibited on glutamic acid, aspartic acid, and potassium nitrate. The strain hardly grows in urea and ammonium sulfate.

Antibiotic resistance. SBSr003^T was resistant to gentamycin, ampicillin, and neomycin, whereas sensitivity was observed in apramycin, tobramycin, kanamycin, spectinomycin, hygromycin B, tetracycline, oxytetracycline, streptomycin, carbenicillin, and rifampicin. Antibiotic resistance of SBSr002^T was seen in gentamycin, apramycin, tobramycin, streptomycin, ampicillin, neomycin and hygromycin B, whereas sensitivity was observed in kanamycin, spectinomycin, tetracycline, oxytetracycline, carbenicillin, and rifampicin.

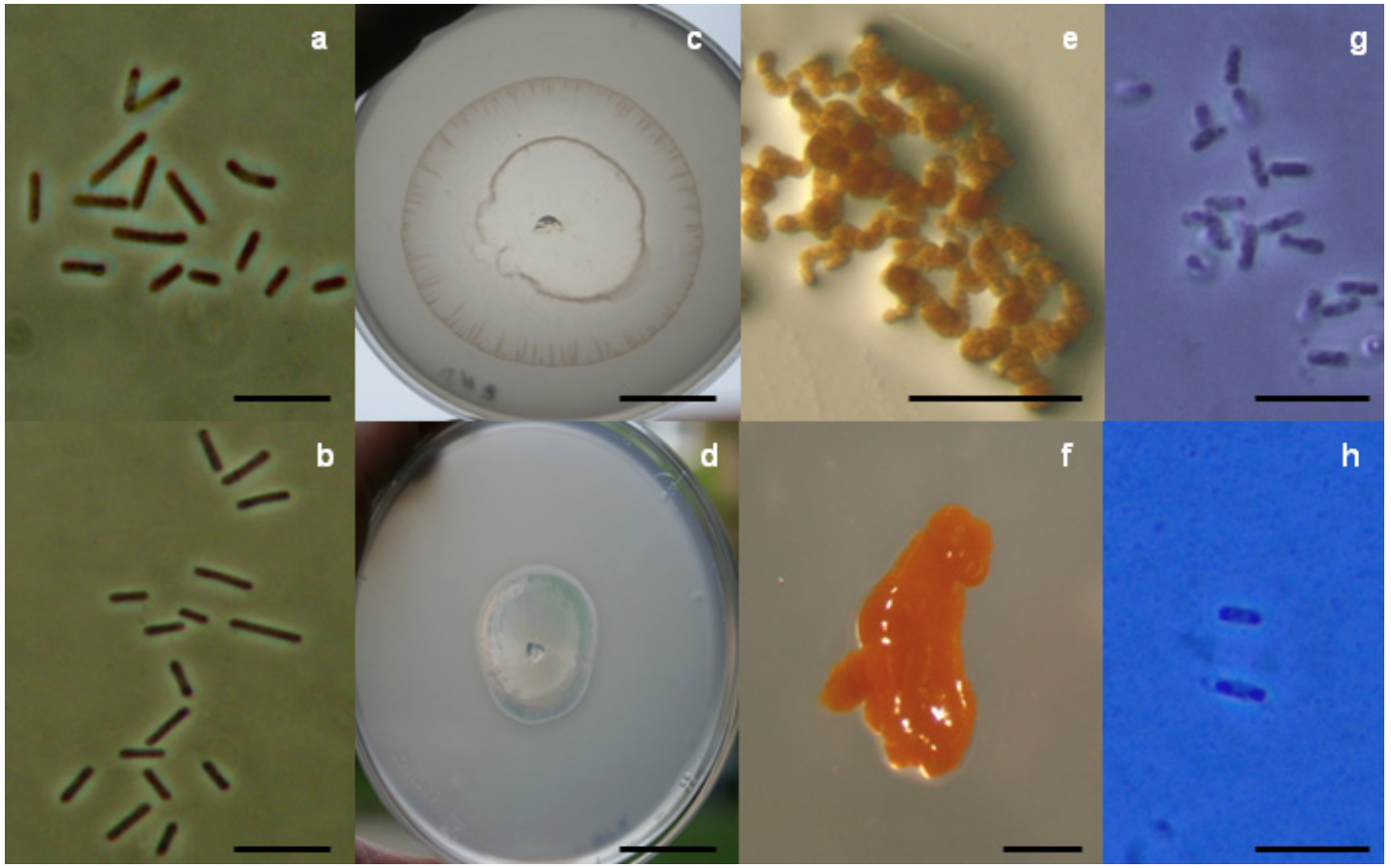


Figure 1. Growth stages of *Aetherobacter fasciculatus* SBSr002^T (top) and *Aetherobacter rufus* SBSr003^T (bottom). Phase dark vegetative cells. Bar, 10 µm (a-b). Dissecting photomicrographs of swarming colony on buffered yeast agar. Bar, 15mm (c-d). Dissecting photomicrographs of fruiting bodies. Bar, 300 µm (e-f). Phase contrast photomicrograps of myxospores. Bar, 10 µm (g, h).

Fatty acid profile and mol % G + C content

Major fatty acids for both strains were *iso*-$C_{15:0}$, and $C_{22:6}$ (*n*-3) (docosahexanoic acid, DHA) (Table 1). The presence of $C_{17:1\ 2OH}$ indicates that the isolates belong to *Sorangiineae*, a proposed hallmark for this suborder (Garcia *et al.,* 2011). Their position in that suborder was further supported by the predominance of straight-chained fatty acids. The uniqueness of the isolates was reflected in the presence of DHA; to date, they are the only group of myxobacteria capable of producing this type of fatty acid. Surprisingly, other PUFAs, such as eicosapentaenoic acid (EPA) and arachidonic acid, were also detected. In order to allow comparative analysis among members of the order, the differences in the FA profile among myxobacteria has recently been extensively studied (Garcia *et al.,* 2011).The DNA G + C content of the novel bacteria is between 68.0 – 69 mol %.

16S rRNA gene sequence and phylogenetic analysis

Complete 16S rRNA gene sequences of the isolates revealed their closest similarity (95% – 98%) to uncultured bacteria [GenBank accession: EU881332 (95%), FJ479473 (96%), FN421522 (98%)], while their similarity to the culturable bacteria was determined to be between 95% - 96% among the cellulose degrading species [*Byssovorax cruenta* DSM 14553^T (AJ833647), *Sorangium cellulosum* strains So9857, So ce56]. We note that a 96% similarity in the 16S rRNA gene was previously used as justification for the erection of a new genus, *Enhygromyxa* (Iizuka *et al*., 2003), and thus also consider here the creation of the new genus *Aetherobacter*. This proposal is further supported by the phylogenetic clustering of the novel isolates to clones of uncultured bacteria in the neighbour-joining tree obtained via BLASTn.

The clustering of $SBSr002^T$ and $SBSr003^T$ in the phylogenetic tree suggests that they belong together in a common taxon, whereas their phylogenetic divergence to *Byssovorax* and *Sorangium* implies that they belong to a novel genus (Fig. 2). *Aetherobacter* has been previously considered as belonging to a novel genus in a comprehensive myxobacterial phylogenetic study (Garcia *et al.,* 2010).

Table 1. Fatty acid characteristic of *Aetherobacter*.

