Novel myxobacteria as a potential source of natural products and description of inter-species nature of C-signal

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Ram Prasad Awal

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Dekan: .....Prof. Dr. Guido Kickelbick.....

Berichterstatter: .....Prof. Dr. Rolf Müller.....

.....Prof. Dr. Manfred J. Schmitt.....

.....

Vositz: .....Prof. Dr. Uli Kazmaier.....

Akad. Mitarbeiter: .....Dr. Jessica Stolzenberger.....

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List of Publications

1. Sakshi Sood*, **Ram Prasad Awal***, Joachim Wink, Kathrin I. Mohr, Manfred Rohde, Marc Stadler, Peter Kämpfer, Stefanie P. Glaeser, Peter Schumann, Ronald Garcia and Rolf Müller (2015). *Aggregicoccus edonensis* gen. nov., sp. nov., an unusually aggregating myxobacterium isolated from a soil sample. *Int J Syst Evol Microbiol* **65**, 745-753. (* equally contributed)
2. **Ram Prasad Awal**, Ronald Garcia and Rolf Müller (2016). *Racemicystis crocea* gen. nov., sp. nov., a soil myxobacterium in the family *Polyangiaceae*. *Int J Syst Evol Microbiol* **66,** 2389-2395**.**
3. **Ram Prasad Awal**, Ronald Garcia, Katja Gemperlein, Bikram Kunwar, Niranjan Parajuli and Rolf Müller (2016). *Paucitangium cumulatus* gen. nov., sp. nov. and *Paucitangium subalbus* sp. nov., novel soil myxobacteria from Nepalese soil samples. Submitted to *Int J Syst Evol Microbiol.*
4. **Ram Prasad Awal**, Carsten Volz and Rolf Müller. Co-culture studies of myxobacteria reveal an inter-species signal for the induction of fruiting body formation. To be submitted to the ISME Journal.

Other publication

5. Azam Moradi, Gholam Hosein Ebrahimipour, Kathrin Mohr, Peter Kämpfer, Stefanie Glaeser, Fabienne Hennessen, Katja Gemperlein, **Ram Prasad Awal**, Corinna Wolf, Rolf Müller and Joachim Wink (2016). *Racemicystis iranensis* sp. nov., a novel myxobacterium from Iranian soil. Submitted to *Int J Syst Evol Microbiol*.

Oral presentation

1. *Aggregicoccus edonensis*, a novel genus and species of myxobacterium isolated from soil sample 41st International Conference on the Biology of Myxobacteria, Nice, France. May 25-28, 2014
2. Myxobacteria- fascinating bacteria with potential application (2013). Summer symposium of the Interdisciplinary Graduate School of Natural Products Research, Saarbrücken, Germany

Poster presentation

1. MSr9521- a novel genus and species of myxobacterium in the suborder *Sorangiineae*. July, 2015. HIPS-Symposium, Saarbrücken, Germany.
2. Discovery of three novel genera in Myxobacteria, VAAM Workshop 'Biology of Bacteria Producing Natural Products' University of Freiburg, Freiburg, Germany. September 28-30, 2016.

Zussamenfassung

Als Produzenten von vielfältigen und neuartigen Sekundärmetaboliten sind Myxobakterien wichtig für potentielle klinische Anwendungen. In der vorliegenden Studie wurden mithilfe unterschiedlicher Isolierungstechniken aus verschiedenen Umweltproben Vertreter von drei neuen Gattungen entdeckt. Durch die polyphasische Charakterisierung dieser drei neuen Bakteriengattungen (*Aggregicoccus*, *Paucitangium* und *Racemicystis*) wurden diese in den Unterordnungen *Cystobacterineae* und *Sorangiineae* eindeutig von bekannten Gattungen abgegrenzt.

Die Dissertation beschäftigt sich auch mit der Interaktion zwischen verschiedenen myxobakteriellen Spezies, bei der *Myxococcus xanthus* DK1622 eine zelluläre Differenzierung des Bakteriums *Chondromyces crocatus* DSM 14714^{T} veranlasst. In *M. xanthus* spielt CsgA eine wichtige Rolle ber der Entwicklung. Diese Studie zeigt, dass p25, eine unprozesierte Form von CsgA, als ein aktives Enzym agieren könnte und mit den Substraten Cardiolipin und Phosphatidylglycerol reagiert, um ein Signal für die Fruchtkörperbildung von *C. crocatus* zu erzeugen. Diese Arbeit beschreibt zum ersten Mal wie eine myxobakterielle Spezies diese Entwicklung in anderen Myxobakterien aktiviert, wobei vermutlich die aktive p25-Spezies von CsgA beteiligt ist.

Abstract

Myxobacteria are fascinating bacteria that have gained recognition as producers of diverse and novel secondary metabolites with potential clinical applications. In this study, different isolation methods have been applied leading to the discovery of three novel genera from different environmental sources. By polyphasic characterizations, these three novel genera (*Aggregicoccus*, *Paucitangium*, and *Racemicystis*) were clearly delineated in the suborder *Cystobacterineae* and *Sorangiineae*.

This study also highlights the interaction between myxobacterial species where *Myxococcus xanthus* DK1622 induces *Chondromyces crocatus* DSM 14714^{T} to undergo cellular differentiation. In *M. xanthus*, a contact-dependent morphogen called CsgA plays a key role in development. This study shows that p25, an unprocessed form of CsgA, might act as an active enzyme and reacts with the substrates cardiolipin and phosphatidylglycerol to generate a signal for the fruiting body formation of *C. crocatus*. This study reports for the first time, how one myxobacterial species can activate development in another myxobacterial species, which most probably involves the activity of the p25.

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Chapter: 1 Introduction

1.1. An introduction to the myxobacteria

Myxobacteria are a unique group of Gram-negative prokaryotes representing the monophyletic order of the *Myxococcales* within the Deltaproteobacteria with high a G + C mol % content (Shimkets *et al.*, 2006). Myxobacteria are best known for their unique and complex social behavior in the bacterial kingdom. They exhibit a coordinated and cooperative movement known as swarming in their search of growth substrates. Myxobacteria are able to degrade biological macromolecules and prey on other microorganisms by secreting hydrolytic enzymes (Reichenbach, 1999). An individual cell does not produce enough enzymes, but the group as a whole is capable of breaking down the substrates which is commonly known as wolfpack feeding. Myxobacteria enter multicellular morphogenesis upon starvation leading to the formation of multicellular fruiting bodies in which many of the cells differentiate into environmentally resistant myxospores (Reichenbach, 2005).

The name of myxobacteria derives from the Greek word *myxo*, meaning slime or mucus, underlining their ability to secrete a slime consisting of polysaccharides (Reichenbach, 2005). In general, myxobacteria are mesophilic bacteria which exhibit a low salt tolerance and very sensitive to desiccation in a vegetative state (Reichenbach, 1999). Based on catabolic abilities, myxobacteria can be divided into two major groups: 1) cellulolytic species (decomposing cellulose), e.g. *Sorangium* genera and *Byssovorax cruenta* By c2^{T}, and 2) proteolytic species (being able to hydrolyze proteins), with the majority of the isolated species belonging to the latter group (Shimkets *et al.*, 2006).

Myxobacteria have gained attention for their ability to produce novel and diverse secondary metabolites exhibiting interesting biological activities (Wenzel & Müller, 2009a; Weissman & Müller, 2010). Most of the discovered secondary metabolites from myxobacteria so far have unique structures with unique modes of action in the bacterial kingdom. The Helmholtz Center for Infection Research, Braunschweig and the Helmholtz Institute for Pharmaceutical Research Saarland, Department of Natural Products (MINS), Saarbrücken have isolated more than 9,000 myxobacterial strains from different environmental sources. The distribution of isolated strains in different families is depicted

in Fig. 1. Unpublished data show that there are now around 900 compounds belonging to 140 compound families derived from these strains.

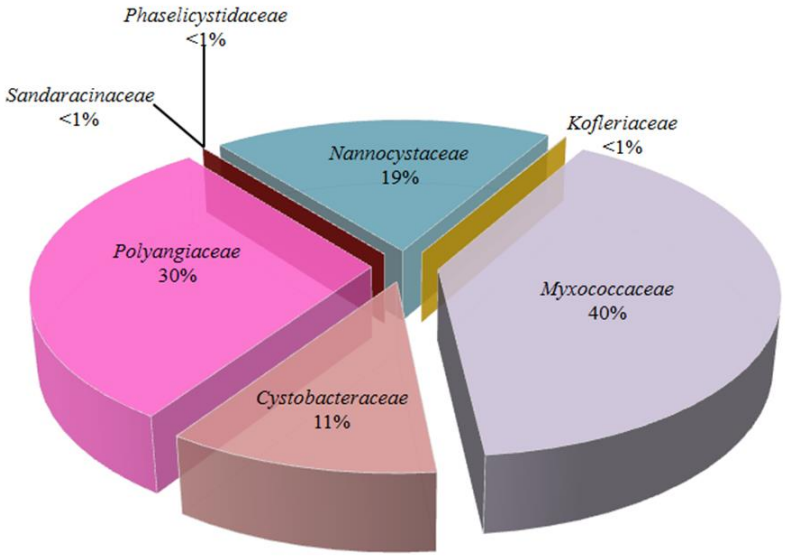

Fig. 1. Distribution of myxobacteria in different myxobacterial families in the combined HIPS-MINS and HZI collection [replotted from (Garcia & Müller, 2014e)].

1.2. The systematics of myxobacteria

1.2.1. The taxonomy of myxobacteria

Initially, myxobacteria were considered to belong to the fungi due to their ability to form fruiting bodies. In 1809, the German botanist H. F. Link first isolated myxobacteria and named them "gasteromycete" (fungi). Nowadays, this strain is known as *Kofleria flava* (previously known as *Polyangium vitellinum*) (Reichenbach, 2005). Two more species, *Stigmatella aurantiaca* and *Chondromyces*, were classified as "hyphomycetes" (fungi imperfecti) by the British mycologist M. J. Berkeley (Berkeley, 1857). In 1892, American botanist, Roland Thaxter, first recognized them as a distinct group of bacteria and coined the name of "*Myxobacteria*" describing the astonishing and unexpected life cycle of these bacteria (Thaxter, 1892).

The taxonomy of myxobacteria is based primarily on the morphology of the vegetative cells, of their swarms, of fruiting bodies, and of myxospores (Reichenbach, 2005). The phenotypic characteristics, such as shape and size of vegetative cells and myxospores, and

the shape, size, colour and structure of fruiting bodies are expressions of the genotype. The fruiting body formation may be lost during artificial cultivation in the laboratory leading to confusion in identification and classification of such species (Reichenbach, 2005)

1.2.2. Myxobacterial phylogeny

On the basis of 16S rRNA sequences, myxobacteria form a phylogenetically coherent cluster in the *Deltaproteobacteria* (Shimkets & Woese, 1992; Spröer *et al.*, 1999) with the bacterivorous *Bdellovibrionales* and the Gram-negative sulfate- and sulfur-reducing organisms as their closest relatives (Oyaizu & Woese, 1985; Stackebrandt *et al.*, 1988; Reichenbach, 2005). In 1992, Shimkets and Woese published the first comprehensive phylogenetic analysis of myxobacteria determining the almost complete 16S rRNA sequences of 12 strains belonging to 11 species of 10 genera (Shimkets & Woese, 1992). The phylogenetic classification of myxobacteria was further expanded with the study of more myxobacterial strains representing different morphological species (Spröer *et al.*, 1999; Garcia *et al.*, 2010). The phylogenetic studies have entirely analyzed the culture collections from terrestrial as well as marine habitats (Shimkets & Woese, 1992; Spröer *et al.*, 1999; Garcia *et al.*, 2010). The topologies of phylogenetic trees based on 16S rRNA sequences have been found to agree with morphological characteristics of myxobacterial strains (Reichenbach, 1993; Shimkets *et al.*, 2006). Based on the morphological and the phylogenetic characteristics, and after several events of renaming, today, the order of *Myxococcales* can be divided into the three subgroups *Cystobacterineae*, *Sorangiineae* and *Nannocystineae* (Reichenbach, 2005; Shimkets *et al.*, 2006). Improvements in sequencing techniques have made it easier to genetically identify novel strains. The housekeeping genes such as *gyrB*, *rpoB*, *fusA* and *lepA* have been successfully used and established for genotypic classification of myxobacteria, especially *Corallococcus* (Stackebrandt & Pauker, 2005; Stackebrandt *et al.*, 2007). Type II DNA topoisomerase (DNA gyrase) is ubiquitous in bacteria and is encoded by the *gyrB* and *gyrA* genes. Studies have shown that the DNA gyrase subunit B (*gyrB*) is useful to discriminate closely related strains due to a high genetic variation in protein coding genes (Stackebrandt *et al.*, 2007). Up to date, three suborders, 10 families, 29 genera, and 61 species exist within the order *Myxococcales* (Fig. 2).

Taking into account available genotypic, chemotaxonomic, and phenotypic characteristics will deliver considerable information for establishing the reliable taxonomic position of a novel organism and thus constitute a polyphasic approach for taxonomic studies. Novel methods, such as the chemophylogenetic of myxobacteria (Garcia *et al.*, 2011) in polyphasic approaches have become an important tool in bacterial systematics. More such markers need to be determined for elucidating accurate relationships between closely related taxa. Therefore, application of polyphasic taxonomic approaches will enhance the modern myxobacterial systematics that is rooted in the genetic lineages of the organisms.

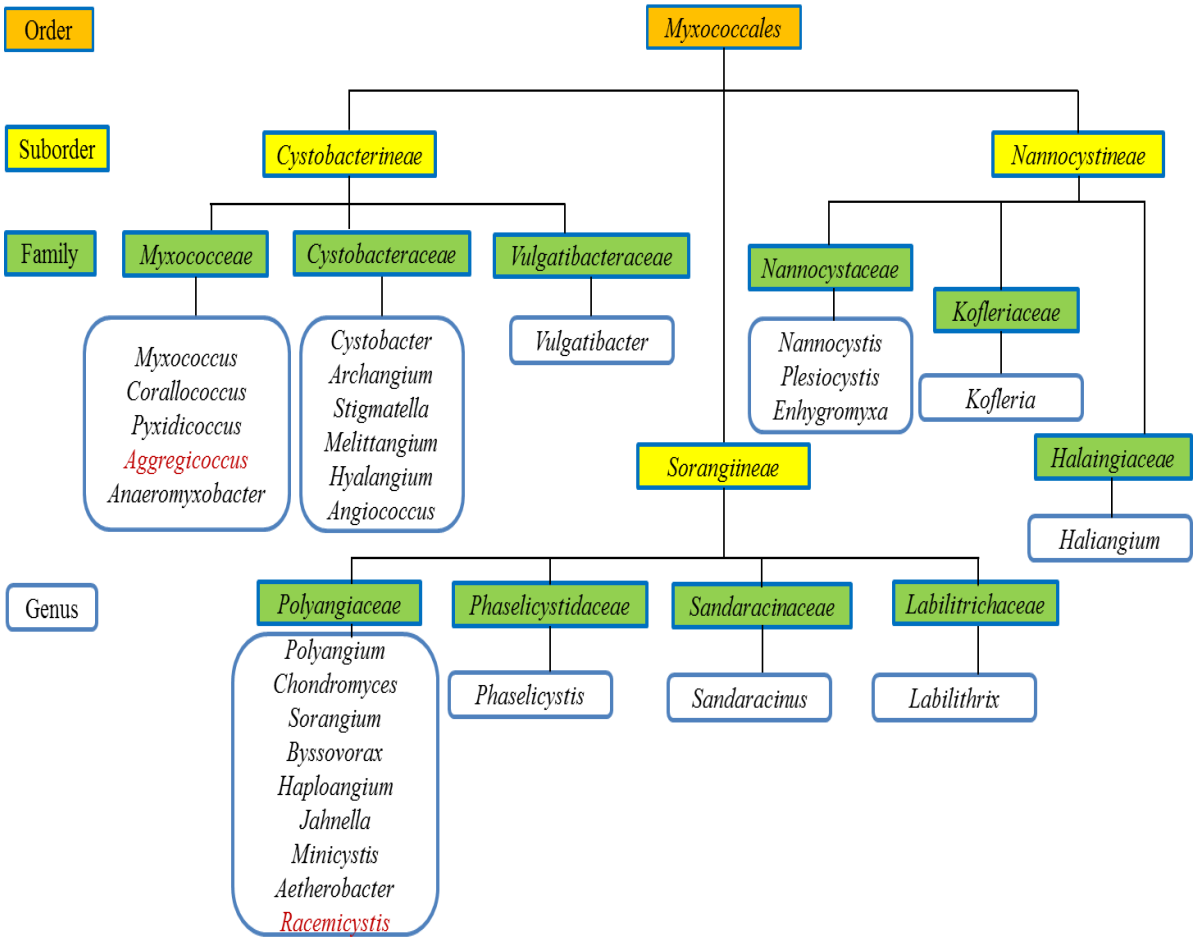

Fig. 2. Systematic classification of myxobacteria within order *Myxococcales*. (Reichenbach, 2005; Mohr *et al.*, 2012; Garcia *et al.*, 2014, Garcia & Müller, 2014a, 2014b, 2014c, 2014d, 2014e; Yamamoto *et al.*, 2014; Sood *et al.*, 2015; Awal *et al.*, 2016; Garcia *et al.*, 2016)

1.3. Myxobacterial strains from different habitats and approach to novel strain discovery

Myxobacteria have been isolated from a wide range of geographical regions around the globe and appear in temperate and tropic soils containing rotting plant materials (Dawid, 2000). Myxobacterial species are isolated form soil samples, dung of herbivores, barks of trees, rotting woods, marine sediments or marine-associated samples (Reichenbach, 1999; Dawid, 2000; Sanford *et al.*, 2002; Iizuka *et al.*, 2003b; Iizuka *et al.*, 2003a). Studies have shown that some species are restricted to certain sample types which could be due to their nutritional requirements. *Stigmatella aurantiaca* was isolated from rotting wood bark. Similarly, *Myxococcus fulvus*, *Myxococcus virescens*, *Cystobacter fuscus*, *Stigmatella erecta* and a representative of the genus *Jahnella* were isolated from herbivore dung (Reichenbach, 2005).