Fatty acids	**SBSr002^T**	**SBSr003^T**
Straight-chain	%	%
$C_{13:0}$	0.04	0.2
$C_{14:0}$	0.21	1.23
$C_{15:0}$	5.23	8.88
$C_{15:1}$	0.2	0.19
$C_{16:0}$	3.93	8.65
$C_{16:0\ 9,10\ CH2}$	0.42	0.13
$C_{16:1\omega5c}$	2.94	5.19
$C_{17:0}$	3.95	3.64
$C_{18:0}$	0.61	7.04
$C_{20:4\omega6}$ ARA	0.64	-
$C_{20:5\omega3}$ EPA	6.99	3.95
$C_{22:6\omega3}$ DHA	**18.9**	**14.96**
$C_{16:0\ 2\text{-}OH}$	0.75	0.36
$C_{17:0\ 2\text{-}OH}$	5.29	2.61
$C_{17:1\ 2\text{-}OH}$	0.55	4.26
O-Alkylglycerols (OAG)		
OAG	3.67	2.47
OAG	0.46	-
OAG	0.99	-
OAG	1.68	-
OAG	1.11	-
Branched-chain		
iso-$C_{13:0}$	0.07	0.08
iso-$C_{14:0}$	0.49	0.13
iso-$C_{15:0}$	**16.44**	**23.9**
iso-$C_{16:0}$	5.93	1.76
iso-$C_{17:0}$	6.8	3.12
iso-$C_{17:1\omega5c}$	0.19	-
iso-$C_{18:0}$	0.08	-
anteiso-$C_{15:0}$	0.01	-
iso-$C_{17:0\ 2\text{-}OH}$	0.56	0.07
anteiso-$C_{15:0}$	-	0.3
anteiso-$C_{17:0}$	8.32	-
iso-$C_{15:0}$ OAG	-	1.64

* Major fatty acids (> 10 %) are marked in boldface.

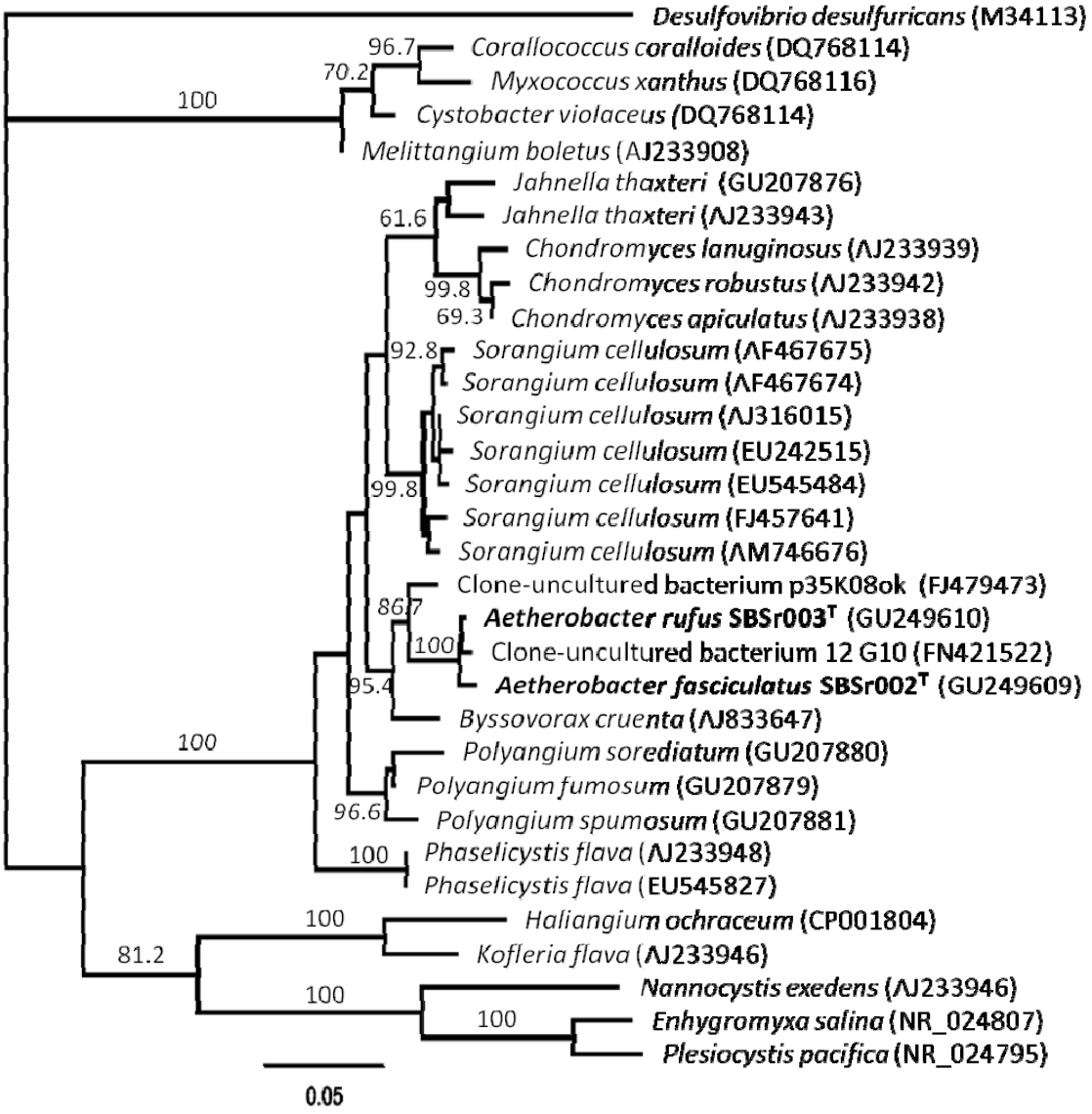


Figure 2. Phylogenetic position of *Aetherobacter* showing the clustering to clones of unculturable bacteria. The tree was constructed based on 16S rRNA gene sequences using the maximum likelihood method (PHYML). The numbers at branch points indicate the percentage bootstrap support based on 1000 resamplings. Values greater than 60 % are shown. Bar, 0.05 substitution per nucleotide position.

Description of *Aetherobacter* gen. nov.

Garcia and Müller

Aetherobacter [Ae.the.ro.bac'ter. Gr. masc. n. *Aether Greek God of Light* (refers to clear and transparent swarming); Gr. fem. n. *bacter* from Gr. neut.. n. *bakterium* small rod, stick; M.L. masc. n. *Aetherobacter* clear swarming rod].

Vegetative cells are moderately long and slender cylindrical rods with blunt ends; movement occurs by gliding on surface and under the agar. *Swarm* is film-like to transparent-clear, with ring-like colony shaped in yeast agar. Congo-red-negative, colony edges characterised by coherent migrating cells penetrating deep into the medium; agar slightly depressed. *Myxospores* are refractive slender rods with blunted ends, shorter than vegetative cells, enclosed in sporangial wall. *Fruiting bodies* appear as tiny ovoid sporangioles, usually compact or clustered. Yeasts and bacteria are strongly degraded. Contain high amount of *iso*-$C_{15:0}$ and omega-3 polyunsaturated fatty acid. Percent G + C, 68.0 – 70 mol %. The type species is *Aetherobacter rufus*.

Description of *Aetherobacter fasciculatus* sp. nov.

Garcia and Müller

Aetherobacter fasciculatus [*fasc.i.cu'la.* L. masc. n. *fasciculum* little bundles or packets (refers to the arrangement of sporangioles)].

Exhibits all characteristics of the genus. *Vegetative cells* are fat rods, 1.2−1.3 x 2.9−5.7 μm in size and phase dark. *Swarms* are yellowish-orange, showing complete clearing of yeast cells, with shallow depressions on the surface of agar, often deeply penetrating the medium. *Fruiting bodies* are yellow-orange in colour, often under the agar, with sori (30 x 50 μm) composed of 5-20 tiny sporangioles (10.4 x 11.4 μm), tightly arranged as bunches. *Myxospores* are refractive, stout rods, with rounded ends similar but shorter (1.0−1.2 x 3.2−4.0 μm) than vegetative cells, enclosed in a sporangial wall. Nutritional type is bacteriolytic, yeast degrader. Cellulose and chitin not degraded. Good growth in saccharose, fructose, D- mannose, and L-arabinose. Resistant to a broad spectrum of antibiotics: gentamycin, apramycin, tobramycin, streptomycin, ampicillin, neomycin, and

hygromycin B. Sensitive to kanamycin, spectinomycin, tetracycline, oxytetracycline, carbenicillin, and rifampicin. Major cellular fatty acid components are *iso*-$C_{15:0}$ and DHA. Produce EPA. Mol percent G + C is 68.9.

The type strain is SBSr002^T (= DSM 24601^T = NCCB 100377^T), isolated in November 2007 from an Indonesian soil sample taken from the Landsweiler-Reden collection, Germany.

Description of *Aetherobacter rufus* sp. nov. **Garcia and Müller**

Aetherobacter rufus (ru.fus. L. masc. adj. *rufus* red).

Exhibits all characteristics of the genus. *Vegetative cells* are fat rods, 1.0−1.2 x 3.0−6.0 μm in size, and phase-dark. In yeast agar, the *swarm* moves coherently in a ring or circular shape, with white cells concentrated at the edges of the ring and the middle of the swarm appearing clear and transparent. Exhibits shallow agar depressions. *Fruiting bodies* are red to vermilion in colour, appearing as a mound (120 x 140 μm) or long rolls (340 x 400 μm – 1900 x 2900 μm). Composed of tiny sporangioles (6-12 μm) compacted in a sorus (14 x 15 μm – 16 x 26 μm). *Myxospores* are slightly refractive, stout and short rods (1·0−2·0 μm) with rounded ends, enclosed in a sporangial wall. Lyse bacteria and yeast cells. Cellulose and chitin not degraded. Shows good growth in the presence of sugars: arabinose, fructose, galactose, glucose, mannose, molasses, sorbitol, xylose, cellobiose, lactose, maltose, saccharose, and soluble starch. Resistant to ampicillin, neomycin, and gentamycin. Sensitive to apramycin, tobramycin, kanamycin, spectinomycin, hygromycin B ampicillin, tetracycline, oxytetracycline, streptomycin, carbenicillin, and rifampicin. Major cellular fatty acid components are *iso*−$C_{15:0}$ and DHA. Also produce EPA. Mol percent G + C is 68.0.

The type strain is SBSr003^T (= DSM 24628^T = NCCB 100378^T), isolated in December 2007 from an Indonesian soil sample, taken from the Landsweiler-Reden collection, Germany.

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Expanded phylogeny of myxobacteria and evidence for cultivation of the 'unculturables'

Ronald Garcia, Klaus Gerth, Marc Stadler, Irineo J. Dogma Jr., and Rolf Müller (2010)

Molecular Phylogenetics and Evolution **57:**878–887.

Fatty acid Related Phylogeny of Myxobacteria as an Approach to Discover Polyunsaturated Omega-3/6 Fatty Acids

Ronald Garcia, Dominik Pistorius, Marc Stadler, and Rolf Müller (2011)

Journal of Bacteriol **193:** 1930–1942.

This article is available online at:

http://jb.asm.org/cgi/content/abstract/JB.01091-10v1

Chapter III. Discussion

A. General Scope of this Study

This work aims mainly on the discovery of natural products from myxobacteria. In order to find novel bioactive compounds, the study focused on strain discovery by combining microbiological, chemical, molecular, and phylogenetic techniques. Several new strains representing new genera and a new family were isolated and characterised. Their 16S rRNA genes were analysed and compared to representative strains to determine their position in the expanded phylogenetic tree. In addition, the significance of fatty acid correlation with each strain was illustrated for the first time. Furthermore, the study also describes the surprising discovery of diverse polyunsaturated fatty acids in myxobacteria. Lastly, the work highlights the isolation of bioactive compounds from the novel genus *Aetherobacter* which could be potential candidates for novel potent anti-cancer therapy.

B. Introduction to myxobacteria and guide to novel strain and compound discovery

1. Discovering Natural Products from Myxobacteria with an Emphasis on Rare Producer Strains in Combination with Improved Analytical Methods

Myxobacteria produce structurally diverse natural products with unique modes of action. This study discusses an approach encompassing the discovery of novel myxobacterial strains, bioactivity screening, and mining for novel compounds that are possible candidates for drug development. Samples have shown that environments exhibiting significant biological complexity yield the highest probability of isolating novel taxa. This was in concurrence with a previous study on samples representing all geographical locations (Dawid, 2000). The interaction and competition by organisms for common resources in an environment seem to be important factors for the development of bioactive secondary metabolites. As exemplified by compounds acting on the cytoskeleton and nucleic acid polymerases, these metabolites are hypothesised to be produced by myxobacteria to inhibit eukaryotic competitors in the environment (Reichenbach, 2009).

Strain recognition, a non-selective approach, efficient and detailed purification techniques, and genetic characterisation have led to the systematic identification of novel taxa. The discovery of novel strains with unknown metabolites revealed that the potential of myxobacterial diversity and natural products are far from exhausted. Sophisticated high resolution mass spectrometry and guided bioassays play key roles in natural product discovery, as will be discussed towards the end of this chapter.

Sorangiineae hold great promise for novel myxobacterial discovery, as members of this suborder are difficult to culture and purify; it is likely that many members have been missed by earlier investigators. The isolation methods employed here were designed to mimic the natural predatory environment of myxobacteria, simulating a natural environment during cultivation, with the knowledge that all myxobacteria are highly specialised in biomacromolecule degradation (Reichenbach, 1999b). Using these methods, a large number of novel strains belonging to a novel family and to proposed novel genera were isolated. Some of these bacteria were previously described ones but never cultivated (Thaxter, 1904) and thus considered to be rare (Reichenbach, 2005). The rediscovery of *Chondromyces catenulatus* illustrates this method and this strain is proposed here as a novel genus and species (*Pseudochondromyces catenulatus*). This group, which includes the *Phaselicystis* (Garcia *et al.,* 2009b), *Aetherobacter,* and *Minicystis* appear to represent the ‘‘uncultured’’ taxa of myxobacteria. The unusual characteristics of these isolates will be discussed in their respective sections. Fundamentals of the success of this method in unearthing novel taxa were elaborated, including methods of preservation. Data suggest that tropical and subtropical samples represent the best source of novel myxobacteria capable of producing biologically active metabolites. Previous study supports the diversity of myxobacteria in those communities (Dawid, 2000).

Using LC-coupled high resolution mass spectrometry, myxobacterial natural products were identified. Novel bioactive compounds are uncovered after bioassay-guided fractionation against sensitive microorganisms or eukaryotic cell lines. Lymphoma

(U937) and cervical carcinoma (KB3.1) cell lines are used as preferred test cell lines due to their sensitivity and reproducibility in this study.

Statistical tools represent another approach to mine novel secondary metabolites. In Principal Component Analysis (PCA), significant differences between numbers of multivariate LC-MS data sets are extracted, thereby creating groups of observations, trends, and outliers. Novel compounds are then identified based on significant differences in a given set of samples. In combination with a compound-finding algorithm, a chromatographic elution profile of the compounds can be differentiated from background noise (Krug *et al.,* 2008). This technique is elaborated upon in the following section of this manuscript.

2. Efficient Mining of Myxobacterial Metabolite Profiles Enabled by Liquid Chromatography–Electrospray Ionisation-Time-of-Flight Mass Spectrometry and Compound-Based Principal Component Analysis

Modern spectrometry is an important and reliable tool in the identification and characterisation of diverse microbial metabolites. This study specifically focuses on diverse *Myxococcus* isolates and a control strain *M. xanthus* DK1622. Using accurate mass data from high-resolution ESI-TOF measurements, target screening has facilitated the rapid identification of known myxobacterial metabolites. At least one of the five compound families (myxalamide, myxocheline, DK xanthene, myxochromide, and cittilin) was identified in new *Myxococcus* isolates, suggesting the robustness of the target analysis in finding known compounds.

Statistical tools using principal component analysis (PCA) implement an advanced compound-based bucketing approach to reveal the presence of other compounds which contribute to variation among the metabolite profiles being investigated. Molecular formulae generation for potential novel compounds uses exact mass position and isotopic patterns, emphasising their significant role in de-replication and compound characterisations.

Although the tools presented here contribute significantly to the identification of novel compounds, they do not reveal anything regarding bioactivity. Conventional approaches to novel metabolite discovery, coupled to bioassays, point more directly to a targeted compound of interest. The last section of this chapter tackles the discovery of new bioactive compounds using the classical approach.

C. Isolation of Novel and Rare Myxobacteria

1. *Phaselicystis flava* gen. nov., sp. nov., an Arachidonic Acid-Containing Soil Myxobacterium, and the Description of *Phaselicystidaceae* fam. nov.

Unusual myxobacteria were discovered to represent a novel family. The strains (NOSO-1, SBKo001^T) were obtained from a soil sample of decaying plant material, and isolated at different times (1988, 2006), places (Namibia, Philippines), and continents (Africa, Asia). Although they show cigar-shaped rods with blunted ends, extraordinarily, they exhibit white radial vein swarming atypical for the *Sorangiineae*. So far, this combined characteristic has never been described before in this suborder. The thick-walled yellow sporangioles distinguish *P. flava* from members of related *Polyangiaceae* family.