The discovery of the halophilic myxobacteria *Plesiocystis*, *Enhygromyxa*, and *Haliangium* from marine-associated samples led to a large interest in a more intense exploration of poorly examined habitats such as marine sediments (Fudou *et al.*, 2002; Iizuka *et al.*, 2003b, Iizuka *et al.*, 2003a, 2003a). Marine myxobacteria have been reported to be phylogenetically distinct from terrestrial species (Jiang *et al.*, 2010). However, no pure culture of such strains could be isolated due to the lack of suitable isolation protocols not including fruiting body formation. Myxobacteria have been isolated from unusual extreme habitats like alkaline peat bogs (Hook *et al.*, 1980), soil samples from Antarctic desert (Dawid *et al.*, 1988) and hot springs (Iizuka *et al.*, 2006). A novel approach using oxygen-free conditions for isolating *Anaeromyxobacteria* (Sanford *et al.*, 2002) also led to the discovery of a novel strain. Nevertheless, novel isolation procedures are necessary to obtain isolates from these underexplored sources. New approaches could also lead to the discovery of novel strains even from terrestrial sample sources. At this, the knowledge on the biology and systematics of myxobacteria are valuable for them to be recognized and identified. Fruiting body formation and swarming are important characteristics to be considered during isolation.

1.4. Characteristic features of myxobacteria

Myxobacteria exhibit a unique social lifestyle; they show complex and cooperative social behaviours that are exceptional in the bacterial kingdom. Active growth occurs in the direction of their long axis on solid agar surfaces in multicellular swarms without the use of flagella. This multicellular swarm allows them to gather and to lyse other microorganisms cooperatively producing hydrolytic enzymes and antimicrobial compounds. Under nutrient limitation, myxobacteria can undergo complex cellular morphogenesis through the activation of the developmental program with the formation of dormant myxospores within macroscopic multicellular fruiting bodies (Fig. 3). The structure and size of fruiting bodies is species specific exhibiting a large diversity from simple mounds (e.g. *Myxococcus xanthus*) to complex structures (e.g. *Chondromyces* spp.).

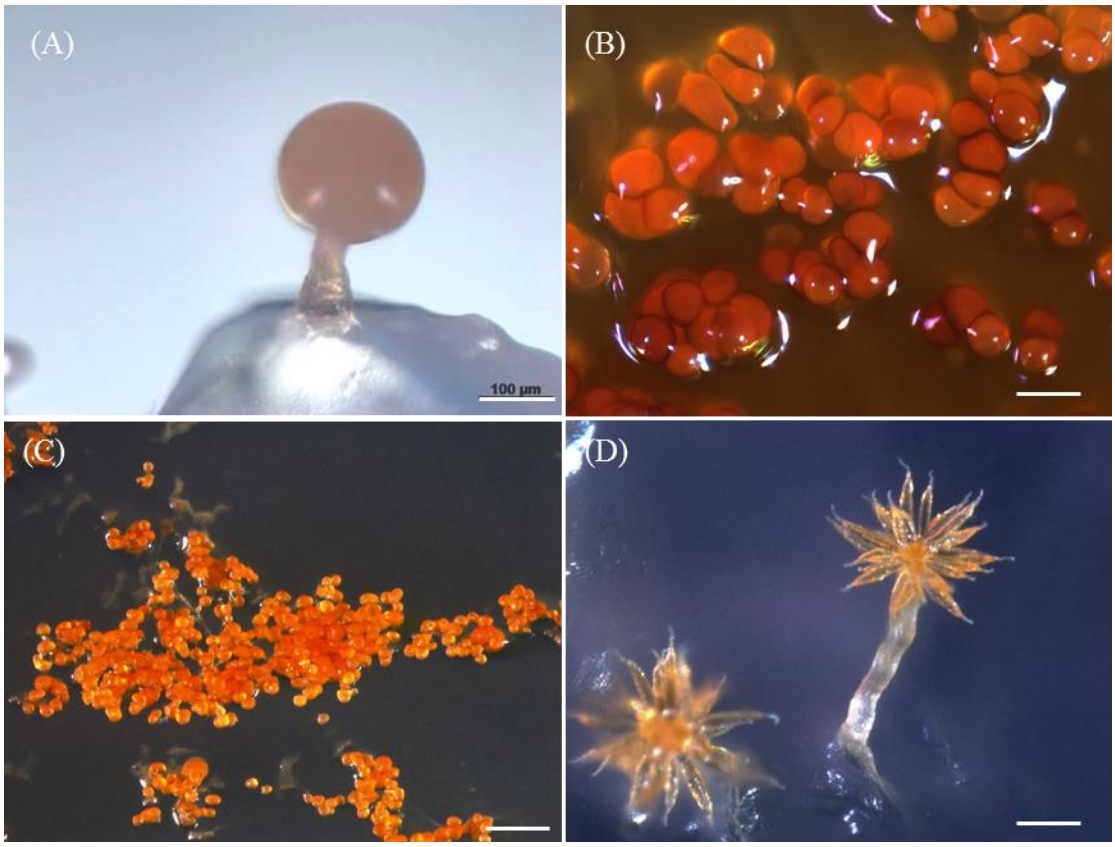

Fig. 3. Stereophotomicrographs of myxobacterial fruiting bodies on an agar. A) A Fruiting body of *Myxococcus stipitatus* B) *Cystobacter* sp. aggregates of sporangioles C) Fruiting bodies of *Melittangium* sp. D) Fruiting bodies of *Chondromyces apiculatus*. Bars, 100 µm.

1.4.1. Gliding Motility

The development of the best studied *Myxococcus xanthus* into multicellular fruiting bodies depends on gliding motility of cells on a solid surface (Reichenbach & Dworkin, 1992). *M. xanthus* has two separate systems for gliding, adventurous (A)- and social (S)-motility (Hodgkin & Kaiser, 1979a). S-motility, also called the social system, is responsible for the movement of cells in groups, whereas A-motility is required for the movement of individual cells (Hodgkin & Kaiser, 1979b). A large number of gene products play a role in A- and S-motility. The motility that provides the twitching motility force for S-motility is generated by the extension and retraction of type IV pili (Kaiser, 1979; Wu & Kaiser, 1995). Type IV pili are usually present only at one pole of the cell at one time and are polarly localized (Kaiser, 1979). Lipopolysaccharides (LPS) O-antigen and extracellular matrix fibrils, which consist of equal amounts of polysaccharide and protein, play important roles in S-motility (Arnold & Shimkets, 1988; Bowden & Kaplan, 1998). A-motility is propelled by an uncharacterized engine hypothesized to be associated with slime secretion, and the motility engines are located at the cell posterior, pushing cells forward (Yu & Kaiser, 2007).

1.4.2. The development of myxobacteria

Under starvation, ribosomes are stalled due to a lack of charged tRNAs that influences the ribosome-associated protein RelA to synthesize guanosine-5′-diphosphate-3′-diphosphate (ppGpp) and guanosine-5′-triphosphate-3′-diphosphate (ppp5pp). These compounds are commonly abbreviated as (p)ppGpp (Kaiser *et al.*, 1979; Harris *et al.*, 1998). Genetic evidence suggests that fruiting body development is regulated by at least five signals designated as the A-, B-, C-, D-, and the E-signal (Hagen *et al.*, 1978; Downard *et al.*, 1993). Up to date, the function of the A- and C- signals has been extensively investigated (Shimkets, 1999; Kaiser, 2004; Konovalova *et al.*, 2012b). The roles that B-, D- and E-signals play in the development of myxobacteria are less clear and, as a result, they are assumed to play a role as cell-cell signals (Kaiser, 2004).

Accumulation of (p)ppGpp is required to induce the cells to undergo a development and activates A-signaling which begins with the secretion of proteases. These proteases hydrolyze cell surface proteins to produce amino acids that serve as a signal resulting in aggregation of starving cells (Kuspa *et al.*, 1992; Singer & Kaiser, 1995; Crawford,E. W.

,Jr. & Shimkets, 2000). Once the concentration of A-signaling reaches a threshold, starving cells induce the expression of A-signal-dependent genes, and the developmental program begins. Five genes are known to be involved in the A-signal production. These genes are *asgA*, *asgB*, *asgC*, *asgD* and *asgE*. *asgA* and *asgD* encode hybrid histidine protein kinases (Plamann *et al.*, 1995; Cho & Zusman, 1999); *asgB* encodes a putative DNA-binding protein (Plamann *et al.*, 1994, 1994); *asgC* encodes the major sigma factor, RpoD (Davis *et al.*, 1995) and *asgE* has sequence homology to a ubiquitous family of amidohydrolases and possesses potential membrane-spanning domains (Garza *et al.*, 2000). The C-signal acts as a morphogen that becomes important after 6 h of development. The C-signal plays an important role in fruiting body formation: aggregation of cells into fruiting bodies, expression of developmental genes and differentiation of vegetative cells into spores (Shimkets *et al.*, 1983). C-signal functions in a dose-dependent manner. Low concentrations induce aggregation whereas sporulation is induced at a higher level of signaling (Kim & Kaiser, 1991; Kruse *et al.*, 2001). Studies have shown that it is essential for rippling and also plays a role in motility (Shimkets & Kaiser, 1982; Sager & Kaiser, 1994). The C-signaling system consists of only one protein named CsgA that might act as an enzyme to generate the C-signal (Lee *et al.*, 1995; Shimkets, 1999). CsgA has sequence homology with members of small-chain alcohol dehydrogenase (SCAD) and encodes a 25 kDa protein (Lee *et al.*, 1995). SCAD proteins contains two conserved sequence motifs, both of which are encoded by *csgA*: an N-terminal NAD (P) coenzyme binding pocket and a C-terminal catalytic domain (Lee *et al.*, 1995; Oppermann *et al.*, 2003). Lee et al. (1995) showed that affinity purified MalE-csgA protein binds to NAD^+ in vitro. An exogenous CsgA protein containing amino acid substitutions in either the putative coenzyme binding pocket (T6A or R10A) or catalytic site (S135T or T155R) failed to rescue the developmental defects in *csgA* mutant cells (Lee *et al.*, 1995). Overproduction of SocA, a CsgA and SCAD homolog, restored development in a *csgA* mutant in vivo (Lee & Shimkets, 1994).

Immunoblot analysis during development has shown the presence of two forms of CsgA, one with a molecular mass of ca. 25 kDa (full-length protein, designated as p25) and another with a molecular mass of ca. 17 kDa (C-terminally processed form, designated as p17) (Lobedanz & Sogaard-Andersen, 2003). It was reported that the CsgA NAD^+ binding pocket is non-essential for the signaling suggesting p17 could be the C-signal. Studies suggest that C- signaling is generated either via a receptor on a cell surface that

binds p17, generated from p25 cleaved by the protease PopC or via some enzymatic products of CsgA binds to a protein that resides on a cell surface (Rolbetzki *et al.*, 2008; Konovalova *et al.*, 2012a). Up to date, C-signaling is not clearly understood. Cell-cell contact is necessary for C-signaling, but the factors that trigger the signaling in cells are still a matter of debate.

1.4.3. The predatory nature of myxobacteria

Myxobacteria live in complex ecological habitats where they can interact and prey on other soil inhabitants, including both bacteria and eukaryotic organisms like yeast and grow on the nutrients released (Berleman & Kirby, 2007; Xiao *et al.*, 2011). Myxobacteria have an extracellular predatory behaviour that has been compared to multicellular 'wolfpack', but the mechanism of predation and its connection to multicellularity are still under study (Berleman & Kirby, 2009). The predatory nature of myxobacteria is non-species-specific one (Mendes-Soares & Velicer, 2013) and may need cell-cell contact, but without prey-cell invasion (Berleman & Keane, 2016). Myxobacteria secrete a cocktail of secondary metabolites with antibiotic properties and hydrolytic enzymes that kill and digest prey cells, so that molecular components are released into the environment and taken up again by myxobacteria to integrate these molecules into the metabolism (Reichenbach & Höfle, 1993; Berleman & Kirby, 2009; Mendes-Soares & Velicer, 2013).

1.5. Myxobacteria - a reservoir of natural products

Myxobacteria are known for their exceptional ability to produce structurally diverse secondary metabolites. The number of compounds identified from myxobacteria is much less compared with those found in actinomycetes and fungi. This comparably smaller number can perhaps be attributed to difficulty in cultivating myxobacterial strains and lack of suitable media for screening. Despite a limited number of myxobacterial species, more than 100 different basic structures and approximately 500 derivatives have been isolated. Most of these secondary metabolites were novel at the time of their discovery (Wenzel & Müller, 2009a). Therefore, myxobacteria are regarded as outstanding secondary metabolite producers (Weissman & Müller, 2010). The compound diversity in myxobacteria appears as associated with the discovery of more novel isolates from different environments.

Myxobacterial compounds exhibit a variety of biological activities, mostly against fungi (54%) and bacteria (29%), which reflects the competitive pressure of their natural habitats (Weissman & Müller, 2009). Most of the antifungal compounds inhibit the electron flow within the mitochondrial respiratory chain by specifically targeting NADH dehydrogenase and cytochrome bc1 in a unique fashion compared to natural products known from other bacterial sources. Several antifungal compounds isolated from myxobacteria interrupt DNA replication and transcription (leupyrrin A) (Bode *et al.,* 2003), disrupt the integrity of the cell membrane (ambruticin, jerangolid) (Knauth & Reichenbach, 2000; Gerth *et al.,* 1996), inhibit acetyl CoA carboxylase activity (soraphen A) (Shen *et al.,* 2004) and block the electron flow in the respiratory chain (stigmatellin A) (Thierbach *et al.,* 1984), Fig. 4.

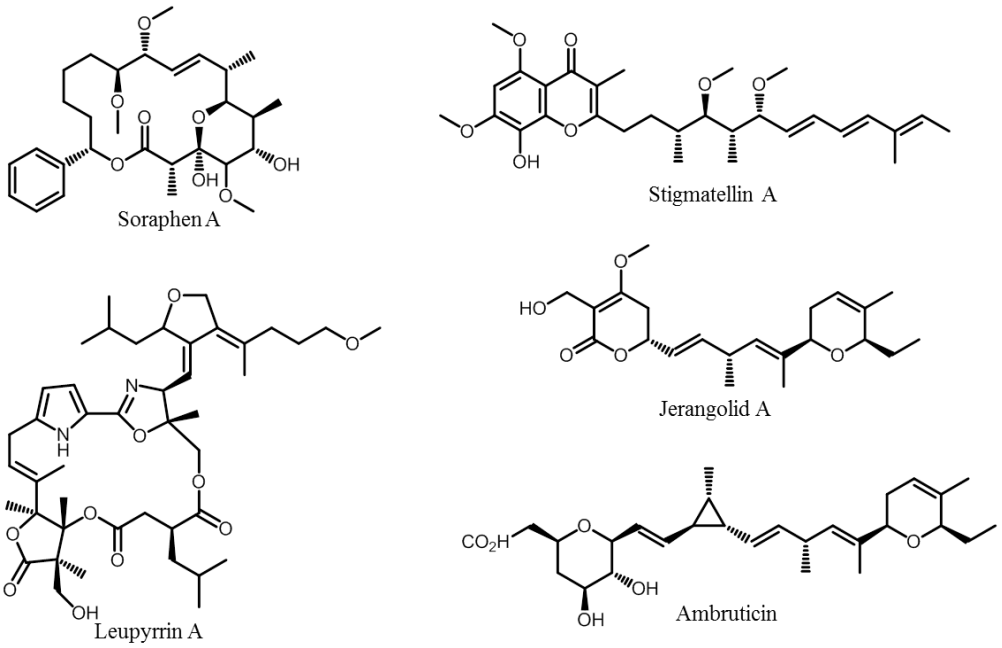

Fig. 4. Myxobacterial natural products with antifungal activities. Soraphen A, Jerangolid A, Leupyrrin A and Ambruticin from *Sorangium cellulosum* and Stigmatellin A from *Stigmatella aurantiaca*.