The uniqueness of the isolates was also reflected in their fatty acid (FA) composition. In myxobacteria, the occurrence of $C_{20:4}$ FA was first documented in 'halophilic isolates' (Iizuka *et al.,* 2003a, 2003b), although this also appears to be produced by non-related marine gliding bacteria (Hosoya *et al.,* 2006; Hosoya *et al.,* 2007). The abundance (12 – 36%) of $C_{20:4\omega6}$ (arachidonic FA) was first time identified here in strains SBKo001^T and NOSO-1, while the abundance of $C_{17:1\ 2\text{-}OH}$ and straight-chain FA confirms that both strains belong to the *Sorangiineae* suborder (Garcia *et al.,* 2011).

Although the novel isolates were unable to degrade filter paper, 16S rRNA gene sequence analysis revealed their closest similarity (94%) to be with cellulose degrading *Sorangium* and *Byssovorax*. A bifurcation from *Polyangiaceae* in the phylogenetic tree suggests that the novel clade represent a novel family (*Phaselicystidaceae*), genus (*Phaselicystis*) and

species (*Phaselicystis flava*), and its position was confirmed in the expanded phylogenetic tree of myxobacteria (Garcia *et al.,* 2010). *Phaselicystidaceae* was the 6th family discovered in myxobacteria, and is so far composed of only one genus.

2. *Minicystis rosea*, gen. nov., sp. nov., a Pink Myxobacterium

A novel myxobacterial strain, SBNa008^T, was isolated in 2007 from Philippine soil sample taken in Landsweiler-Reden collection, Germany. The bacterium was noted for its loose swarming pattern and undefined colony edges, a characteristic unusual for cigar-shaped myxobacteria; *Sorangiineae* normally produced ridge ends with coherent swarming. SBNa008^T produced an exceptionally thin and almost transparent colony, although in higher cell density resulted in pinkish swarming. Unlike most myxobacteria, no rippling and veins could be found in the colony. Fruiting bodies show similarity with *Nannocystis*; however the novel myxobacterium produced fruiting bodies much smaller in size and often as clusters on agar. Absence of deep agar corrosions and holes in the novel isolate additionally emphasise its difference to *Nannocystis*. Morphological features indicate that SBNa008^T is a *Sorangiineae*-type bacterium.

The presence of *iso*-$C_{15:0}$, $C_{17:1\ 2\text{-}OH}$ and $C_{20:4\ \omega6}$ (arachidonic acid) as major FA cell constituents indicates its clustering within the *Sorangiineae* suborder. Polyunsaturated $C_{20:5\omega3}$ (ω-3 eicosapentaenoic acid: EPA) and trace amount of $C_{18:3\omega6}$ (ω-6 γ linolenic acid) were also found. The ability of the novel bacterium to produce an ergostane steroid (Gawas *et al.,* 2011) seems to be a unique characteristic for this taxon. To our knowledge, only a few genera of myxobacteria were described as producing steroids (Bode *et al.,* 2003; Shimkets *et al.,* 2006); this ability does not seem to be common in the bacterial kingdom.

BLASTn analysis of 16S rRNA gene sequences revealed 95 - 96% similarity to clones of uncultured bacteria (e.g. EU662572, EU104167, AM490752), whereas 94 - 95% similarity was found to members of *Sorangiineae* [(95% to *Byssovorax cruenta* (AJ833647), 94% to *Phaselicystis flava* (EU545827), *Chondromyces lanuginosus*

(FJ176774), and *Sorangium cellulosum* (AM746676)]. The clustering of the novel isolate to uncultured bacteria and its novel branch in the phylogenetic tree suggests that SBNa008^T represents a novel genus (*Minicystis*) and species (*rosea*).

3. *Pseudochondromyces catenulatus* gen. nov., sp. nov., nom. rev., A Rediscovery of '*Chondromyces catenulatus*' Thaxter 1904

The surprising discovery of strain SBCm007^T unwinds the mystery of Thaxter's uncultured *Chondromyces catenulatus*. This novel myxobacterium was isolated in 2008 from a piece of wood collected along a riverbank in China. Isolation began with the appearance of *Chondromyces*-like fruiting bodies, which is composed of a stalk and elegant sporangioles. To date, six *Chondromyces* species are known (Reichenbach, 2005), although only five were cultivated axenically. *C. catenulatus* represents the uncultured species of *Chondromyces*, wherein its description relies mainly on its appearance in crude culture (Thaxter, 1904). Its second occurrence was documented from environmental sample by scanning electron microscopy (SEM) in Australia (McNeil & Skerman, 1972). Although the bacterium was never been cultivated, it was still included in the approved lists of bacterial names (Skerman *et al.,* 1980). The bacterium was not once encountered in their decades-long myxobacteria isolation-screening and thus reported to be rare (Reichenbach, 2005).

This study describes the isolation and characterisation of SBCm007^T, a strain initially identified as *Chondromyces catenulatus*, which, for more than a century (107 years), had never been brought into axenic culture. Further characterisation of the isolate based on morphology, chemo-physiology, genetics, and phylogenetics provides convincing evidence that the bacterium belongs to a novel genus.

SBCm007^T exhibits dual fruiting stages, one as a typical *Chondromyces* on surface of the agar – composed of a stalk and sporangioles, and second as *Polyangium*-like sporangioles in the agar. This bi-phasic feature has never been described before in myxobacteria. Pseudoplasmodium, which has only previously been observed in *Byssovorax*

(Reichenbach *et al.,* 2006), was also seen in the novel isolate but differs in its orange band-shaped swarm edges penetrating deeply into the agar. It also differs from *Byssovorax* in its absence of wrinkled parchment-like slime trails on filter paper. The novel bacterium was distinguished from *Chondromyces* by its agar liquefaction and cellulose degradation ability. The latter characteristic was supported by high protein similarity (59 %) to CelA and CelB in *Sorangium cellulosum* after BLASTp search of a translated nucleotide sequence in contig 00219 of the SBCm007 genome.

The difference of SBCm007^T to *Chondromyces* was also mirrored in $C_{16:0}$ and $C_{18:1\omega9c}$ as major FAs. The former was absent in *Chondromyces*, while the latter was generally found in low levels except for one species (*C. robustus*). Phylogenetic analysis revealed SBCm007^T divergence to *Chondromyces*, surprisingly finding it to be clustered with *Polyangium*, supporting a previous study (Garcia *et al.,* 2010). Taken together, these data suggest that SBCm007^T belongs to a new taxon which represents a novel genus and species (*Pseudochondromyces catenulatus*).

4. *Aetherobacter fasciculatus* sp. nov., *Aetherobacter rufus* sp. nov., Omega-3-Rich Polyunsaturated Fatty Acid-Producing Myxobacteria, and the Description of *Aetherobacter,* gen. nov.

The myxobacterial strains designated as SBSr002^T and SBSr003^T were isolated from dried soil samples taken from Landsweiler-Reden collection, Germany, collected in Indonesia. The organisms share common remarkable characteristics for producing transparent colonies with swarm edges burrowing deep into the agar (Fig. 3a). This feature had not been observed before in myxobacteria and seems to be an indicative of microaerophilic or facultative anaerobic behaviour. This is not surprising, given that several related taxa have been noted to be capable of anaerobic growth (Coates *et al.,* 2002; Sanford *et al.,* 2002). As with many other myxobacteria, the novel isolates exhibit a bacteriolytic-type of nutrition and mesophilic growth. Although these two novel species share many similarities, they differ physiologically and morphologically in some stages of development. *A. fasciculatus* SBSr002^T produces bundles of sporangioles (Fig. 3b),

whereas SBSr003^T exhibit more compact and denser fruiting bodies. They also have varying degrees of resistance to antibiotics. In addition, this group is also unusual for its fatty acid constituents. Although major FA *iso*-$C_{15:0}$ and $C_{17:1\ 2OH}$ appear typical for this family, $C_{20:5\omega3}$ (eicosapentaenoic acid, EPA) and $C_{22:6\omega3}$ (docosahexaenoic acid) are unique to this genus. In hundreds of myxobacterial strains screened, including most representative type strains, the production of EPA appears exclusive to some genera, whereas DHA appears specific for *Aetherobacter*. Lately, $C_{20:4\omega6}$ identified as arachidonic acid (omega-6 FA), was also found in trace amounts (Garcia *et al.,* 2011). These PUFAs, particularly the *n*-3 family, appear to be rare in prokaryotes and are usually produced only by a few groups of marine bacteria (Fang *et al.,* 2004; Nichols *et al.,* 1999; Nichols & McMeekin, 2002).

The BLASTn similarity (95-99%) of the strains in 16S rRNA gene sequence was found closest to clones of uncultured bacteria (e.g. FJ479473, FN421522), suggesting that they represent the uncultured group of myxobacteria. Phylogenetic analysis revealed the novelty of the isolates, forming a unique cluster in *Polyangiaceae*, with *Byssovorax cruenta* and *Sorangium cellulosum* as their sister taxa.

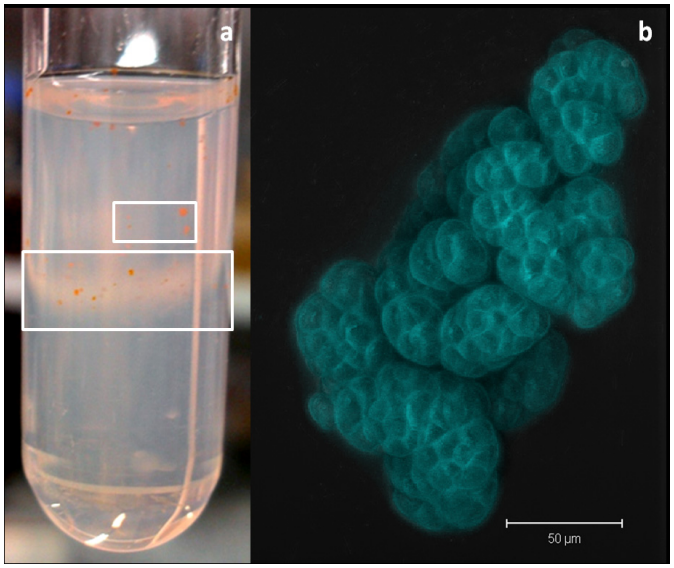

Figure 3. *Aetherobacter fasciculatus* SBSr002^T flourescent lasser scanning photomicrograph of fruiting body showing the characteristic bundle-shaped appearance of the sporangioles and deep agar growth (white box).

D. Phylogeny and Fatty Acids of Myxobacteria

1. Expanded Phylogenetic Tree of Myxobacteria and Evidence for Cultivation of the "Unculturables"

The increasing number of isolates discovered in the past decade has led this study to construct a new phylogenetic tree of myxobacteria based on 16S rRNA gene sequence. To date, 3 suborders, 6 families, 20 genera and 46 species are recognised culturable in *Myxococcales*, as shown by the phylogenetic positions, and distinctions among suborders were clearly delineated. This work also covers all species previously studied (Shimkets & Woese, 1992; Kaiser 1993; Pradella *et al.,* 2002; Spröer *et al.,* 1999; Ludwig *et al.,* 1983). The phylogenetic positions of the anaerobes (*Anaeromyxobacter*, myxobacterium strain KC), novel *Cystobacterineae* (*Pyxidicoccus* and *Cystobacter* spp.), the marine taxa *Plesiocystis* (Iizuka *et al.,* 2003a)*, Haliangium* (Fudou *et al.,* 2002), and *Enhygromyxa* (Iizuka *et al.,* 2003b) were clearly demarcated into clusters. Other undescribed isolates (*'Paraliomyxa miuraensis'*, brackish-water strain SYR-2), which may represent the 'slightly halophilic' taxa, were also included. Changes in the nomenclature of several isolates (*Polyangium vitellinum* Pl vt1^{T}, *Polyangium thaxteri* Pl t3, *Polyangium cellulosum*, NOSO-1, NOCB-2, NOCB-4) were also emphasised. Overall, the phylogenetic placements of these isolates and many other strains were re-affirmed.

The discovery of 9 novel isolates highlights the suborder *Sorangiineae*, in which they may represent new genera or even higher taxa. They appear to represent the so-called viable but not culturable (VBNC) group of myxobacteria, as suggested by their high 16S rRNA sequence similarity. This supports previous findings in metagenomic studies derived from the natural environment and show phylogenetic relatedness to myxobacteria (Wu *et al.,* 2005; Jiang *et al.,* 2007; Jiang *et al.,* 2010). 'VBNC' may perhaps be a mere reflection of the yet unknown physiological and nutritional growth requirements of the organism. The nine novel isolates significantly contribute to a clearer distinction between the *Sorangiineae* and *Nannocystineae* suborders.

Myxobacteria are phylogenetically coherent group. Based on at least 4% phylogenetic distance, new clades were formed and, on the basis of bracket distance, myxobacteria could be divided into 16 clusters supported by tree topology. It is hoped that the proposed phylogenetic tree will serve as a key guide to the genetic- and morphology-based taxonomy of myxobacteria and also as an important tool for the discovery of interesting novel compounds from unexplored novel strains. In addition to the unexplored environmental sources, high expectations that novel groups of myxobacteria could possibly be isolated by changing the isolation and baiting methods. Novel screening of known strains by co-cultivation with other soil bacteria and by simulation of cultivations to that of the natural environment are seem to be new stratigies that could lead to discovery of potential bioactive compounds.

2. Fatty Acid-Related Phylogeny of Myxobacteria as an Approach to Discover Polyunsaturated omega-3/6 Fatty Acids

A comprehensive report of the cellular fatty acid (FA) content in the order *Myxococcales* is presented here for the first time. Unlike other microorganisms, myxobacteria synthesise diverse and more complex FAs. These are represented by a vast number of different FA types (Fautz *et al.,* 1979; Fautz *et al.,* 1981; Schröder & Reichenbach, 1970; Stackerbrandt *et al.,* 2007; Ware & Dworkin, 1973). As much as 58 different FAs have been detected in myxobacteria. *Sorangiineae* appears to be the most sophisticated among the three suborders, with the discovery of 49 FAs. In addition, *Sorangiineae* also appears unrepresented in most myxobacterial FA studies. This is perhaps attributed to accessibility of the strain, maintained only by a few laboratories, and also appears associated to difficulties in handling due to their unknown nutritional metabolism.

The distribution of straight-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs) supports the clustering of strains in the phylogenetic tree based on 16S rRNA gene sequence. *Sorangiineae* contains higher amounts of SCFA than BCFA, whereas *Cystobacterineae* displays the opposite. In *Nannocystineae*, the ratio of BCFA and SCFA seems dependent on the taxonomic clade. Myxobacteria could also be classified using

their predominant FAs and biomarkers. *Nannocystineae* is remarkably unique among the suborders for its absence of hydroxy FAs. In myxobacteria, only the 2-OH and 3-OH FAs were found. *Cystobacterineae* seems more creative in synthesising diverse FAs of the 3-OH type compared to *Sorangiineae*. The latter suborder is distinguished by absence of straight-chained hydroxy FA and, so far, only the *iso*-$C_{17:0\ 3\text{-}OH}$ has been found. However, *Sorangiineae* are also unique for the presence of $C_{17:1\ 2OH}$ FA, which appears to be a marker for this suborder.

The study also highlights the FA profile of *Sorangiineae* and the discovery of 8 PUFAs, all reportedly new in myxobacteria. Many studies have shown and supported the importance and health benefits of PUFAs to humans (Funk *et al.,* 2001; Horrocks & Yeo, 1999). The PUFAs were identified as linoleic acid, γ-linolenic acid, homo-γ-linolenic acid, eicosadienoic acid (all ω6), α-linolenic, eicosapentaenoic acid, docosapentaenoic acid, and docosahexaenoic acid (all ω3). Commercially-valuable EPA appears restricted to certain genera in *Sorangiineae* and *Nannocystineae*, whereas DHA appears exclusive to *"Aetherobacter."* All isolates within this genus were discovered to be producing significant amounts of DHA, EPA and other PUFAs (Stadler *et al.,* 2010). The demand for the omega-3 PUFAs in pharmaceutical and food industry is increasing due to their health-related benefits (Horrocks & Keo, 1999), and therefore there is a continuous search for alternative sources (Fang *et al.,* 2004; Jøstensen & Landfald, 1997; Nichols *et al.,* 1999; Singh *et al.,* 1996; Ward & Singh, 2005; Warude *et al.,* 2006; Yano *et al.,* 1997).