The approval of a semi-synthetic analogues epothilone, "ixabepiolne" (Ixempra®) for the treatment of breast cancer resulted in new efforts to search for novel drug leads from myxobacteria. Epothilone was isolated form *Sorangium cellulosum* So ce 90 that showed activity against multidrug resistant cancer cells, Fig. 5 (Reichenbach & Höfle, 2008). Thirty-two major classes of cytotoxic compounds have been identified from myxobacteria (Weissman & Müller, 2010).

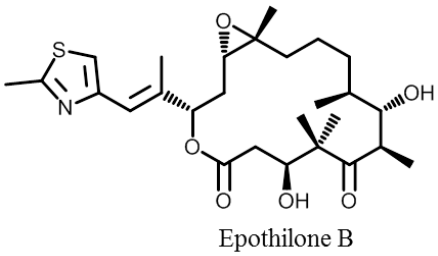

Fig. 5. Myxobacterial natural products with antitumor activity. Epothilone B from *Sorangium cellulosum*.

Corallopyronin A (Fig. 6) and Sorangiacin A (Fig. 6) are known for their antibacterial activity due to their ability to inhibit bacterial RNA polymerase. Myxovirescin (Fig 6) interferes cell wall synthesis (Irschik *et al.*, 1985; Irschik *et al.*, 1987). Thuggacin A (Fig. 6) acts on the electron transport system of *Mycobacterium tuberculosis* making this compound an interesting potential candidate for anti-tuberculosis therapy (Steinmetz *et al*., 2007). Cystobactamids (Fig. 6) are a new compound with activity against Gram-negative pathogens inhibiting gyrase (Baumann *et al.*, 2014).

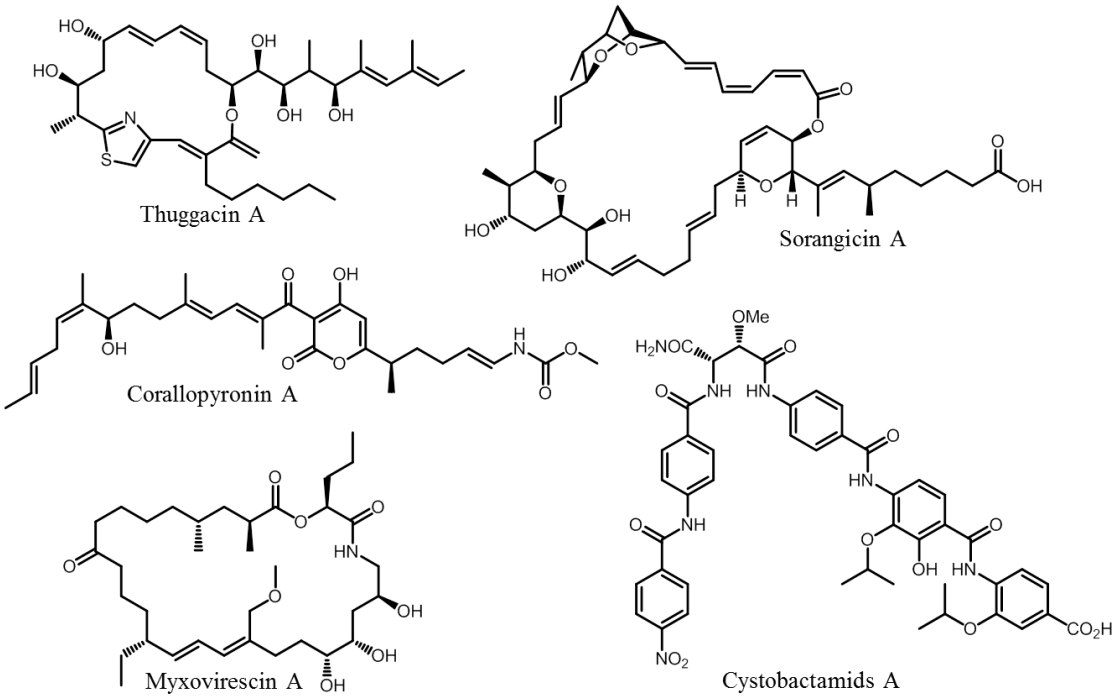

Fig. 6. Myxobacterial natural products with antibacterial activity. Thuggacin A and Sorangicin A from *Sorangium cellulosum*, Corallopyronin A from *Corallococcus coralloides*, Myxovirescin A from *Myxococcus virescens*, Cystobactamids A from *Cystobacter* sp.

In the last decade, a lot of effort has been made to identify and characterize numbers of secondary metabolites gene clusters from various myxobacterial strains and that has contributed to a deeper understanding of the complex biosynthetic machinery in secondary metabolites production. However, the numbers of silent or poorly expressed genes under routine laboratory conditions are more than anticipated in Myxobacteria. For example, the products of roughly only one third of the biosynthetic gene clusters present in *Myxococcus xanthus* have been identified (Wenzel & Müller, 2009b). Studies have reported that inter-species interactions can be utilized to activate cryptic gene clusters dedicated to the production of secondary metabolites (Bassler & Losick, 2006; Nett *et al.*, 2009; Schroeckh *et al.*, 2009). Thus, one strategy to trigger the expression of genes that are not expressed in an axenic culture would be the co-cultivation of two microorganisms in a single confined environment and activation of the silent gene clusters could be most probable via possible interspecies interactions (Scherlach & Hertweck, 2009). However, this has not been much explored in myxobacteria.

Furthermore, myxobacteria harbour the largest known genomes for bacteria and contain numerous natural product biosynthetic genes which are usually organized into clusters within the genome (Wenzel & Müller, 2010). These biosynthetic gene clusters can be manipulated or transferred into heterologous hosts to obtain a better yield and produce new analogs with biotechnological advantages.

1.6. Outline of the study

This study has a focus on the discovery of novel myxobacterial strains from soil samples from our HIPS-HZI soil collection using classical, as well as new isolation techniques for the primary purpose of discovering novel secondary metabolites with anti-infectives. The characterization of three novel genera (*Aggregicoccus*, *Racemicystis* and *Paucitangium*) including four species (*Aggregicoccus edonensis, Racemicystis crocea, Paucitangium cumulatus, Paucitangium subalbus*) using polyphasic taxonomic approaches is described (Sood *et al.*, 2015; Awal *et al.*, 2016). In addition, a part of this work focuses on the interaction of selected myxobacterial strains revealing that a well-known inducer of multicellular morphogenesis is not only an intra-species signal: A new role of this inducer as an inter-species signal for fruiting body formation is described for the first time.

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Chapter 2

Aggregicoccus edonensis gen. nov., sp. nov., an unusually aggregating myxobacterium isolated from a soil sample

Sakshi Sood[1, 2*], Ram Prasad Awal[3, 4*], Joachim Wink[1], Kathrin I Mohr[1], Manfred Rohde[5], Marc Stadler[1], Peter Kaempfer[6], Stefanie P. Glaeser[6], Peter Schumann[7], Ronald Garcia[3] and Rolf Müller[3, 4]

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*Contributed equally

Affiliations

[1]Department of Microbial Drugs, Helmholtz Centre for Infection Research (HZI), Inhoffenstraße 7, D-38124 Braunschweig, Germany

[2]Genomic and Applied Microbiology, Institute of Microbiology and Genetics, Georg-August University, Grisebachstraße 8, 37077 Göttingen, Germany

[3]Department of Microbial Natural Products, Helmholtz Institute for Pharmaceutical Research Saarland (HIPS) – Helmholtz Centre for Infection Research (HZI), Saarland University, Campus C2 3, 66123 Saarbrücken, Germany

[4] Department of Pharmaceutical Biotechnology, Saarland University, Campus C2 3, 66123 Saarbrücken, Germany

[5]Department of Medical Microbiology, Central Facility for Microscopy, Helmholtz Centre for Infection Research (HZI), Inhöffenstraße 7, B-38124 Braunschweig, Germany

[6]Department of Applied Microbiology, Justus Liebig University Gießen, Heinrich-Buff-Ring 26, D-35392 Gießen, Germany

[7]Central Services, Leibniz institute DSMZ-German Collection of microorganisms and cell cultures, Inhöffenstraße 7, B-38124 Braunschweig, Germany

Contributions

Author's efforts

The author isolated *Aggregicoccus edonensis* MCy10622 from the soil sample collected by the author. The author characterized the strain MCy10622 by morphological, physio-chemical, molecular and phylogenetic characteristic. After knowing the existence of MCy1366^T, The strain MCy10622 was re-characterized along with MCy1366^T in the same condition. The author did physio-chemical characterization, 16S rRNA analysis and phylogenetic analysis. The author conceived and wrote the paper.

Contributions by others

Sakshi Sood performed antibiotic susceptibility test and API ZYM test of the strains described. Sakshi Sood also took part in writing the paper. Manfred Rohde prepared SEM images. Peter Kämpfer and Stefanie P. Glaeser did the DNA-DNA hybridization and its analysis. Peter Schumann performed MALDI-TOF measurement. Ronald Garcia did the fatty acid analysis. Kathrin I. Mohr, Joachim Wink, Marc Stadler and Rolf Müller supervised Sakshi Sood. Ronald Garcia and Rolf Müller supervised the author. Kathrin I. Mohr, Ronald Garcia and Rolf Müller designed the study. All author contributed in writing the paper.

Chapter: 2 *Aggregicoccus edonensis* gen. nov., sp. nov., an unusually aggregating myxobacterium isolated from a soil sample

2.1. Abstract

A novel myxobacterium, MCy1366^T (Ar1733), was isolated in 1981 from a soil sample collected from a region near Tokyo, Japan. It displayed general myxobacterial features like Gram-negative-staining, rod-shaped vegetative cells, gliding on solid surfaces, microbial lytic activity, fruiting-body-like aggregates and myxospore-like structures. The strain was mesophilic, aerobic and showed a chemoheterotrophic mode of nutrition. It was resistant to many antibiotics such as cephalosporin C, kanamycin, gentamicin, hygromycin B, polymyxin and bacitracin, and the key fatty acids of whole cell hydrolysates were iso-$C_{15:0}$, iso-$C_{17:0}$ and iso-$C_{17:0}$ 2-OH. The genomic DNA G+C content of the novel strain was 65.6 mol%. The 16S rRNA gene sequence showed highest similarity (97.60 %) to '*Stigmatella koreensis*' strain KYC-1019 (GenBank accession no. EF112185). Phylogenetic analysis based on 16S rRNA gene sequences and MALDI-TOF MS data revealed a novel branch in the family *Myxococcaceae*. DNA–DNA hybridization showed only 28 % relatedness between the novel strain and the closest recognized species, *Corallococcus exiguus* DSM 14696^T (97 % 16S rRNA gene sequence similarity). A recent isolate from a soil sample collected in Switzerland, MCy10622, displayed 99.9 % 16S rRNA gene sequence similarity with strain MCy1366^T and showed almost the same characteristics. Since some morphological features like fruiting-body-like aggregates were barely reproducible in the type strain, the newly isolated strain, MCy10622, was also intensively studied. On the basis of a comprehensive taxonomic study, we propose a novel genus and species, *Aggregicoccus edonensis* gen. nov., sp. nov., for strains MCy1366^T and MCy10622. The type strain of the type species is MCy1366^T (=DSM 27872^T=NCCB 100468^T).

2.2. Introduction

Myxobacteria are known for their unique lifecycle and the ability to produce natural products with unique structures and bioactivities (Weissman & Müller, 2010). They form a phylogenetically coherent group within the class Deltaproteobacteria based on 16S

rRNA gene sequence analysis (Garcia *et al.*, 2010). Three suborders, seven families, 23 genera and 55 species with valid names have been published within the order *Myxococcales* (Approved List of Bacterial Names). Myxobacterial systematics is mainly based on morphological characteristics like shape and size of vegetative cells and myxospores, swarming colonies and fruiting bodies. However, their morphological characteristics are often unstable and may vary or be lost on repeated sub-culturing under laboratory conditions (Garcia *et al.*, 2010). 16S rRNA gene sequences are also quite conservative for fine discrimination between closely related taxa (Fox *et al.*, 1992). Hence, a polyphasic approach should be followed in order to clearly delineate a potential novel taxon from its closest relatives. Recently, fatty acid analysis with respect to different taxonomic clades within the myxobacteria helped to establish chemotaxonomic–phylogenetic correlations (Garcia *et al.*, 2011). In the future, other molecular markers and approaches also need to be identified and applied to establish modern myxobacterial systematics.

Using divergent approaches based on genotypic, phenotypic and chemotaxonomic analysis performed together with the reference strain MCy10622 and selected type and neotype strains of species belonging to the genera *Myxococcus*, *Corallococcus*, *Pyxidicoccus* and *Melittangium*, we propose a novel species in a new genus for the soil bacterium (strain MCy1366^T) from Japan.

2.3. Result and Discussion

Two strains described here were isolated from different geographical locations at different times. Strain MCy1366^T was isolated at the Helmholtz Centre for Infection Research (HZI; formerly GBF, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany) in 1981 from a soil sample collected in 1980 near Tokyo, Japan. The reference strain MCy10622 was isolated in July 2013 at the Helmholtz Institute for Pharmaceutical Research Saarland (HIPS, Saarbrücken, Germany) from a soil sample collected in Zürich, Switzerland. Standard isolation and purification protocols were used for both strains (Reichenbach & Dworkin, 1992). The type and neotype strains used in this study are listed in Table S1, available in the Supplementary Material. All strains were continuously maintained on CY/H medium supplemented with 1 ml l^{-1}, 500-fold-diluted vitamin solution after autoclaving (Schlegel, 1992; Mohr *et al.*, 2012) and VY/2 agar (Shimkets *et*

al., 2006). All cultures were incubated at 30 °C and liquid cultures were grown at 160–200 r.p.m. unless specified otherwise.

Morphology of vegetative cells and myxospores were studied with phase-contrast microscopy (BX51; Olympus). Fruiting-body-like aggregates (for MCy1366^T), fruiting bodies (for MCy10622) and swarming colonies were observed under an Olympus SZX12 stereomicroscope and Zeiss Discovery-V20 stereomicroscope on P (Garcia *et al.*, 2009), VY/2 and CY agar (Shimkets *et al.*, 2006). All photographs were taken using the image analysis software 'AxioVision LE' and AxioCam MRc camera (Carl Zeiss). Fruiting body aggregate morphology of strain MCy1366^T was also studied by field emission scanning electron microscopy (SEM) using a Zeiss Merlin with aldehyde-fixed, critical-point-dried and gold-palladium-sputtered samples. Images were taken with an Everhart-Thornley SE-detector and Inlens-SEM detector in a 25:75% ratio applying the SEMSmart software version 5.05. Formation of myxospore-like structures by strain MCy1366^T was observed in myxo media broth (10 g casein peptone, 1 mg $CoCl_2$, 0.05 g $CaCl_2.2H_2O$, 0.25 g $MgSO_4.7H_2O$, 23.6 g HEPES, 1 l distilled water; pH 7.0) after incubation for a minimum of 3 days.

The swarm colony of both isolates on VY/2 agar was transparent, soft, thin and surrounded by a clear halo due to the lysis of yeast cells as often described for most members of the family *Myxococcaceae* (Reichenbach, 2005), and the salmon orange colour of MCy1366^T cells was evident upon scraping the swarm with a loop. The swarm colony on P agar was yellow to orange with large flame-like outward extensions at the periphery (Fig. 1d) and surrounded by a clear halo suggesting starch hydrolysis. Swarm on CY agar contained shades of orange to brown (Fig. 1b), ripples and flat ridge-like structures in the centre, and intricate wavy veins at the edges (Fig. 1c). Such an undulating vein structure is typical for *Myxococcaceae* swarms on peptone-containing agar (Reichenbach, 2005). Radiating veins in the swarm or corrosion of agar, typical for members of the families *Cystobacteraceae* and *Nannocystaceae*, respectively, were not observed on any medium.