Based on the chemo-phylogeny tree of myxobacteria, the production of omega-3/6 PUFAs appear to be associated to certain taxa or clades. This correlation seems to be more specific to omega-3 FAs, as exemplified by docosahexaenoic acid and eicosapentaenoic acid. DHA appears to only be produced by the novel *Aetherobacter* cluster. Using this innovative approach, commercially important omega-3 FAs can be predicted in novel myxobacterial isolates. The clustering of three additional strains (SBSr001-SBSr003) to *Aetherobacter* sp. SBSr008 supports the validity of this approach, all of which produced significant amounts of EPA and DHA.

Although myxobacteria are morphologically related through their gliding ability with *Herpetosiphon* and *Flexibacter,* FA profile revealed huge differences, agreeing with previous studies (Intriago & Floodgate, 1991; Johns & Perry, 1977). In addition, these gliding bacteria do not produced PUFAs, except for linoleic acid in *Herpetosiphon*.

The study concludes that myxobacterial FAs complement findings from 16S rRNA gene phylogeny, and is therefore considered to be a valuable tool for the chemo-taxonomic classification of myxobacteria, especially for the discovery of new omega-3 FAs producer strains.

Chapter IV. Production, Isolation, and Biological Activity of Novel Secondary Metabolites

New biologically active compounds from *Aetherobacter* (Myxobacteria): Production, isolation, and biological activity

Abstract

In the course of screening for new myxobacterial producer strains, a novel isolate proposed to novel genus and species (*Aetherobacter rufus* strain SBSr003) was isolated. Two novel compounds with a molecular weight of 718.3829 were isolated. High-resolution LTQ-Orbitrap analysis revealed similarities in their chemical formulae, $C_{41}H_{54}N_2O_9$. Both compounds show selective antifungal activity and, more interestingly, exhibit strong cytotoxicity to the human colon tumor cell line HCT-116. Compound A shows an IC_{50} of 81.3 ng/ml, whereas compound B exhibits a slightly lower IC_{50} value (84.8 ng/mL).

Introduction

Myxobacteria have gained recognition as proficient sources of novel natural products with a wide range of biological activities (Weissman & Müller, 2010; Wenzel & Müller, 2009). Many of the compounds known from this group are derived from *Myxococcus* and *Sorangium*, and the number of compounds discovered from these two genera appears to reflect the number of isolated strains. To date, the latter genus produces the highest number of known compounds amongst myxobacteria. Secondary metabolites produced by the suborder *Sorangiineae* account for nearly 64% (48.4% from *Sorangium*, 10.3% from *Chondromyces*, 5.2% from *Polyangium*) of the total number of myxobacterial compounds (Gerth *et al.,* 2003). The richness of natural products and the great potential in this suborder is also reflected in the novel isolate described in this study, *Aetherobacter rufus* SBSr003.

Materials and Methods

Microorganism and Cultivation Condition

The myxobacterium of interest, *Aetherobacter rufus* strain SBSr003, was isolated in 2007 from a soil sample containing decaying plant material (see *Aetherobacter* section). This bacterium produces a whitish-cream colony, later developing into aggregated clusters of red fruiting bodies. *A. rufus* SBSr003 and other strains within this proposed new genus are noted for their unusual deep agar penetration and simultaneous yeast medium clearing as coherent cells glide underneath the solid medium. Due to good growth yielding high cell densities at the colony edges, the novel bacterial isolate was maintained in this yeast medium (Garcia *et al.,* 2009b).

Production

From an actively growing agar plate culture, swarm colony edges were excised and inoculated into a 100 mL flask containing 50 mL medium. After 3-4 days cultivation, cells clumps were broken into smaller pieces by pipetting up and down several times. Homogenised cells were then transferred into 100 mL medium in a 300 mL flask. Pre-culture was then obtained through 2–3 subcultivations of the homogenised cells and production cultivations were performed in 1L flasks containing 500mL medium and incubated for 1 month with constant daily medium renewal. All cultivations were performed in buffered yeast (bufVY/2) medium (Garcia *et al.,* 2009a), shaken at 160rpm at 30°C.

HPLC-MS screening

Initial screening of *A. rufus* SBSr003 was performed in MD1G medium (0.35% Casitone, 0.3% glucose, 0.05% $MgSO_4 \cdot 7\ H_2O$, 0.025% $CaCl_2 \cdot 2\ H_2O$, pH 7.0, adjusted with KOH before autoclaving) supplemented with 2.0% Amberlite resin XAD-16 (Sigma-Aldrich). At the end of the 5 d cultivation, cells and resins were harvested together by centrifugation (5000 rpm, 10 min) and extracted with 50 mL methanol. After the sample was dried by rotary evaporation, the dried extract was resuspended in 1 mL methanol. 100 µL samples of crude extract were prepared for HPLC-MS analysis (Agilent 1100

Series LC coupled to Bruker HCT Plus MS). 5 uL aliquots were injected into a CC125/2 Nucleosil C18 gravity column (ID, 3µm, Macherey-Nagel). A constant flow rate of 0.4 ml/min was maintained. The elution gradient using solvents A and B was as follows: 0-2 min 5% B, 2-32 min 5-95% , 32-36 min 95% B, 36-39 min 95-5% B, 39-44 min 5% B. Solvent A was ddH_2O with 0.1% formic acid and solvent B was acetonitrile with 0.1% formic acid. Ion detection was set to alternate between positive and negative modes. Masses were analysed in the base peak chromatograms in positive, negative and UV modes.

Extraction and Compound Isolation

After the end of cultivation, cells clumps were harvested from 500 mL culture by pipetting and centrifugation at 5000 rpm for 10 min at 20°C. The supernatant was discarded and the wet cell pellet (30 mL) was defatted with 400 mL hexane followed by eight successive extractions using 200 mL ethyl acetate. The crude extract was then concentrated *in vacuo* by rotary evaporation. Semi-preparative separations of the ethyl acetate was performed in a HPLC system (Agilent Technologies) fitted with a Jupiter Proteo C_{12} 250 x 10mm column, using methanol as solvent A and ddH_2O with 0.05% triflouroacetic acid as solvent B. Using a constant flow rate of 2.5 mL/min, the extract was separated using the following gradient: 0 min, 50% B; 40 min, 20% B; 50 min, 5% B; yielding pure compound A [retention time, 40.58 min; *m/z*, 718.3829] and compound B [RT 42.94 min; *m/z*, 718.3829] (Fig. 4). Both compounds were isolated manually based on retention time and UV absorption at 220 nm and 280 nm.

Biological Assays

A standardised MTT test was done in a 96-well plate using the human colon carcinoma HCT-116 cell line at a density of 1 x 10^5 cells/mL. The cells were inoculated into McCoy's medium (Gibco) containing 10% fetal bovine serum (Gibco). The pure compounds were prepared at a concentration of 1 mg/mL, dissolved in dimethyl sulfoxide (DMSO), and serially diluted (1:3) in the plate. DMSO was used as the solvent control. After incubation for 3 d at 37°C and 5% CO_2 saturation, 5 mg/mL MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dissolved in phosphate buffer

solution was added and the cells further incubated for 30 min under the same conditions. The reagent was then carefully discarded and the cells lysed with 100 µL DMSO. The IC_{50} value was determined by measuring absorption at 590 nm using the plate reader (Bio-Tek Instruments, Inc., U.S.A.).

An antimicrobial assay of the pure compounds was performed using a range of bacteria, yeasts, and mould. Gram-negative *Escherichia coli* 2-DC, *Escherichia coli* TolC, *Pseudomonas aeroginosa*, *Chromobacterium violaceum*, Gram-positive *Staphylococcus aureus* MRS3, *Micrococcus luteus*, acid-fast *Mycobacterium diernhoferi*, yeast *Candida albicans, Hansenula anomala*, and filamentous fungus *Mucor hiemalis* were used as test indicator strains. The bacteria were grown in EBS medium [0.5% peptone (Marcor), 0.5% proteose peptone, 0.1% yeast extract (Difco), 0.1% meat extract (Merck), 1.0% HEPES, pH adjusted to 7.0 before autoclaving] while the fungi were grown in MYC medium [1.0% Phytone Peptone (Difco), 1.0% glucose, 50mM HEPES, pH adjusted to 7.0 before autoclaving], and incubated for 24 h (bacteria) and 36 h (yeasts and mould) at 30ºC. These microorganisms were adjusted to an OD_{600} of 0.015. Using the Kirby-Bauer agar diffusion method, 20 µg/mL of the air-dried compound were impregnated on 6 mm sterile standard blank paper discs. Growth inhibition of the test microorganism was determined by measuring the diameter of the inhibition zone.

Results and Discussion

Initial screening based on a 50 mL culture containing resin XAD-16 has shown cytotoxicity of the crude extract against cervical carcinoma (KB3.1) and mouse fibroblast cells (L929) that could not be correlated to any known myxobacterial compound. The HPLC-MS chromatogram revealed unknown compounds [e.g. $(M + H)^+$ 717.9344] in the negative mode with UV absorption at 230 nm. In addition, no known compound could be identified from this strain. Comparative HPLC-MS analysis of the resin and cell extracts revealed that the compounds of interest were bound to the latter, and isolation was therefore performed from cell biomass. This is not the first time that a compound from myxobacteria was reported as bound to the cell. The secondary metabolite crocacin from *Chondromyces crocatus* Cm c3 was also detected in cell pellet (Kunze *et al.,* 1994).

LTQ-Orbitrap high resolution analysis indicated a chemical formula of $C_{41}H_{54}N_2O_9$ for both compounds, suggesting possible isomerism. The compounds appear to belong to the same family, as supported by identical mass fragmentation and UV absorption patterns observed in HPLC-MS.

From 30 mL wet cell biomass, 250 mg of yellowish crude ethyl acetate extract were obtained. Semi-preparative HPLC separations yielded 3.97 mg and 4.81 mg of an oily white substance corresponding to compounds A and B, respectively. The yield is unsurprising, as myxobacteria tend to initially produce low amounts (0.1 – 20mg/L) of secondary metabolites (Reichenbach & Höfle, 1993). In general, secondary metabolite production in myxobacteria can be increased through growth optimisation (Gerth *et al.,* 2003). As with many other myxobacteria, the production of compound families appears common. *Sorangium* exemplifies this characteristic in the biosynthesis of many derivatives of disorazol. Myxalamides and myxothiazols derivatives are likewise commonly produced by different species and genera of the *Cystobacterineae* suborder. The production of intriguing compounds by the novel isolate *A. rufus* reaffirms a previous study (Gerth *et al.,* 2003) suggesting that *Sorangiineae* is one the richest sources of secondary metabolites in myxobacteria.

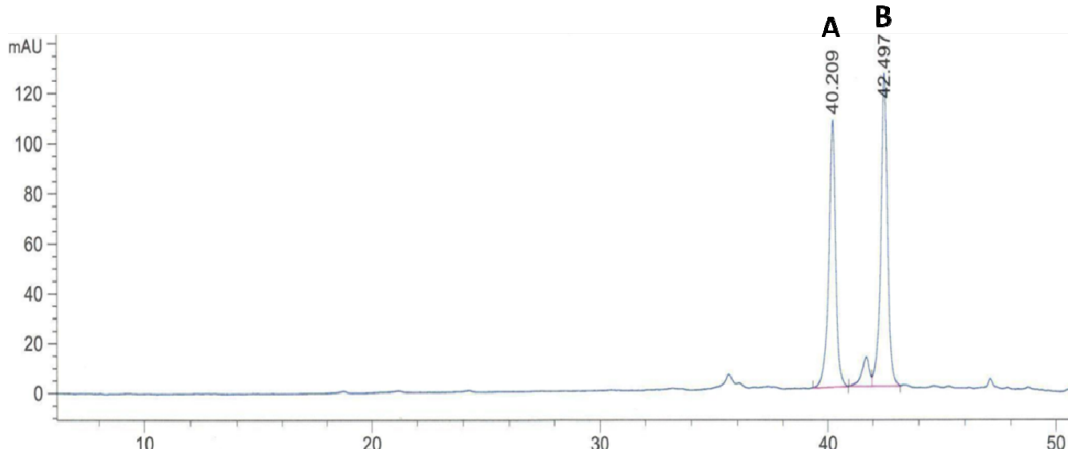

Figure 4. Semi-preparative HPLC separation of compounds A and B. Compounds are detected at 280 nm.

Interestingly, compounds A and B show biological activity against a human colon tumour (HCT) cell line, with compound A (IC_{50} = 81.3ng/mL) exhibiting nearly the same activity as compound B (IC_{50} = 84.8ng/mL) (Fig. 5). Myxobacteria are known for the production of many active compounds against eukaryotic cells, and their ability to produce these compounds appears to play a role in their micropredatory lifestyle, colonisation of a niche, and out-competition of other microorganisms (Reichenbach & Höfle, 1993). It is hypothesised that many of the secondary metabolites of *Sorangiineae*, as represented by the cellulose-degrading *Sorangium* and *Byssovorax* and the common wood-colonising *Chondromyces*, are produced to inhibit yeast and mould competitors on wood substrates. As a member of *Sorangiineae* suborder and closely related to other previously mentioned genera (Garcia *et al.,* 2010; Garcia *et al.,* 2011), it is clear as to why *Aetherobacter* is producing these bioactive compounds.

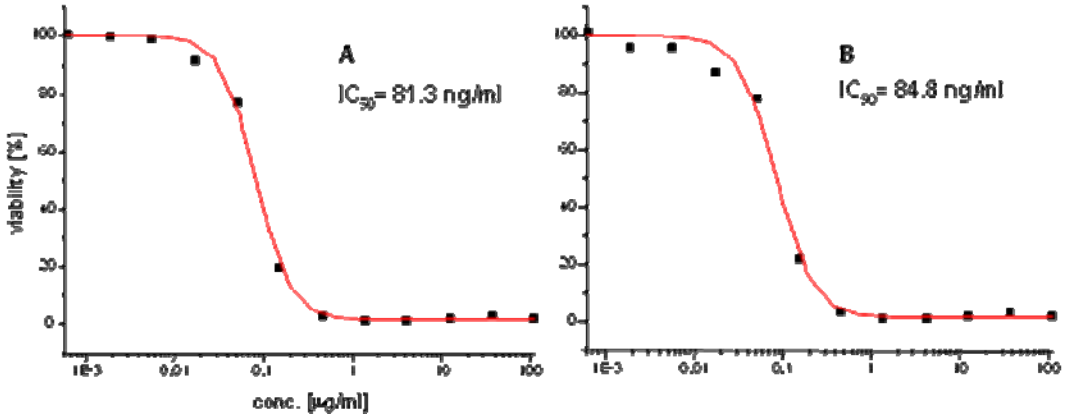

Figure 5. Cytotoxic effect of compounds A and B against HCT-116 human colon tumour cells and their corresponding IC_{50} values.

An MTT test revealed that the novel compounds were cytotoxic to some cell lines. This is not surprising as some myxobacterial compounds (10%) act upon the cytoskeleton (Reichenbach, 2001). In U-20S human osteosarcoma cells (data not shown) and human fibroblasts, actin fibre remodelling, suspected to be a result of stress, was observed (Figs. 6a-b). This activity is different from the known actin-stabilising chondramides (Sasse *et al.,* 1998; Figs. 6c-d). Actin-related activity resulting from treatment with other myxobacterial compounds was also determined in chivosazol (Diestel *et al.,* 2009; Irschik

et al., 1995) and rhizopodin (Jansen *et al.,* 2008), produced by members of *Sorangium* and *Myxococcus*, respectively. Although there is no clear evidence yet as to the real target and mode of action of either compound, studies are on-going. The actin stress fibre effect observed in the treated cell lines are perhaps a secondary effect of a yet unknown mechanism of action. Although a mode of action could also be predicted on the basis of structural similarity to closely related substances, this is only possible after complete structure elucidation. There are no indications of antibacterial activity, as suggested by negative results against a panel of Gram-positive and Gram-negative bacteria; however, mild antifungal activity was observed in the yeast *Hansenula anomala*. The activity of the newly isolated compounds reflects the original environmental source, where there is stark competition and dynamic interaction for common resources.