Vegetative cells were slender rods with slight tapering ends, measuring 0.72–0.82 µm × 5.71–10.73 µm except a few which were as long as 20 µm after cultivation in myxo media for 2 days (Fig. S1). Numerous optically refractile, irregular spherical to ellipsoidal

myxospore-like structures (1.3–1.7 µm in size) surrounded by a conspicuous dark capsule were observed in the same media after a longer (10–20 days) incubation period (Fig. 1h). Cultivation in both CY/H and myxo media broth produced salmon orange and beige vegetative cells of strains MCy1366^T and MCy10622, respectively, which often gathered into a huge aggregate of cells. Such a massive aggregation of cells with a branching morphology is a peculiar characteristic of both isolates in liquid media (Fig. 1a).

Fruiting body-like aggregates of strain MCy1366^T could only be observed on P and CY agar after prolonged incubation (8–10 weeks) (Fig. 1e). They mostly appeared solitary as yellowish-orange spherical heads and lacked sporangioles and stalks. Immature fruiting bodies had the appearance of humps or knobs on the agar, as in species of the genus *Myxococcus* (Reichenbach, 2005). Cell aggregation on the agar, presumably in an attempt to form the fruiting bodies, was clearly visible under the stereomicroscope as well as by SEM imaging (Fig. 1g). No fruiting bodies were observed on buffered VY/2 agar; conversely the novel isolate MCy10622 could form reproducible fruiting bodies on the same agar within 1 week of incubation (Fig. 1f). However, the fruiting bodies of strain MCy10622 were irregularly shaped, cushion-like cell mounds formed in groups clearly distinguishable from fruiting bodies of strain MCy1366^T (Fig. 1f). Myxospores similar to spherical structures observed in liquid myxo media were also observed upon squashing the fruiting bodies and fruiting body-like aggregates on a glass slide.

The reaction of 3-week-old swarming colonies of MCy1366^T and MCy10622 on VY/2 agar to Congo red stain was determined using the McCurdy method (McCurdy, 1969). Enzyme activity was determined using API ZYM strips (bioMérieux) inoculated with the cell suspension in sterile water (1 : 10) and incubated aerobically at 30 °C overnight. Starch hydrolysis, cellulose and agar degradation was studied as described by (Mohr *et al.*, 2012). The chitin degradation assay was performed using CT-6 (Reichenbach, 2006) and synthetic S agar (0.5 g $CaCl_2.2H_2O$, 0.5 g $MgSO_4.7H_2O$, 0.06 g K_2HPO_4, 8 mg Ferric EDTA, 50 mM HEPES, 1 l distilled water; pH 7.2) with 0.7% (w/v) chitin as the sole nutrient source. Salt tolerance to 1.5 % (w/v) NaCl was tested on CY agar after incubation for 3–4 weeks at 30 °C.

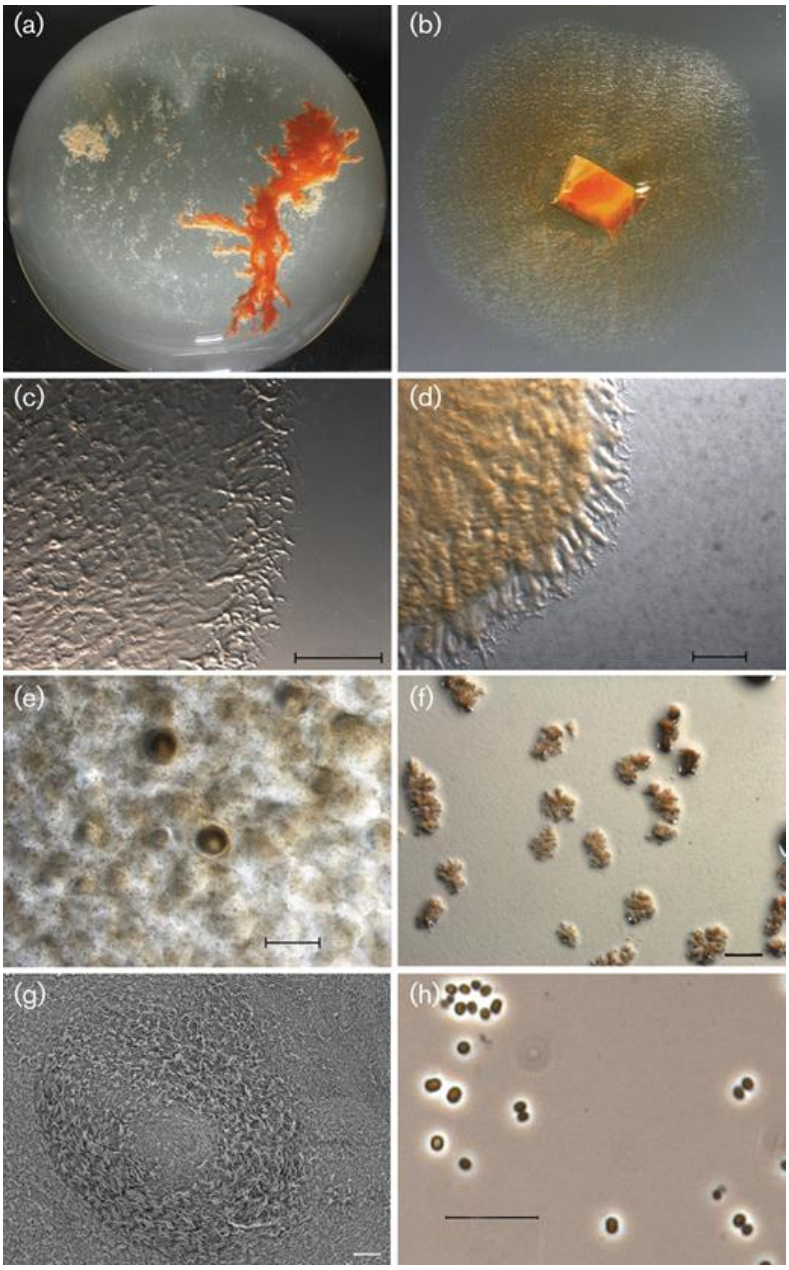

Fig.1. Photographs showing morphology of strain MCy1366^T and MCy10622. (a) Photograph of huge bright orange cell aggregate of MCy1366^T in 11 day old culture in CY+H broth. (b) Saffron orange coloured 4-week-old swarming colony of MCy1366^T on CY agar. Stereophotomicrograph of (c) 4-week-old swarm of MCy1366^T on CY agar (bar, 2000 μm), (d) 3 week old swarm of MCy1366^T on P agar (bar, 2000 μm), (e) fruiting body-like aggregates of MCy1366^T on P agar (bar, 500 μm), (f) mature fruiting bodies of MCy10622 on VY/2 agar (bar, 200 μm). (g) SEM image showing cell aggregation of MCy1366^T on agar surface for initiation of fruiting body formation (bar, 20 μm) (h) Phase contrast image of myxospore-like structures in liquid myxo media (bar, 20 μm).

The Congo red reaction was very weak and displayed a light pink colour (Fig. S1) in contrast to strong reactions observed for species of the genera *Corallococcus* and *Pyxidicoccus*, and some species of the genus *Myxococcus*. Neither isolate could degrade cellulose, chitin or agar. No cell growth was observed for strains MCy1366^T and MCy10622 with 1.5 % NaCl, which was also observed for species of the genus *Corallococcus* and *Melittangium lichenicola* DSM 2275^T, while fair to good growth was observed for *Pyxidicoccus fallax* DSM 14698^T and all species of the genus *Myxococcus* except *Myxococcus fulvus* DSM 16525^T (Table S2).

The colourless haloes on P agar plates after staining with Lugol's solution was limited to the size of the swarms of MCy1366^T and MCy10622 as opposed to species of the genus *Corallococcus*, depicting weak starch hydrolysis ability (Fig. S1). Ability to degrade starch and salt tolerance are often used as characteristics to differentiate between species of the genera *Myxococcus* and *Corallococcus* (Lang & Stackebrandt, 2009).

The reactions on the API ZYM strips by strain MCy1366^T were reasonably similar to the reactions observed for species of the genus *Corallococcus* suggesting a close relationship (Table S3). Positive reactions were observed for alkaline phosphatase, esterase lipase, leucine arylamidase and acid phosphatase activities while *β*-galactosidase, *α*-mannosidase and *α*-fucosidase activities were negative for strain MCy1366^T along with all the other strains used during this study (Table S1). Trypsin activity was negative for strain MCy1366^T along with type strains of species of the genus *Corallococcus* and *Melittangium lichenicola* DSM 2275^T but it was positive for three species of the genus *Myxococcus* and *Pyxidicoccus fallax* DSM 14698^T. All the reactions for strain MCy1366^T on API ZYM strips were the same as strain MCy10622 except for cysteine arylamidase activity, which was found to be negative for the former and positive for the latter. Results from all the members of the family *Myxococcaceae* strengthened the supposition that both novel isolates belong to the same family. Detailed results from the API ZYM test system for all strains are given in Table S3.

Cross-streaked microbial baits of live bacteria (*Paenibacillus polymyxa* DSM 36^T), yeasts (*Candida albicans* DSM 1665 and *Wickerhamomyces anomalus* DSM 6766) and autoclaved *Escherichia coli* K-12 cells on water agar were used for testing the ability of strains MCy1366^T and MCy10622 to lyse micro-organisms as described by (Mohr *et al.*,

2012). *E. coli* and *C. albicans* cells were completely lysed by both strains. *W. anomalus* and *Paenibacillus polymyxa* supported the growth of strain MCy1366^T on water agar but clearing of cells could not be observed making it difficult to assess the ability of strain MCy1366^T to lyse the cells of these organisms.

The growth response of strains MCy1366^T and MCy10622 to different temperatures was tested at 20, 30, 37 and 42 °C on VY/2 agar. The optimum growth temperatures for strains MCy1366^T and MCy10622 were near 30 °C like most myxobacteria, and neither strain could grow at 42 °C. The pH optimum for both strains was tested between pH 5.0 and 10.0 at intervals of 0.5 pH units in CY liquid media. Both strains tolerated a wide pH range, from pH 6.0–10.0, but maximum growth was seen at pH 6.5–7.0 (Table S8) and there was no evidence of growth below pH 5.5.

Antibiotic resistance of strains MCy1366^T and MCy10622 and other related strains was tested as described by (Mohr *et al.*, 2012) against 13 antibiotics. The final concentration was adjusted to 50 (μg ml^{-1}) for most antibiotics except oxytetracycline (10), chloramphenicol (30), ampicillin (100) and hygromycin B (150). Strain MCy1366^T was sensitive to spectinomycin, thiostrepton and chloramphenicol and resistant to cephalosporin C, kanamycin, gentamicin, hygromycin B, polymyxin and bacitracin, similar to species of the genus *Corallococcus* used in this study. However, it was sensitive to fusidic acid and ampicillin unlike the species of the genus *Corallococcus*, which showed some growth on fusidic acid and good growth on ampicillin. Strain MCy10622 was found to be sensitive to spectinomycin and thiostrepton but resistant to most of the other antibiotics tested. Both MCy1366^T and MCy10622 grew poorly on trimethoprim. A detailed description of the antibiotic resistance test is given in Table S4.

Synthetic liquid medium S containing 0.25 % (w/v) casitone supplemented with 0.25 % (w/v) of various sugars and starch was used for testing the carbon sources favoured for growth. Nitrogen requirement was tested on a minimal casitone medium (C) (Mohr *et al.*, 2012) with the addition of 0.25 % (w/v) of some organic and inorganic nitrogen sources. Utilization of various peptones was also tested by their addition to synthetic S medium individually at a concentration of 0.25 % (w/v). These experiments were performed in duplicate and all flasks were incubated at 30 °C, 200 r.p.m. for 7 days. Cell pellets were

collected and washed with sterile water in order to remove salt contents, dried and weighed.

Table 1. Differential characteristics of strains MCy1366^T and MCy10622 and closely related type strains

Characteristic **No. of strains used for study**	**MCy1366^T**	**MCy10622**	***Myxococcus* spp.** **5**	***Corallococcus* spp.** **2**	***Pyxidicoccus fallax*** **1**	***Melittangium lichenicola*** **1**
Resistance to (μg ml^{-1}):						
Ampicillin (100)	−	+	v(3)	+	+	−
Kanamycin (50)	+	+	v(2)	+	−	+
Spectinomycin (50)	−	−	+	−	+	+
Trimethoprim (50)	+	+	v(2)	+	+	+
Fusidic acid (50)	−	+	v(3)	+	+	−
Growth with 1.5% salt	−	−	v(4)	−	+	−
Starch hydrolysis	Weakly positive	Weakly positive	Weakly positive	Strongly positive	Positive	nd
Congo red reaction	Very weak	Very weak	Weak/Strong	Strong	Strong	Weak
Trypsin (API ZYM test)	−	−	v(3)	−	+	−
Fatty acid analysis						
$C_{15:0}$	1.8	1.6	0.0–2.7	0.2–0.8	1.1	0
$C_{15:1}$ isomer 2	3.3	0	0.0–2.5	0.1–0.4	0.9	0
$C_{16:1}$ isomer 2	1.1	0	6.1–14.5	0.2–0.6	21	5.5
$C_{17:1}$ω7cis	0.4	0	0.0–1.0	1.5–2.2	0.2	0
iso-$C_{15:0}$	23.2	25.3	13.5–39.7	17.5–22.3	14	13
iso-$C_{17:0}$	16.6	10.4	1.3–11.7	4.3–10.1	7.7	0.8
iso-$C_{17:0}$ 2-OH	26.1	14.1	0.2–12.4	32.9–34.4	9.3	0.5
iso-$C_{15:0}$ OAG	1.9	1.3	0.0–10.3	3.4–7.2	10.8	0
iso-$C_{15:0}$ DMA	2	4.4	0.0–2.1	1.6–4.2	4.7	0

*Results for individual strains are given in Supplementary Tables S2 - S4 and S7. V, Variable results (numbers of positive strains given in parentheses); n.d., not determined; for reactions to antibiotics, + indicates resistance, and – indicates sensitivity; fatty acids analysis, OAG, O-alkylglycerol fatty acids; DMA, Dimethylacetal fatty acids. Strains used for the study: *Myxococcus* spp. (*M. xanthus* DSM 16526^T, *M. fulvus* DSM 16525^T, *M. stipitatus* DSM 14675^T, *M. virescens* DSM 2260^T, *M. macrosporus* DSM 14697^T); *Corallococcus* spp. (*C. exiguus* DSM 14696^T, *C. coralloides* DSM 2259^T); *Pyxidicoccus fallax* DSM 14698^T and *Melittangium lichenocola* DSM 2275^T.

Strain MCy1366^T as well as strain MCy10622 could not utilize any of the monosaccharides or disaccharides under the tested conditions (Table S5) which is also true for most members of the family *Myxococcaceae* (Reichenbach, 2005). Interestingly, growth was observed on molasses (Juchem), which could be due to presence of other supplements. Both strains could utilize polysaccharides viz. potato starch, soluble starch and pyruvate but the two strains differed slightly in their peptone requirements; strain MCy1366^T yielded high cell densities in neopeptone (Becton Dickinson), phytone peptone (BD) and skimmed milk (Oxoid) whereas strain MCy10622 grew better in tryptone enzymic digest from casein (BD). No growth was observed in synthetic media S supplemented with Casamino acids (BD) while all the organic and inorganic nitrogen sources tested resulted in moderate to fair growth of both strains (Table S6).

For fatty acid analysis, all the strains used in the study (Table S7) were cultivated in 50 ml CY medium in 300 ml flasks shaken at 160 r.p.m. at 30 °C. Cellular fatty acid extraction, GC-MS analysis and peak identification were done in duplicate using the method described by (Garcia *et al.*, 2011). All strains had substantially high amounts of iso-C $_{15:0}$, which is true for most myxobacteria (Garcia *et al.*, 2009). The fatty acid patterns of strains MCy1366^T and MCy10622 were significantly distinct from all other strains used in the study. Strain MCy10622 differed from strain MCy1366^T due to the presence of significant amounts of iso -C $_{17:1}$ (10.9 %), which was completely absent in the fatty acid profile of the latter. The highest percentage of the branched-chain fatty acids (BCFAs) observed in strain MCy1366^T was for iso-C $_{17:0}$ 2-OH (26.1 %) followed by iso-$C_{15:0}$ (23.2 %) (Table 1). Indeed, high amounts of iso-$C_{17:0}$ 2-OH has been recommended as a determinative chemotaxonomic marker for species of the genus *Corallococcus* in the family *Myxococcaceae* (Garcia *et al.*, 2011). Strain MCy1366^T possessed a significant amount of iso-C $_{17:0}$ (16.6 %) and higher amounts of $C_{15:1}$ isomer 2 (3.3 %) compared to all other strains and differed from species of the genus *Corallococcus* with lower amounts (1.9 %) of O-alkylglycerol (OAG) fatty acids. In addition, strains MCy1366^T and MCy10622 were distinguishable from *Melittangium lichenicola* DSM 2275^T, which contains higher amounts of $C_{16:0}$ (9.8 %), $C_{18:0}$ (20.6 %) and unspecified fatty acid alcohol (32.1 %) as well as much lower amounts of iso-$C_{15:0}$ (13.0 %) and iso-$C_{17:0}$ 2-OH (0.5 %). The total amount of straight-chain fatty acids (SCFAs) and BCFAs for strain MCy1366^T was 15.4 % and 84.4 %, respectively, while strain MCy10622 showed 10.0 % and 76.3 %, respectively (Table S7).

For 16S rRNA gene amplification, genomic DNA was extracted from actively growing cells of MCy1366^T and MCy10622 using the protocol for Gram-negative bacteria from the Puregene Core Kit A (Qiagen). The 16S rRNA gene was amplified by PCR using universal primers (forward 5′-GAGTTTGATCCTGGCTCAGGA-3′; reverse 5′-AAGGAGGTGATCCAGCCGCA-3′). The PCR product was purified using the Nucleospin Gel and PCR clean-up kit (Macherey Nagel) following analysis of the PCR product by gel electrophoresis using a 0.8 % (w/v) agarose gel at 95 V for 35 min. Forward primers F27, F945, F357, F1100 and reverse primers R336, R518, R1078 and R1525 in addition to the primers used for PCR were applied for sequencing of the 16S rRNA gene (Lane, 1991; Stackebrandt & Liesack, 1993; Weidner *et al.*, 1996; Turner *et al.*, 1999). The consensus sequences were produced using the Cap contig assembly function of the BioEdit Sequence Alignment Editor Version 7.1.3.0. and compared to the NCBI- blast nucleotide database using the FASTA search tool and showed the highest similarity (MCy1366^T, 97.60 %, and MCy10622, 97.47 %) to '*Stigmatella koreensis*' KYC-1019 (GenBank accession no. EF_112185).

The complete sequences of MCy1366^T and MCy10622 were aligned with sequences of their close relatives using CLUSTAL W (Larkin *et al.*, 2007). A phylogenetic analysis with type and representative myxobacterial strains was calculated using two tree-making algorithms, neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Guindon & Gascuel, 2003), using the software package Geneious version 7.0.3 (Biomatters). A sulfate-reducing bacterium, *Desulfovibrio desulfuricans* DSM 642^T (GenBank accession no. NR_036778) was chosen as an out-group to root the phylogenetic tree. The Jukes–Cantor model (Jukes & Cantor, 1969) was applied to calculate evolutionary distance matrices of the neighbour-joining and maximum-likelihood methods and the topology of the phylogenetic tree was evaluated by bootstrap support based on 1000 resamplings (Felsenstein, 1985).

The tree reconstructed by the maximum-likelihood method (Fig. 2) showed that both strains (MCy1366^T and MCy10622) formed a separate cluster within the family *Myxococcaceae* close to *Corallococcus exiguus* DSM 14696^T, *Corallococcus coralloides* DSM 2259^T and *Melittangium lichenicola* DSM 2275^T. Their separate branching from the known myxobacterial taxa in the suborder *Cystobacterineae* was also clear in the phylogenetic tree reconstructed with the neighbour-joining method (Fig. S2). The

delineation of the two isolates from closely related strains clustering within the family *Myxococcaceae* clearly indicated that they represent a novel taxon in the same family.

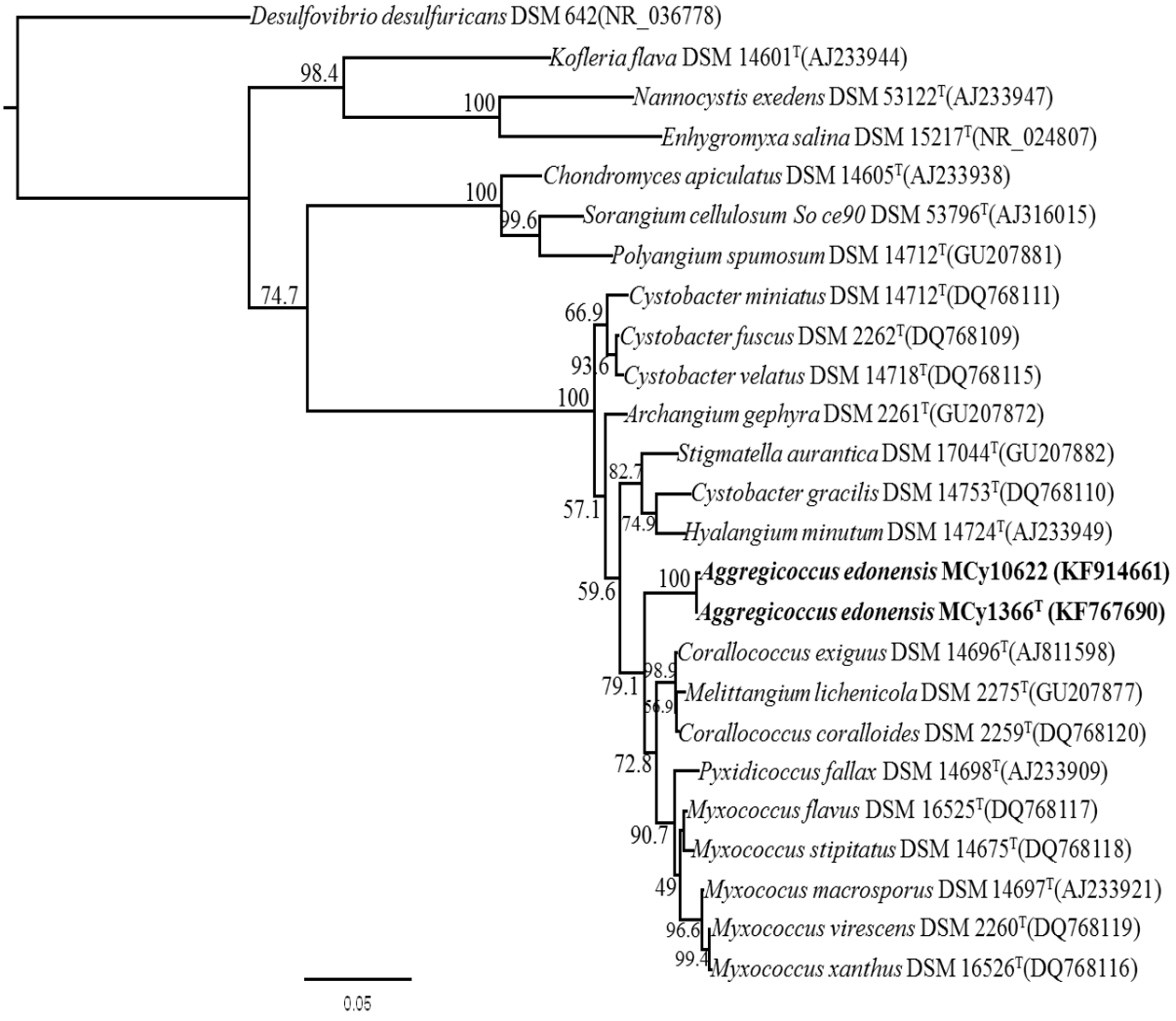

Fig. 2. Maximum-likelihood phylogenetic tree (PHYML) based on 16S rRNA gene sequences of strains *Aggregicoccus edonensis* MCy1366^T and *Aggregicoccus edonensis* MCy10622 and related taxa. Numbers at branch points indicate bootstrap support based on 1000 resamplings. GenBank accession numbers are shown in parentheses. *Desulfovibrio desulfuricans* DSM 642^T was used as an out-group. Bar, 0.05 substitutions per nucleotide position.

The two closest relatives of MCy1366^T and MCy10622 according to the 16S rRNA gene sequence-based phylogenetic tree, *C. exiguus* DSM 14696^T and *C. coralloides* DSM 2259^T, were chosen for DNA–DNA hybridization assays and DNA G+C content analysis. A method adapted from (Kieser *et al.*, 2000) but modified and optimized especially for myxobacteria was used for isolation of high yields of genomic DNA. Cultures (100 ml) in myxo media, CY+H media and A media (8 g starch, 4 g soymeal, 2 g yeast, 1 g $CaCl_2$, 1 g $MgSO_4$, 100 mM HEPES, 8 mg Ferric EDTA, 4 ml glycerol, 1 l distilled water; pH 7.4) were harvested by centrifugation at 11000 r.p.m. (15286 g) for 5 min. The cell pellets were resuspended in 10 ml Tris buffer after discarding the supernatant and the cell aggregates were dispersed and homogenized with an Ultraturrax (Art Moderne Labortechnik). After addition of 200 µl proteinase K (20 mg ml^{-1} in 50 mM Tris-HCl, 1 mM $CaCl_2.2H_2O$; pH 7.5) and 200 µl SDS (20 %, w/v, solution), the solution was mixed gently and kept in a hybridization oven for continuous shaking and incubation at 41 °C for 30 min 2 h until the solution became slightly clear. NaCl (3.5 ml of a 5 M solution) was added to the solution with gentle mixing followed by 4 ml CTAB extraction buffer [4.1 % (w/v) NaCl, 10% (w/v) cetyltrimethylammonium bromide, water]. The cell solutions were shaken and incubated at 65 °C for another 20 min inside the hybridization oven. The cell solution was then incubated at room temperature from 1 h to 12 h in an overhead shaker after addition of chloroform up to 35 ml. Phase separation of the solution was then achieved by centrifugation at 12000 r.p.m. for 20 min and the upper phase was carefully transferred into new falcon tubes. The acquired upper phase was added to an equal volume of 2-propanol and mixed gently. The precipitated DNA was aspirated, rinsed twice with 70 % (v/v) ethanol, air-dried and dissolved in 2 ml TE buffer.

DNA–DNA hybridization was performed as described by (Ziemke *et al.*, 1998), with the modification that for nick translation, 2 µg DNA was labelled during a 3 h incubation at 15 °C. The G+C content of the DNA was determined using a fluorimetric thermal denaturation temperature determining method described by (Gonzalez & Saiz-Jimenez, 2002). Analysis was performed using 5 µg genomic DNA in a total volume of 20 µl, with 0.1× SSC buffer (0.03 M NaCl, 0.03 M sodium citrate), 10 % (v/v) deionized formamide and 0.25× SYBR Green I (Molecular Probes). Thermal denaturation was performed in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad) starting with 15 min at 25 °C followed by a ramp from 65 to 98 °C with an increase of 0.1 °C cycle $^{-1}$. Each cycle held for 5 s and included a fluorescence measurement (SYBR channel). Analysis was

performed at least in duplicate. Five reference strains were used to generate a standard curve of G+C mol% versus melting temperature (T_m) and a linear regression analysis was used to calculate the DNA G+C content (mol%) of the novel strains.

The DNA G+C content of strain MCy1366^T was found to be same as *Corallococcus coralloides* DSM 2259^T, i.e. 65.6 mol%, in our analysis. *C. exiguus* DSM 14696^T and *C. coralloides* DSM 2259^T are very closely related with 99.9 % 16S rRNA gene sequence similarity and sharing 61.5 % DNA–DNA relatedness (Stackebrandt *et al.*, 2007). However, these high values did not hold true for the pairs MCy1366^T and *C. exiguus* DSM 14696^T (28 %; with 97 % 16S rRNA gene sequence similarity) and MCy1366^T and *C. coralloides* DSM 2259^T (35.15 %; with 96.9 % 16S rRNA gene sequence similarity) clearly revealing that strain MCy1366^T does not belong to the species *C. exiguus* or *C. coralloides* (Wayne *et al.*, 1987). Strains MCy1366^T and MCy10622 showed 99.9 % 16S rRNA gene sequence similarity and 100 % DNA–DNA relatedness.

Biomass for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was collected from one-week-old, 100 ml cultures of MCy1366^T, MCy10622 and the reference strains in CY+H broth and centrifuged at 11000 r.p.m. (15286 g) for 10 min. Whole-cell protein extracts were analysed by using a Microflex L20 mass spectrometer (Bruker Daltonics) equipped with a N2 laser. Sample preparation for MALDI-TOF MS protein analysis was carried out according to the ethanol/formic acid extraction protocol recommended by Bruker Daltonics as described in detail by (Toth *et al.*, 2008). The MALDI-TOF mass spectra were analysed with the BioTyper software (version 3.1; Bruker Daltonics). The dendrogram generated on the basis of MALDI-TOF mass spectra (Fig. 3) confirms the close relationship of strains MCy1366^T and MCy10622 as revealed by DNA–DNA hybridization and demonstrates that both strains can be differentiated by MALDI-TOF MS from type strains of related species of the genera *Corallococcus*, *Myxococcus*, *Pyxidicoccus* and *Melittangium*.

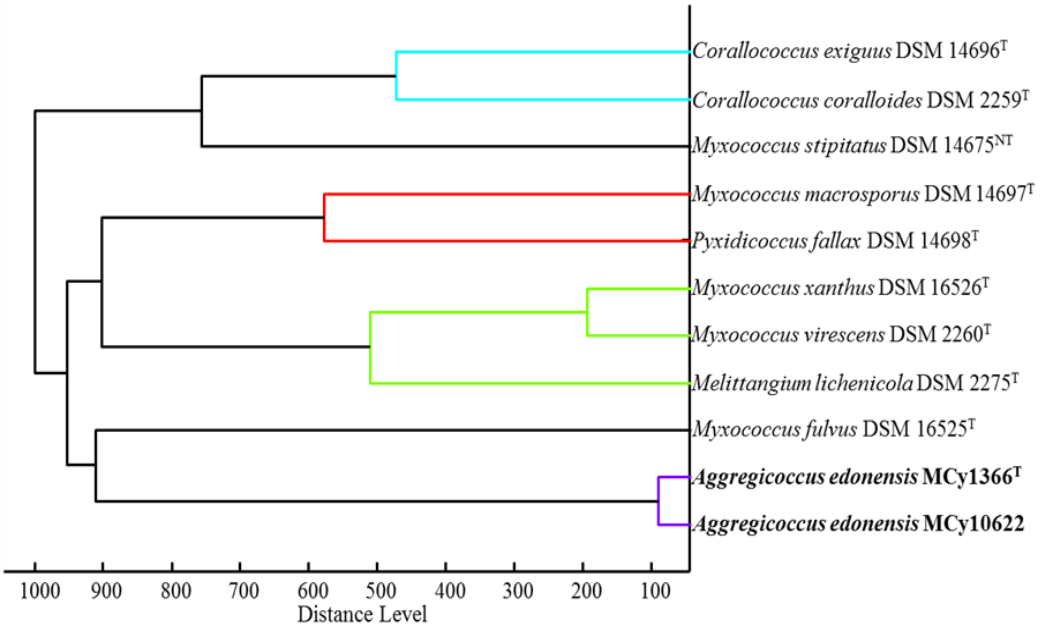

Fig.3. Score-oriented dendrogram generated by the BioTyper software (version 3.1, Bruker Daltonics) showing the similarity of MALDI-TOF mass spectra of cell extracts of strains MCy1366 species within the genera *Corallococcus*, *Myxococcus*, *Pyxidicoccus* and *Melittangium*.

In addition to the 3 % phylogenetic distance based on 16S rRNA gene sequences and low DNA–DNA relatedness to the closest known myxobacterial neighbours (*C. exiguus* and *C. coralloides*), strain MCy1366^{T} also delineates into a novel branch in the MALDI-TOF MS dendrogram and shows a distinct cellular fatty acid profile and biochemical characteristics compared to other members of the family *Myxococcaceae*. Therefore, we propose strain MCy1366^{T} represents a novel species of a new genus, *Aggregicoccus edonensis* gen. nov., sp. nov., based on these distinguishing characteristics.

2.4. Description of *Aggregicoccus* gen. nov.

Aggregicoccus gen. nov. [Ag.gre.gi.coc′cus. L. v. *aggrego* to aggregate, form clumps; N.L. masc. n. *coccus* (from Gr. masc. n. *kokkos* grain, seed, coccus); N.L. masc. n. *Aggregicoccus* an aggregating coccus, referring to massively aggregating cells in liquid culture].