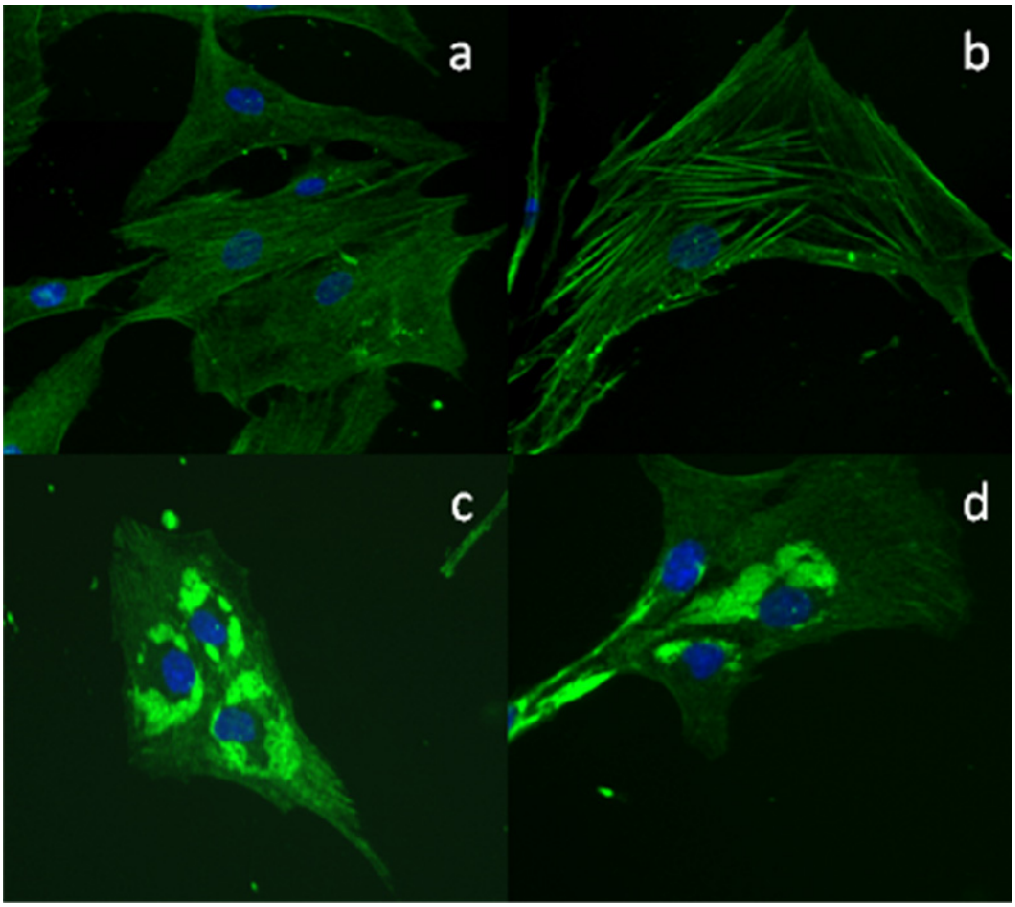

Figure 6. Effect of compound A on HSF-1 human fibroblasts as determined by fluorescent microscopy. Untreated control cells (a), treated cells exhibiting pronounced accumulation of long green fluorescent stress fibres (b), cells treated with chondramide A (c) and chondramide C (d).

Novel isolates, as represented by *Aetherobacter*, clearly represent a good source of new bioactive natural products. A recent study on a yet undescribed *Sorangiineae* also supports this hypothesis (Gawas *et al., 2011*). Through continuous isolation and screening of new isolates, novel compound discovery in myxobacteria appears far from exhaustion.

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Curriculum Vitae

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Professional Memberships
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International Society for Infectious Diseases (ISID)
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Patents

1. Synthetic enzymes for the production of Argyrins
Rolf Müller, Silke Wenzel, and Ronald Garcia
2008. European Patent: 08159743.7 – 2405

2. Production of omega-3 fatty acids by myxobacteria
Marc Stadler, Ernest Roemer, Rolf Müller, Ronald Garcia, Dominik Pistorius, Alexander Brachmann. June 2010. International World Patent: WO/2010/063451

Publications

1. Garcia, R. O., D. Krug, and R. Müller. 2009a. Discovering natural products from myxobacteria with emphasis on rare producer strains in combination with improved analytical methods, p. 59–91. *In* D. Hopwood (ed), Methods in enzymology: complex enzymes in microbial natural product biosynthesis. vol. 458., part A. Academic Press, Burlington.

2. Krug, D., G. Zurek, B. Schneider, R. Garcia, and R. Müller. 2008. Efficient mining of myxobacterial metabolite profiles enabled by liquid chromatography–electrospray ionisation-time-of-flight mass spectrometry and compound-based principal component analysis. Anal. Chim. Acta 624:97–106.

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10. Gawas, D., R. O. Garcia, V. Huch, and R. Müller. 2011. A highly conjugated dihydroxylated C_{28} steroid from a myxobacterium. J. Nat. Prod. 74:1281–1283.

11. Simmons, L., R. Garcia, K. Kaufmann, G. Schwär, V. Huch, and R. Müller. Bendigoles D-F, novel anti-inflammatory sterols from the marine sponge-derived *Actinomadura* sp. SBMs009. 2011. Bioorgan. Med. Chem. DOI:10.1016/j.bmc.2011.05.044.

12. Gawas, D., R. O. Garcia, J. Hermann, and R. Müller. A family of tyramine glycosides with cytotoxic activity from myxobacterial strain SBNa008. J. Nat. Prod. Manuscript submitted.

Short Lectures / Oral Presentations

1. Pyxidicoccus: A novel source for anti-infectives. 34th International Conference on the Biology of the Myxobacteria. Granada, Spain. July 14 -18, 2007.

2. Cystobacter as multi- producer of cytotoxic and novel secondary metabolites. VAAM Workshop 'Biology of Bacteria Producing Natural Products.' Nonnweiler, Germany. October 4-6, 2007.

3. Search for novel myxobacteria: Possibilities and prospects for novel compounds. VAAM Workshop 'Biology of Bacteria Producing Natural Products.' Technical University, Berlin, Germany. September 28-October 1, 2008.

4. Biology of myxobacteria. The Graduate School, University of Santo Tomas Manila, Philippines. February 2009.

5. Myxobacteria as proficient source of novel secondary metabolites. First life science PhD student day. Saarland University, Saarbrücken, Germany. August 21, 2009.

6. Comprehensive chemo-phylogeny of myxobacteria based on 16S rDNA and fatty acids. 37th International Conference on the Biology of Myxobacteria. European Academy, Otzenhausen, Nonnweiler, Germany. September 1, 2010.

7. Novel compounds from novel genera of myxobacteria. Australian Society for Microbiology Annual Scientific Meeting & Exhibition. Sydney Convention & Exhibition Centre, Sydney, Australia. July 4-8, 2010.

8. *Discovery and biotechnological potential of Aetherobacter gen nov ined. (Myxobacteria) for production of omega-3-polyunsaturated fatty acids (PUFAs) and novel secondary metabolites.* GenoMik-Transfer Statusseminar 2011, Göttingen, May 12-13, 2011.

9. *Novel myxobacteria: Source of new bioactive compounds.* 38th International Conference on the Biology of Myxobacteria, New York, U.S.A. July 18-21, 2011.

Poster Presentations

1. Antimicrobial potentials of Philippine myxobacteria
2. Isolation of myxobacteria by enrichment methods
Annual Scientific Meeting and Exhibition of the Australian and New Zealand Societies for Microbiology. Auckland, New Zealand, September 28 – October 2, 2003.
(Presented during Master's study)

3. Discovery of omega-3 fatty acids in myxobacteria. Australian Society for Microbiology Annual Scientific Meeting & Exhibition. Sydney Convention & Exhibition Centre, Sydney, Australia. July 4-8, 2010.