Vegetative cells are rod-shaped with slight tapering ends (*Myxococcaceae* type). They form transparent swarms on VY/2 yeast agar and produce wavy, rippling structures with intricate veins on edges on CY agar. Colonies are very weakly positive in Congo red stain. Fruiting-body-like aggregates and fruiting bodies are spherical to irregularly shaped, cushion-like, yellow–orange and lack sporangioles and stalks. Myxospores are optically refractive and irregularly spherical. Myxospore-like structures are also produced in peptone-containing liquid media upon longer incubation. Does not utilize monosaccharides or disaccharides. Bacteria and yeast are lysed. Aerobic, chemo-organotrophic and strictly mesophilic. Hydrolyses starch, but not cellulose, chitin or agar. The DNA G+C content is 65.6 mol%. The phylogenetic position is in the family *Myxococcaceae*, suborder *Cystobacterineae*, order *Myxococcales*. The type species is *Aggregicoccus edonensis*.

2.5. Description of *Aggregicoccus edonensis* sp. nov.

Aggregicoccus edonensis (e.do.nen′sis. N.L. masc. adj. *edonensis* belonging to Edo, former name of Tokyo, where the soil sample was collected from which the type strain was isolated).

In addition to the characteristics of the genus, the vegetative cells are 0.72–0.82 µm×5.71–10.73 µm in size. The swarm colony varies from yellow–orange to almost transparent colour depending upon the medium. Myxospore-like structures, 1.3–1.7 µm in size and covered with outer capsule are obtained in liquid media. Cell aggregates are beige to saffron–orange with extreme branching in peptone or casitone media. Optimal growth temperature is between 30 and 37 °C. pH tolerance is between pH 6.0 and 10.0, and optimum at pH 6.0–7.0. Cannot tolerate high salt concentrations. Best nutritional sources are media with peptones. Can utilize L-asparagine monohydrate, ammonium sulfate, potassium nitrate, urea, L-glutamic acid, glycine, L-lysine monohydrate and L-arginine as nitrogen sources in the presence of casitone. Major fatty acids are $C_{17:0}$ 2-OH, iso -$C_{15:0}$ and iso -$C_{17:0.}$ Lipase, valine arylamidase and naphthol-AS-BI-phosphohydrolase production is positive while α-galactosidase, β-glucuronidase, α-glucosidase and cystine arylamidase show negative results in the API ZYM test system.

The type strain is MCy1366^T (=DSM 27872^T=NCCB 100468^T), isolated from soil collected in 1981 from a region near Tokyo, Japan. The DNA G+C content of the type strain is 65.6 mol%. An additional strain of the species is MCy10622.

2.6. References

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2.7. Supplementary information

Table S1. Type strains and neotype strains used as reference in this study

Strain name	**Strain no.**
***Corallococcus exiguus*^{T}**	DSM14696 (Cce167)
***Corallococcus coralloides*^{T}**	DSM2259(M2)
***Myxococcus virescens*^{T}**	DSM2260(M22)
***Myxococcus fulvus*^{T}**	DSM16525(M17)
***Myxococcus xanthus*^{T}**	DSM16526
***Myxococcus stipitatus*^{NT}**	DSM14675(MxS8)
***Myxococcus macrosporus*^{NT}**	DSM14697(Ccm8)
***Pyxidococcus fallax*^{T}**	DSM14698(Pyf1)
***Melittangium lichenicola*^{T}**	DSM2275(M155)

Table S2. Salt tolerance
Assessment of growth levels.-, no growth; +, poor growth; ++, moderate; +++, very good growth

Strain name	**Growth on CY agar with 1.5% NaCl**
MCy1366^{T}	-
***Corallococcus exiguus*^{T}**	-
***Corallococcus coralloides*^{T}**	-
***Myxococcus virescens*^{T}**	+++
***Myxococcus fulvus*^{T}**	-
***Myxococcus xanthus*^{T}**	++
***Myxococcus stipitatus*^{NT}**	+
***Myxococcus macrosporus*^{NT}**	+++
***Pyxidococcus fallax*^{T}**	+
***Melittangium lichenicola*^{T}**	-

Table S3. Differential physiological reactions in API ZYM tests

Enzyme	*M. stipitatus* DSM 14675^{NT}	*M. virescens* DSM 2260^{T}	*M. xanthus* DSM 16526^{T}	*M. fulvus* DSM 16525^{T}	*M. macrosporus* DSM 14697^{T}	**MCy1366^{T}**	**MCy10622**	*C. coralloides* DSM 2259^{T}	*C. exiguus* DSM 14696^{T}	*P. fallax* DSM 14698^{T}	*M. lichenicola* DSM 2275^{T}
Control	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
Esterase(C4)	+	+	+	+	+	**V**	+	-	+	+	+
Lipase(C14)	-	+	+	-	+	+	+	+	+	+	+
Valine arylamidase	+	+	+	+	+	+	+	+	-	-	+
Cystine arylamidase	+	+	+	+	-	-	+	+	-	-	+
Trypsin	+	-	+	-	+	-	-	-	-	+	-
***α*-chymotrypsin**	+	-	+	-	-	**V**	+	+	-	-	-
Naphthol- AS-BI-phosphohydrolase	-	+	+	+	+	+	+	+	+	+	+
***α*-galactosidase**	-	-	-	+	-	-	-	-	-	-	-
***ß*-glucuronidase**	-	-	-	+	-	-	-	-	-	-	-
***α*-glucosidase**	-	-	-	+	-	-	-	-	-	-	+
***ß*-glucosidase**	-	-	-	+	-	-	-	-	-	-	-
N-acetyl-*ß*-glucosaminidase	+	-	-	+	-	-	-	-	-	-	+

Reactions on API ZYM strips: +, positive; -, negative; V, variable. All the tested strains were positive for alkaline phosphatase, esterase lipase, leucine arylamidase, and acid phosphatase and negative for *ß*-galactosidase, *α*-mannosidase, and *α*-fucosidase.

Table S4. Antibiotic resistance test for MCy1366^{T} and MCy10622 along with related type and neotype strains

Antibiotic	*M. stipitatus* DSM 14675^{NT}	*M. virescens* DSM 2260^{T}	*M. xanthus* DSM 16526^{T}	*M. fulvus* DSM 16525^{T}	*M. macrosporus* DSM 14697^{T}	**MCy1366^{T}**	**MCy10622**	*C. coralloides* DSM 2259^{T}	*C. exiguous* DSM 14696^{T}	*P. fallax* DSM 14698^{T}	*M. lichenicola* DSM 2275^{T}
Control VY/2 agar	+	+	+	+	+	+	+	+	+	+	+
Ampicillin	+	-	+	-	+	-	+	+	+	+	-
Kanamycin	+	-	-	+	-	+	+	+	+	-	+
Spectinomycin	+	+	+	+	+	-	-	-	-	+	+
Trimethoprim	+	-	-	-	+	+	+	+	+	+	+
Bacitracin	+	+	+	+	+	+	+	+	+	+	-
Oxytetracyclin	-	-	-	-	-	-	**n.c.**	-	-	-	-
Fusidic acid	+	+	+	-	-	-	+	+	+	+	-
Thiostreptone	+	-	-	-	-	-	-	-	-	+	-
Chloramphenicol	-	+	-	-	-	-	**n.c.**	-	-	+	-

+, Growth; -, Inhibition of growth; n.c., not clear. All the tested strains were resistant to cephalosporin C, gentamycin, hygromycin B and polymyxin.

Table S5. Dry weight biomass (mg) of MCy1366^T and MCy10622 obtained with supplementation of following carbon sources in synthetic S medium. Casitone was used as a peptone source.

Carbon sources	**Average dry biomasses (MCy1366^T)**	**Average dry biomasses (MCy10622)**
1. Sucrose	16.05	14.25
2. Fructose	15.15	16.0
3. Maltose	14.15	16.7
4. Potato starch	41.05	43.85
5. Molasses	29.95	29.85
6. Xylose	15.05	14.4
7. Soluble starch	29.6	31.35
8. Cellobiose	16.1	15.2
9. Lactose	16.35	17.25
10. Mannose	14.35	15.35
11. Galactose	20.5	14.75
12. Glucose	15.45	15.0
13. Pyruvate	20.2	25.35
14. Control	15	14.25

Table S6. Dry weight biomass (mg) of MCy1366^T and MCy10622 in minimal casitone medium supplemented with various nitrogen sources, amino acids or peptone sources.

Nitrogen sources	**Average dry biomasses (MCy1366^T)**	**Average dry biomasses (MCy10622)**
1. L-asparagine monohydrate	16.7	24.55
2. Urea	15.6	15.55
3. L-arginine	13.25	19
4. KNO_3	13.25	12.5
5. $(NH_4)_2SO_4$	11.5	14.85
6. L-lysine monohydrate	14.8	14.5
7. Glycine	12.7	12.45
8. L-glutamic acid	15.3	24.35
9. Probion FM 582	61.1	60.65
10. Tryptone enzymatic digest of casein	7.9	20.35
11. Casitone	19.75	25.75
12. Soytone	23.4	13.9
13. Yeast extract	13.4	21.15
14. Tryptone	18.95	33.05
15. Neopeptone	9.1	4.15
16. Phytone peptone	10.75	3.1
17. Skimmed milk	16.3	7.3
18. Gluten from wheat	32.85	39.6
19. Corn Steep solid	28.65	83.45
20. Meat extract	15.25	14.7
21. Casamino acid	1.5	1.4
22. Control	1.3	1.25

Table S7. Fatty acid analysis of MCy1366^T, MCy10622 and closely related type strains. Values are represented as percentages of the total fatty acid content.

	C. exiguus **DSM 14696^T**	***C. coralloides*** **DSM 2259^T**	**MCy1366^T**	**MCy10622**	***M. xanthus*** **DSM 16526^T**	***M. macrosporus*** **DSM 14697^T**	***M. fulvus*** **DSM 16525^T**	***M. virescens*** **DSM 2260^T**	***M. stipitatus*** **DSM 14675^{NT}**	***P. fallax*** **DSM 14698^T**	***M. lichenicola*** **DSM 2275^T**
SCFAs											
^{C}10:0	-	-	-	-	0.2	0.3	-	0.3	0.2	0.1	-
^{C}11:0	0.2	-	-	0.1	-	-	-	0.1	-	-	-
^{C}12:0	0.0	-	-	-	0.1	0.1	-	-	-	-	-
^{C}13:0	0.1	-	-	-	-	0.2	-	0.1	0.1	-	-
^{C}14:0	-	-	0.1	-	5.4	6.4	0.2	3.5	2.2	0.8	-
^{C}15:0	0.8	0.2	1.8	1.6	2.7	2.5	-	2.5	2.7	1.1	-
^{C}16:0	1.6	1.5	2.4	0.5	4.1	4.3	8.2	4.1	4.1	6.1	9.8
^{C}17:0	0.2	-	0.5	0.4	-	-	-	0.1	-	0.3	-
^{C}18:0	3.7	3.7	4.9	0.5	5.8	4.6	14.1	1.5	5.8	4.1	20.6
$C_{14:1}$ isomer 1	-	-	-	-	1.8	0.5	-	0.5	-	-	-
$C_{14:1}$ isomer 2	-	-	-	-	0.1	0.1	-	0.2	0.2	0.7	-
$C_{14:1}$ isomer 3	-	-	-	-	0.3	0.1	-	0.1	-	-	-
$C_{15:1}$ isomer 1	0.3	0.3	-	4.3	1.9	1.1	-	1.0	0.1	-	-
$C_{15:1}$ isomer 2	0.4	0.1	3.3	-	2.5	1.7	-	1.9	0.5	0.9	-
$C_{16:1}$ isomer 1	-	-	-	1.6	0.5	0.8	8.6	0.6	0.4	-	3.3
$C_{16:1}$ isomer 2	0.6	0.2	2.0	-	7.9	12.6	6.1	13.6	14.5	21.0	5.5
$C_{17:1}$ isomer 1	-	-	-	0.7	-	-	-	-	-	-	-
$^C{}_{17:1\omega7cis}$	1.6	2.2	0.4	-	-	-	-	1.0	-	0.2	-
$C_{18:1}$	0.1	-	-	0.1	-	-	-	-	0.2	-	-

PUFAs											
$C_{16:2}$	-	-	-	-	3.4	3.5	-	3.0	0.6	-	-
$C_{18:3w6,cis9,12}$	-	-	-	0.2	-	-	-	-	-	-	-
Hydroxy FAs											
$C_{13:0}$ 3-OH	-	-	-	-	-	-	-	-	-	-	-
$C_{14:0}$ 3-OH	-	-	-	-	0.4	0.7	-	0.5	0.6	0.6	0.1
$C_{15:0}$ 3-OH	-	-	-	-	0.1	0.1	0.3	0.1	0.1	-	-
$C_{16:0}$ 2-OH	-	-	-	-	0.2	1.1	-	0.2	0.7	1.2	-
$C_{16:0}$ 3-OH	-	-	-	-	0.4	0.6	0.5	0.3	0.6	0.4	0.2
$C_{16:1}$ 3-OH	-	-	-	-	-	-	-	-	-	0.2	-
$C_{24:0}$ 2-OH	-	-	-	-	-	-	-	-	-	-	-
Total SCFAs	**9.6**	**8.2**	**15.4**	**10.0**	**37.8**	**41.3**	**38.0**	**35.2**	**33.6**	**37.7**	**39.5**
BCFAs											
iso-$C_{13:0}$	1.9	0.8	0.2	1.5	0.7	0.4	-	0.5	0.2	0.1	-
iso-$C_{14:0}$	0.7	0.3	-	0.4	-	-	-		0.1	-	-
iso-$C_{15:0}$	17.6	22.3	**23.2**	**25.3**	39.8	34.5	13.5	30.7	16.3	14.0	13.0
iso-$C_{15:1}$	-	-	-	1.0	-	-	-	-	-	-	-
$^{iso\text{-}C}15{:}1\omega9cis$	-	1.1	-		0.8	-	-	0.4	-	-	-
iso-$C_{16:0}$	1.9	1.4	2.6	3.3	0.1	0.8	0.2	0.4	1.1	0.9	0.1
iso-$C_{16:1}$	-	1.1	-	0.4	-	0.3	-	0.1	-	-	-
iso-$C_{17:0}$	10.1	4.3	**16.6**	**10.4**	3.3	3.2	1.4	8.1	11.8	7.7	0.8
iso-$C_{17:1}$	-	-	-	**10.9**	-	-	-	-	-	-	-
$^{iso\text{-}C}17{:}1\omega5cis$	12.8	8.2	9.5	-	1.9	1.2	7.3	2.9	2.5	1.9	6.1
$^{iso\text{-}C}17{:}1\omega11cis$	-	2.0	0.2	-	0.7	0.5	-	-	0.3	-	-
iso-$C_{17:2}$	-	-	-	2.3	-	-	-	-	-	-	-
$^{iso\text{-}C}17{:}2\omega5cis,cis11$	1.0	3.7	-	-	2.4	0.9	-	1.4	0.1	-	-

anteiso-$C_{15:0}$	-	-	0.3	0.3	-	-	6.0	0.2	-	-	0.8
anteiso-$C_{17:0}$	0.1	-	0.4	-	-	-	-	0.1	-	-	-
Branched-chain hydroxy FAs											
$^{iso\text{-}C}15:0\ ^{3\text{-}OH}$	2.8	1.6	1.2	0.7	3.1	3.3	4.4	2.9	3.5	1.9	2.7
$^{iso\text{-}C}16:0\ ^{2\text{-}OH}$	0.1	0.2	0.1	-	-	-	-	-	-	-	-
$^{iso\text{-}C}16:0\ ^{3\text{-}OH}$	-	-	-	-	-	-	-	-	-	-	-
$^{iso\text{-}C}17:0\ ^{2\text{-}OH}$	34.4	33.0	**26.1**	**14.1**	4.2	7.1	0.2	3.3	12.4	9.3	0.5
$^{iso\text{-}C}17:0\ ^{3\text{-}OH}$	0.3	-	0.1	-	1.2	1.3	3.7	1.2	1.1	0.4	3.2
$^{iso\text{-}C}17:1\ ^{2\text{-}OH}$	0.0	-	-	-	-	-	-	-	-	-	-
Branched chain OAG FAs											
$^{C}14:0$	-	-	-	-	-	-	-	0.1	0.3	0.4	-
$^{C}15:0$	-	-	-	-	-	-	-	0.1	-	2.9	-
$^{C}16:0$	-	-	-	-	-	-	-	0.1	0.4	7.4	-
iso-$C_{15:0}$	3.4	7.2	1.9	1.3	3.0	3.9	-	10.3	10.1	10.8	-
Branched chain DMA FAs											
iso-$C_{15:0}$	1.6	4.2	2.0	4.4	1.0	0.7	-	1.7	2.1	4.7	-
Total BCFAs	**88.7**	**91.4**	**84.4**	**76.3**	**62.2**	**58.1**	**36.7**	**64.5**	**62.3**	**62.4**	**27.2**
Fatty alcohols											
iso Pentadecanol	0.1	0.3	0.0	-	-	0.0	0.8	0.0	0.2	0.0	0.9
Hexadecanol	-	-	-	-	-	0.0	0.0	-	-	-	-
Unspecified fatty alcohol	-	-	-	-	-	-	24.4	-	-	-	32.1

(Abbreviations: SCFAs, Short chain fatty acids; PUFAs, Polyunsaturated fatty acids; BCFAs, Branched chain fatty acids; OAG FAs, *O*-alkylglycerol fatty acids; DMA FAs, Dimethylacetal fatty acids) *Percentages of major fatty acids in MCy1366^{T} and MCy10622 are distinguished in boldface type.

Table S8. Dry weight biomass (mg) of MCy1366^T and MCy10622 at different pH

pH	Average dry biomasses (MCy1366^T)	Average dry biomasses (MCy10622)
1. 5.0	0.10	0.25
2. 5.5	0.15	0.20
3. 6.0	34.30	27.50
4. 6.5	50.15	52.00
5. 7.0	41.40	38.05
6. 7.5	24.55	25.15
7. 8.0	12.35	21.30
8. 8.5	12.40	15.15
9. 9.0	11.55	14.50
10. 9.5	9.55	14.35
11. 10.0	9.25	14.25

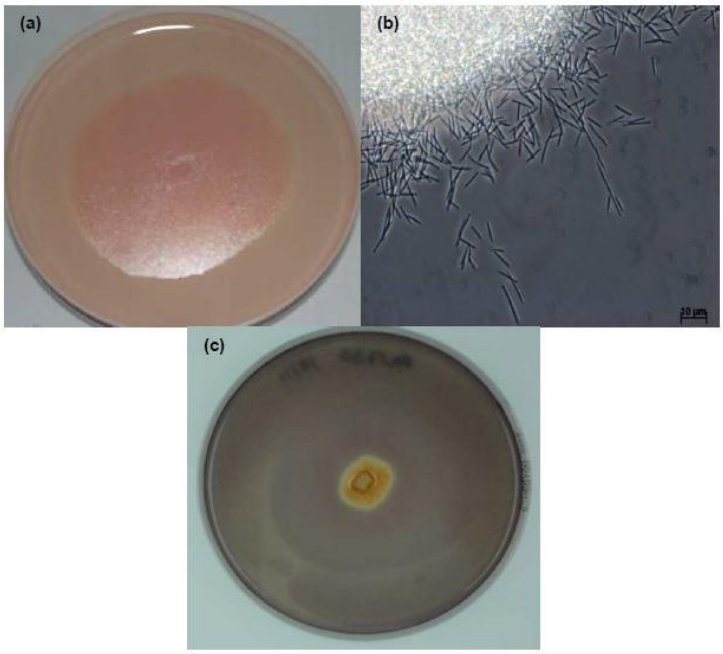

Fig. S1. (a) Congo red staining of 3 week old swarm of strain MCy1366^T on VY/2 agar displaying a weak positive reaction, (b) Phase contrast microscopic image of vegetative cells of MCy1366^T in Myxo-media (Bar, 10 µm), (c) Lugol's solution staining of 2 week old swarm of strain MCy1366^T on P agar displaying a weak positive reaction.

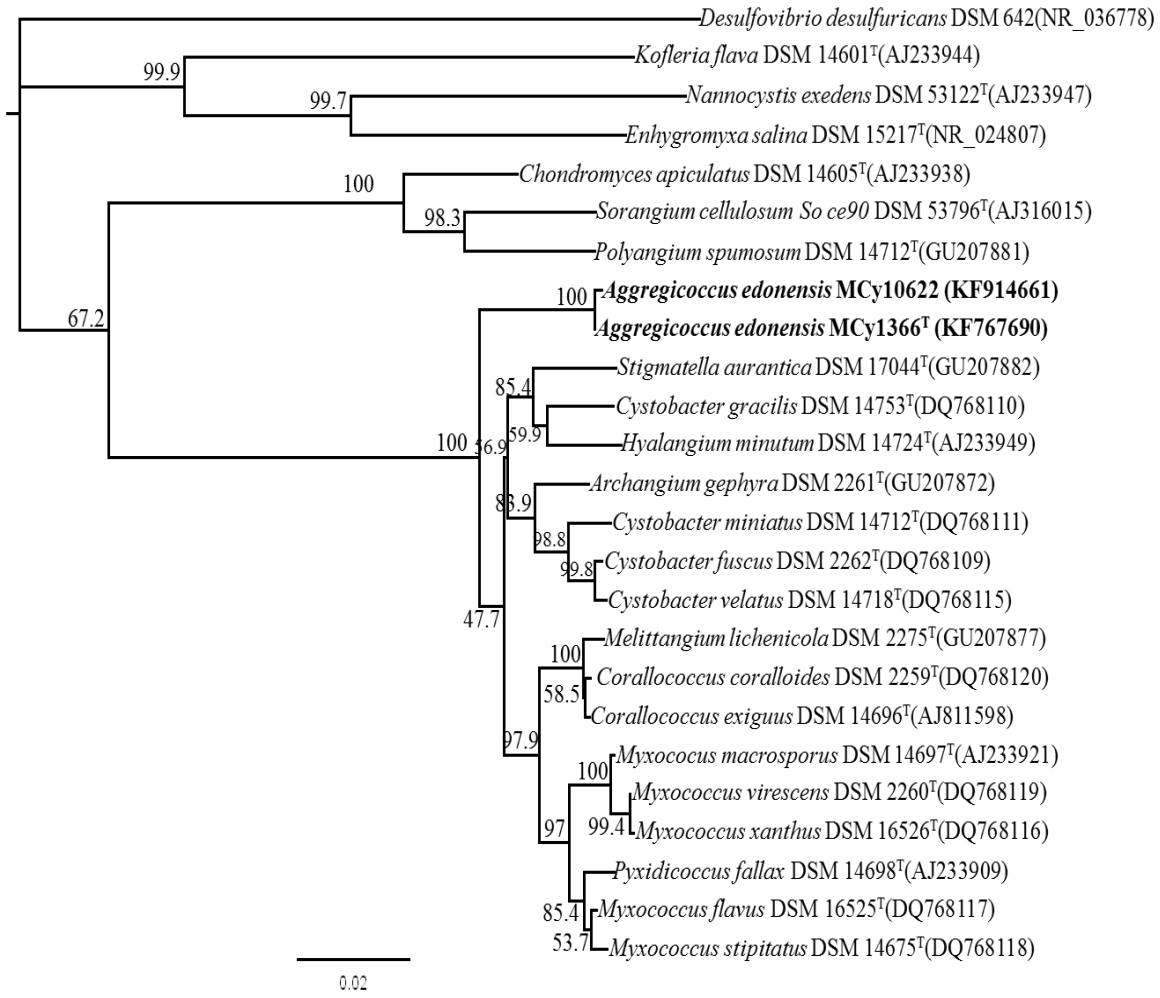

Fig.S2. Neighbor-joining tree showing the phylogenetic position of MCy1366^T and MCy10622 based on 16S rRNA gene sequence. The numbers at branch points indicate bootstrap support based on 1000 resamplings. Bar, 0.02 substitutions per nucleotide position. The GenBank accession number for each strain is shown in parentheses. *Desulfovibrio desulfuricans* DSM 642^T was used as an out group.

Chapter 3

Racemicystis crocea gen. nov., sp. nov., a novel soil myxobacterium in the family *Polyangiaceae*

Ram Prasad Awal, [1, 2] Ronald Garcia, [1, 2, 3] and Rolf Müller[1, 2, 3 *]

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Affiliations

[1]Department of Pharmaceutical Biotechnology, Saarland University, Building E8 1, 66123 Saarbrücken, Germany

[2]Department of Microbial Natural Products (MINS), Helmholtz Institute for Pharmaceutical Research Saarland (HIPS) – Helmholtz Centre for Infection Research (HZI), Saarland University Campus Building E 8 1, 66123 Saarbrücken, Germany

[3]German Center for Infection Research (DZIF), Partner site Hannover-Braunschweig, 38124 Braunschweig, Germany

Author’s efforts

The author isolated the novel strain and performed all of the experiments described and interpreted the obtained result. The author conceived and wrote the paper.

Contributions by others

The project was designed and supervised by Ronald Garcia and Rolf Müller. Co-authors contributed in conceiving and writing of the manuscript.

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Chapter: 3 *Racemicystis crocea* gen. nov., sp. nov., a novel soil myxobacterium in the family *Polyangiaceae*

3.1. Abstract

A novel bacterial strain designated MSr9521^T was isolated in 2014 from a soil sample collected in 1986 from the Philippines. The novel bacterium shows myxobacterial characteristics that include pseudoplasmodial swarming, fruiting body formation and predatory ability to lyse microorganisms. The strain is chemoheterotrophic, mesophilic and aerobic. Major fatty acids are $C_{18:1}$, $C_{17:1}$ 2-OH and iso-$C_{15:0}$, and also contains trace amounts of omega-3/-6 polyunsaturated fatty acids. The G+C content of the genomic DNA is 70.4 mol%. The 16S rRNA gene sequence shows 95–96 % closest similarity to *Sorangium cellulosum* DSM 14627^T, *Polyangium fumosum* Pl fu5^T, *Jahnella thaxteri* Pl t4^T and *Byssovorax cruenta* By c2^T. The molecular phylogenetic analysis shows that the novel isolate forms a novel branch in the family *Polyangiaceae*, suborder *Sorangiineae*. Polyphasic taxonomic characterization suggests that the strain MSr9521^T represents a novel species of a new genus in the family *Polyangiaceae*, for which the name *Racemicystis crocea* gen. nov., sp. nov. is proposed. The type strain of *Racemicystis crocea* is MSr9521^T (=DSM 100773^T=NCCB 100574^T).

3.2. Introduction

Myxobacteria belong to a monophyletic order (*Myxococcales*) in the *Deltaproteobacteria* based on 16S rRNA gene sequences (Garcia *et al.*, 2010). They are commonly isolated from a wide range of environmental sources around the globe and appear diversified in the tropic and temperate soils containing decaying plant materials (Dawid, 2000; Reichenbach, 2005; Garcia & Müller, 2014).Myxobacteria are a unique group of prokaryotes known for their cellulolytic and predatory lifestyle to prey, lyse and out-compete other microorganisms in their environment. They show sophisticated multicellular behaviour by gliding on a solid surface and by formation of complex fruiting bodies filled with environmentally resistant myxospores (Higgs *et al.*, 2014). In the last few decades, and until now, they were considered as outstanding sources of novel secondary metabolites with many potential therapeutic applications (Wenzel & Müller, 2009; Weissman & Müller, 2010; Garcia & Müller, 2014; Plaza & Müller, 2014). We

have isolated a novel strain, MSr9521^T, which is proposed here to represent a novel species of a new genus in the family *Polyangiaceae*.

3.3. Materials and Methods

Strain MSr9521^T was isolated using a mineral salt ST21 agar, overlaid with sterile filter papers (2 × 1cm), containing levamisole (25 µg ml^{-1}) and cycloheximide (25 µg ml^{-1}) to inhibit eukaryotic contaminants (Reichenbach & Dworkin, 1992). Pure culture was obtained by repeated transfers of the swarm edge onto a fresh ST21 agar without a filter paper. The strain was routinely cultivated on buffered VY/2 agar, and cryopreserved by slow freezing using cold isopropanol (Garcia *et al.*, 2014). Phenotypic growth stages were studied, characterised and documented after cultivation of the strain on buffered VY/2 agar (Garcia *et al.*). Swarming colonies and fruiting bodies on agar plates were studied and photographed using a stereomicroscope (Zeiss Discovery-V20) while the vegetative cells and myxospores were observed by phase-contrast microscopy (Zeiss Axio-Star) and photographed using an Axiocam MRC (Zeiss) camera which further analysed by using the AxioVision LE software. Fruiting bodies and vegetative cells of the strain were dried in a desiccator for five months at room temperature to test the tolerance to dry condition.

Gram-staining of vegetative cells was done according to the established protocol (Gerhardt *et al.*, 1981) while Congo red staining was performed according to (McCurdy, 1969, 1969). The test for catalase, oxidase, and enzymatic activities using the API ZYM kit (bioMérieux) was described previously (Garcia *et al.*, 2014). Growth response to oxygen was tested by stab-inoculation in a test-tube containing 10 ml buffered VY/2 agar, while the anaerobic tolerance was performed by incubating the novel strain in an anaerobic jar on buffered VY/2 and R agar (w/v: 0.2 % casitone (BD), 0.2 % soluble starch (Roth), 0.5 % skimmed milk (Oxoid), 0.005 % KNO_3, 0.1 % $MgSO_4.7H_20$, 0.05 % $CaCl_2.2H_20$, 0.0008 % Fe-EDTA, 25 mM HEPES, 1.5 % Bacto agar; adjusted to pH 7.2 with KOH before autoclaving). The pH tolerance was tested at pH 4.0–10.0 with an interval of 0.5 pH unit (adjusted before autoclaving) using 10 mM buffers (MES, pH 4.5–6.5; HEPES, pH 7.0–8.0; Trizma, pH 8.5–9.0; CHES, pH 9.5–10). The pH range was narrowed from pH 7.0–pH 8.0 at intervals of 0.1 pH unit to determine the optimum pH, as indicated by the swarm diameter.

The temperature tolerance was performed by incubation at 4 °C, 18 °C, room temperature (22-23 °C), 30 °C and 37 °C, and was later narrowed from 15–20 °C and 35–40 °C to determine their limits. The antibiotic resistance was described previously (Garcia *et al.*, 2014) but with the exclusion of thiostrepton, rifampicin, trimethoprim, and with the addition of tobramycin (Sigma-Aldrich). NaCl tolerance was assessed in at 0–2 % (w/v), at intervals of 0.2 % (w/v). All these were tested in buffered VY/2 agar.

Predation using live microorganisms (*Escherichia coli, Pseudomonas putida, Bacillus subtilis, Micrococcus luteus, Saccharomyces cerevisiae, Yarrowia lypolitica*) and cellulose degradation were according to the described methods (Garcia *et al.*, 2014) and additionally tested the latter on ST21 agar with filter paper (2 x 1 cm) overlay. The chitin degradation was assessed on CT-6, CT-7 agars (Reichenbach, 2006), and in water agar supplemented with 1 % (w/v) chitin (Sigma). Agar degradation was tested by the cultivation of the strain in buffered VY/2 agar and all solid medium containing agar (BD).

Hydrolysis of casein (w/v: 0.2 % bovine milk casein, Sigma), Tween 80 (w/v: 0.1 %, Sigma), and soluble starch (w/v: 0.2 %, Roth) were all assessed by supplementation on buffered VY/2 agar medium. Hydrolysis of xylan (Roth) and esculin (Sigma) were according to the previously described methods (Lang *et al.*, 2008), while gelatin hydrolysis was performed on modified water agar (w/v: 0.2 % $MgSO_4.7H_2O$, 0.1 % $CaCl_2.2H_2O$, 25 mM HEPES, 0.5 % Bacto agar (BD), adjusted pH to 7.0 before autoclaving) supplemented with 1 % (w/v) gelatin (BD), and was assessed for the clearing zone around the colonies. Except for the temperature tolerance, all tests were incubated for a week at 30 °C.

Carbohydrate, peptone and nitrogen sources were tested based on the previously described methods (Garcia *et al.*, 2014). Filter-sterilised nutrient sources were added individually in 300 ml flask containing 100 ml minimal medium. Potato starch (Sigma) was used as a carbon source for the nitrogen sources test. Cultures were incubated for a week on a rotary shaker at 160 r.p.m. at 30 °C.

Cellular fatty acid extraction was done by fatty acid methyl ester (FAME) method (Garcia *et al.*, 2011) after cultivation of the strain in 50 ml R medium (w/v: 0.2 % Bacto casitone, 0.2 % soluble starch (Roth), 0.5 % skimmed milk (Oxoid), 0.005 % KNO_3, 0.1 %

$MgSO_4.7H_20$, 0.05 % $CaCl_2.2H_20$, 0.0008 % Fe-EDTA, 25 mM HEPES; adjusted to pH 7.2 with KOH before autoclaving) at 30 °C, 180 r.p.m., 5 days. GC-MS analysis and identification of fatty acids including the polyunsaturated types were performed according to the described methods (Garcia *et al.*, 2011; Gemperlein *et al.*, 2014). The DNA mol % G + C content of the novel bacterium was determined by standard HPLC-MS, performed after nuclease P1 digestion of the genomic DNA (Li *et al.*, 2003; Shimelis & Giese, 2006).

Genomic DNA was extracted from an actively growing culture taken from R medium by following the standard protocol for Gram-negative bacteria using the commercial Qiagen Puregene Core Kit A. The 16S rRNA gene was amplified and sequenced using the universal forward 5´-GAGTTTGATCCTGGCTCAGGA-3´ and reverse 5´-AAGGAGGTGATCCAGCCGCA -3´ primers (Lachnik *et al.*, 2002), and further verified with universal primer sets 27F and 1492R (Lane, 1991). Purification of the 16S rRNA gene was performed by using a NucleoSpin PCR clean-up kit (Macherey Nagel) while the sequence alignment was determined using the Cap contig assembly function of the BioEdit Sequence Alignment Editor version 7.1.3.0 software (Hall, 1999). The 16S rRNA gene sequence of the novel isolate was compared with those sequences available in the GenBank/EMBL/DDBJ databases using BLASTN version 2.2.29+ (Zhang *et al.*, 2000; Morgulis *et al.*, 2008). For phylogenetic analysis, the 16S rRNA gene sequences of myxobacteria, of mostly representing the type strains in the three myxobacterial suborders were retrieved from GenBank. Sequence alignments were performed using the MUSCLE software (Edgar, 2004) while the evolutionary distances between sequences were calculated using the Jukes-Cantor substitution model (Jukes & Cantor, 1969). The phylogenetic tree was then constructed using the neighbour-joining (Saitou & Nei, 1987), PHYML (Guindon & Gascuel, 2003) and UPGMA (Sneath & Sokal, 1973) methods applying 1000 bootstrap resampling (Felsenstein, 1985). The sulphate-reducing bacterium *Desulfovibrio desulfuricans* DSM 642^T (GenBank Accession: NR_036778) was chosen as an outgroup to root the tree. All these programs are packed in the Geneious 8.1.2 software (Drummond *et al.*, 2010).

3.4. Result and Discussion

The swarm colony of the novel isolate appears saffron, orange, to golden colour with typical fan-like pseudoplasmodia in buffered yeast agar (Fig. 1a). This pseudoplasmodial swarm pattern reminisce *Polyangium* strains (Garcia & Müller, 2014) and *Byssovorax cruenta* By c2^T but somehow differs in shape, colour and size. The wrinkled parchment-like slime trails behind the bloody-red pseudoplasmodia and the knob-shaped structures produced by *B. cruenta* were not observed in the novel isolate (Reichenbach *et al.*, 2006). The pseudoplasmodial swarms of the novel isolate often merged to form a band-like shape colony edge that partially clear the yeast cells (Fig. 1b). Furthermore, the swarm colony may also appear as slimy and tenacious veins scattered on the agar surface which also reminds some similarity with *Polyangium* strains and many members of the *Cystobacterineae*. Vegetative cells were phase-dark, and typically long and slender rods with blunted ends (1.2–1.5 x 3.0–12.0 µm) common for most *Polyangiaceae* (Fig. 1c). Most of the vegetative cell size was a bit longer compared to the majority of the *Polyangiaceae* (Garcia & Müller, 2014). The novel bacterium commonly formed fruiting bodies that often arranged in sori (140–450 µm) or as mats, consisting of large orange to golden sporangioles (Fig. 1d-f). The sporangioles measure 20–95 µm and commonly appear in spherical, ovoid and cylindrical with sometimes indentations or partial segmentations (Fig. 1d–e). In an older culture, the sporangioles appear peculiarly empty looking and similar to *Hyalangium minutum* in suborder *Cystobacterineae* and some species of *Polyangium* in the suborder *Sorangiineae* (Fig. 1e) (Reichenbach, 2005). The cells within the sporangiole were tightly packed and those released from crushed sporangioles (Fig. 1g) were slightly optically refractile and slender rods (Fig. 1h) measuring 0.8–1.0 x 2.0–5.0 µm, and were desiccation resistant.

The novel bacterium was Gram- and Congo-red negative, catalase- and oxidase-positive. Based on the API ZYM enzymatic test, positive results were obtained in alkaline phosphatase, C4 esterase, C8 esterase lipase, C14 lipase, leucine arylamidase, valine arylamidase, *α*-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *β*-galactosidase and *β*-glucosidase while cysteine arylamidase, trypsin, *α*-galactosidase, *β*-glucuronidase, *α*-glucosidase, N-acetyl-*β*-glucosaminidase, *α*-mannosidase and *α*-fucosidase exhibit negative reactions.

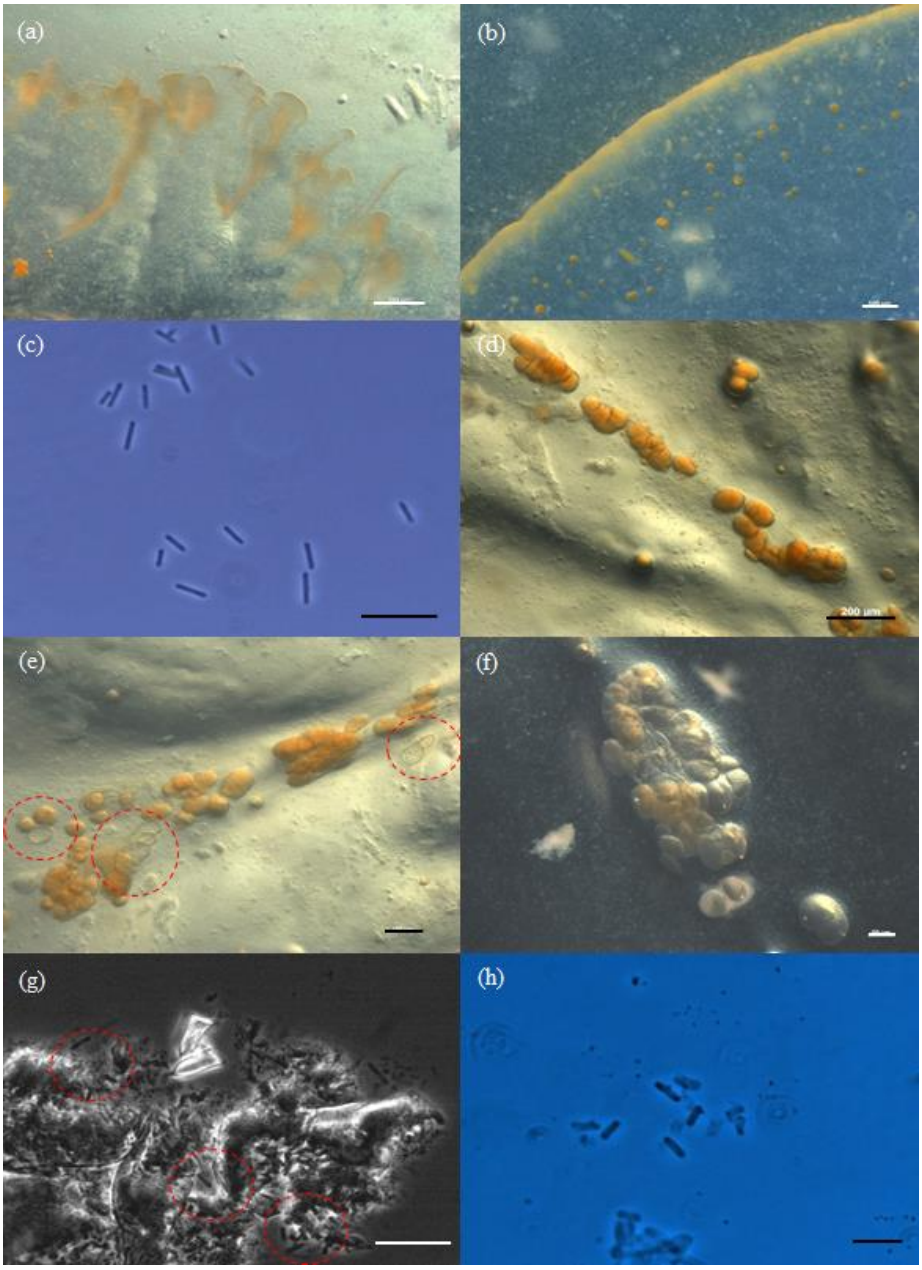

Fig. 1. Photographs of growth stages of MSr9521^{T}, obtained by stereomicroscopy (a, b, d–f) and phase-contrast microscopy (c, g, h). (a) fan-shaped pseudoplasmodial swarms. (b) Coherent swarm with high cell density at colony edges. (c) Slide mount of actively growing vegetative cells taken from the colony edge on buffered VY2 agar. (d) Fruiting bodies arranged in clusters and chains. (e) Fruiting bodies in the agar showing some empty sporangioles (encircled by dotted lines) which reminiscent of *Hyalangium minutum* and some species of *Polyangium*. (f) Sporangioles packed in sori. (g) Myxospores released from pressed sporangioles (encircled by dotted lines). (h) Slightly optically refractile myxospores. Bars, 500 µm (a), 500 µm (b), 20 µm (c), 200 µm (d), 100 µm (e), 50 µm (f), 20 µm (g) and 10 µm (h).

The novel isolate did not grow in an anaerobic jar and therefore was characterised to be aerobic. Strain MSr9521^T exhibited tolerance at pH 5.5–8.5 and with an optimum growth at pH 7.0–7.5. Temperature growth range was at 18-37 °C, and with an optimum at 30 °C.

Strain MSr9521^T was resistant to quite a few antibiotics including polymyxin, bacitracin, hygromycin and tobramycin. The resistance to the latter two antibiotics was shared with members of the *Polyangiaceae* (Garcia & Müller, 2014). Sensitivity was determined with ampicillin, apramycin, carbenicillin, cephalosporin, gentamycin, kanamycin, spectinomycin, fusidic acid, kasugamycin, tetracycline, neomycin, streptomycin and oxytetracycline. The sensitivity of the novel isolate to oxytetracycline was similar with members of the family *Polyangiaceae*, except for *Byssovorax cruenta* By c2^T (Garcia & Müller, 2014).

Sodium chloride is inhibitory at greater than 1 % (w/v). The novel isolate completely lysed Gram-negative bacteria including *E. coli* and *P. putida* whereas incomplete lysis was only observed in *S. cerevisiae* and *Y. lypolitica*, suggesting selectivity towards the microbial bait. Filter paper and chitin were not degraded, indicating a lack of cellulolytic and chitinolytic activities. Agar was depressed and partly corroded suggesting slight agarolytic activity. The novel strain hydrolysed casein, Tween 80, starch, xylan, esculin and gelatin. The hydrolysis of starch and xylan appears common among members of *Polyangiaceae* (Garcia & Müller, 2014).

All carbohydrates supported the growth of the novel myxobacterium, with the best growth exhibited on soluble starch, and lactose showing the least. In peptone supplementations, optimal growth was observed with casitone and soytone while moderate growth was determined with neopeptone, proteose peptone, phytone, tryptone and polypeptone. Casamino acids and inorganic nitrogen sources including ammonium sulphate, potassium nitrate and urea did not induce growth.

The major cellular fatty acids of the novel myxobacterium with nearly 10 % or more of the total were $C_{17:1}$ 2-OH (11.7 %), $C_{18:1}$ (11.8 %) and iso-$C_{15:0}$ (9.1 %). Trace amounts of omega-3/-6 polyunsaturated fatty acids (PUFAs) were identified as $C_{20:4}\omega$3, 6, 9, 12 all cis (eicosatetraenoic acid, ETA), $C_{20:5}\omega$3, 6, 9, 12, 15 all cis (eicosapentaenoic acid, EPA), $C_{20:4}\omega$6, 9, 12, 15 all cis (arachidonic acid, AA), $C_{18:3}\omega$3, 6, 9 all cis (α-linolenic acid,

ALA). The occurrence of these polyunsaturated fatty acids appears common in many myxobacteria (Garcia *et al.*, 2011) although the types and the amounts vary for some members. The anteiso-branched fatty acids which had been previously described as a chemotaxonomic marker for marine myxobacteria (Fudou *et al.*, 2002; Iizuka *et al.*, 2003b; Iizuka *et al.*, 2003a), and recently reported in terrestrial strains (Garcia *et al.*, 2011; Garcia & Müller, 2014), were also found in the novel isolate. Strain MSr9521^T differs with *Byssovorax cruenta* By c2^T, in the much lower amount of straight-chain $C_{16:1}$ fatty acid (Table 1). The novel isolate was remarkable for the elevated amount of iso-$C_{17:1}$ 2-OH in *Polyangiaceae*. Moreover, the predominance of the straight chain fatty acids over the branched-chain fatty acids and the presence of a significant amount of $C_{17:1}$ 2-OH (determined biomarker for *Sorangiineae*), supports the clustering of the novel bacterium into the suborder *Sorangiineae* (Garcia *et al.*, 2011).

Table 1. Cellular fatty acid profile of MSr9521^T after cultivation in R medium in comparison with *B. cruenta* By c2^T grown in VY/2 supplemented with maltose (Garcia *et al.*, 2011).

Fatty acids representing nearly or more than 10 % of the total are marked in bold. -, Not detected; DMA, dimethylacetal; OAG, *O*-alkylglycerol; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; AA, arachidonic acid; ALA, α-linolenic acid.

Straight-chain	**MSr9521^T**	***B. cruenta* By c2^T**
$C_{10:0}$	0.4	-
$C_{12:0}$	0.5	-
$C_{13:0}$	0.3	0.1
$C_{14:0}$	2.9	1.8
$C_{14:1}$	0.6	-
$C_{15:0}$	1.2	1.4
$C_{15:1}$	0.2	0.4
$C_{16:0}$	6.6	5.0
$C_{16:1}$	7.2	**38.6**
$C_{17:0}$	3.7	1.6
$C_{17:1}$	3.6	-
$C_{18:0}$	3.7	2.0
$C_{18:1}$	**11.8**	**13.9**
$C_{19:0}$	0.1	-

$C_{20:0}$	0.1	-
$C_{22:0}$	0.1	-
$C_{23:0}$	0.1	-
$C_{24:0}$	0.1	-
$C_{16:1}$ 2-OH	0.8	-
$C_{17:1}$ 2-OH	**11.7**	5.2
$C_{18:1}$ 2-OH	3.6	-
$C_{14:0}$ OAG	1.2	1.1
$C_{15:0}$ OAG	-	0.8
$C_{16:0}$ OAG	0.9	0.9
$C_{16:1}$ OAG	4.0	0.6
PUFAs		
$C_{16:2}$	0.2	-
$C_{18:2}$	3.3	-
$C_{18:3}\omega$3, 6, 9 $_{\text{all cis}}$ (ALA)	0.5	-
$C_{20:4}\omega$3, 6,9,12 $_{\text{all cis}}$ (ETA)	0.1	-
$C_{20:4}\omega$6, 9, 12, 15 $_{\text{all cis}}$ (AA)	0.2	-
$C_{20:5}\omega$3, 6, 9, 12, 15 $_{\text{all cis}}$ (EPA)	0.5	-
Total straight-chain fatty acids	70.2	73.4
Branched-chain		
iso-$C_{13:0}$	0.1	0.3
iso-$C_{14:0}$	0.4	0.2
iso-$C_{15:0}$	**9.1**	**11.1**
iso-$C_{16:0}$	3.6	2.3
iso-$C_{17:0}$	1.4	7.2
iso-$C_{17:1}$	2.6	0.3
iso-$C_{18:0}$	1.3	0.3
anteiso-$C_{15:0}$	0.4	-
anteiso-$C_{17:0}$	0.7	0.5
iso-$C_{17:1}$ 2-OH	6.9	-
iso-$C_{15:0}$ OAG	3.7	4.9
Total branched-chain fatty acids	30.2	27.1

The BLASTN analysis of the 16S rRNA gene sequence revealed 95–96 % similarity to members of the *Sorangiineae*, 95 % to *Sorangium cellulosum* DSM 14627^T (FJ457641) and *Chondromyces pediculatus* Cm p51^T (GU207875), and 96 % to *Byssovorax cruenta* By c2^T (NR_042341), *Polyangium fumosum* P1 fu5^T (GU207879) and *Jahnella thaxteri* P1 t4^T (NR_117461). Phylogenetic analyses of the 16S rRNA gene sequence showed that MSr9521^T occupied a distinct branch in the family *Polyangiaceae*, suborder *Sorangiineae* (Fig. 2, and Figs S1 and S2, available in the Supplementary Material), with *Byssovorax cruenta* By c2^T appearing to be the closest neighbour. A divergence of 4 % in the 16S rRNA gene sequence from its closest relatives has been used for the proposal of a novel genus in myxobacteria (Garcia *et al.*, 2010). Thus, apart from this sequence divergence, and in addition to its distinct phylogenetic position in the *Polyangiaceae*, the designation of strain MSr9521^T to a novel genus seems justified. Therefore we conclude that strain MSr9521^T represents a novel species of a new genus in the family *Polyangiaceae*, for which the name *Racemicystis crocea* gen. nov., sp. nov. is proposed.

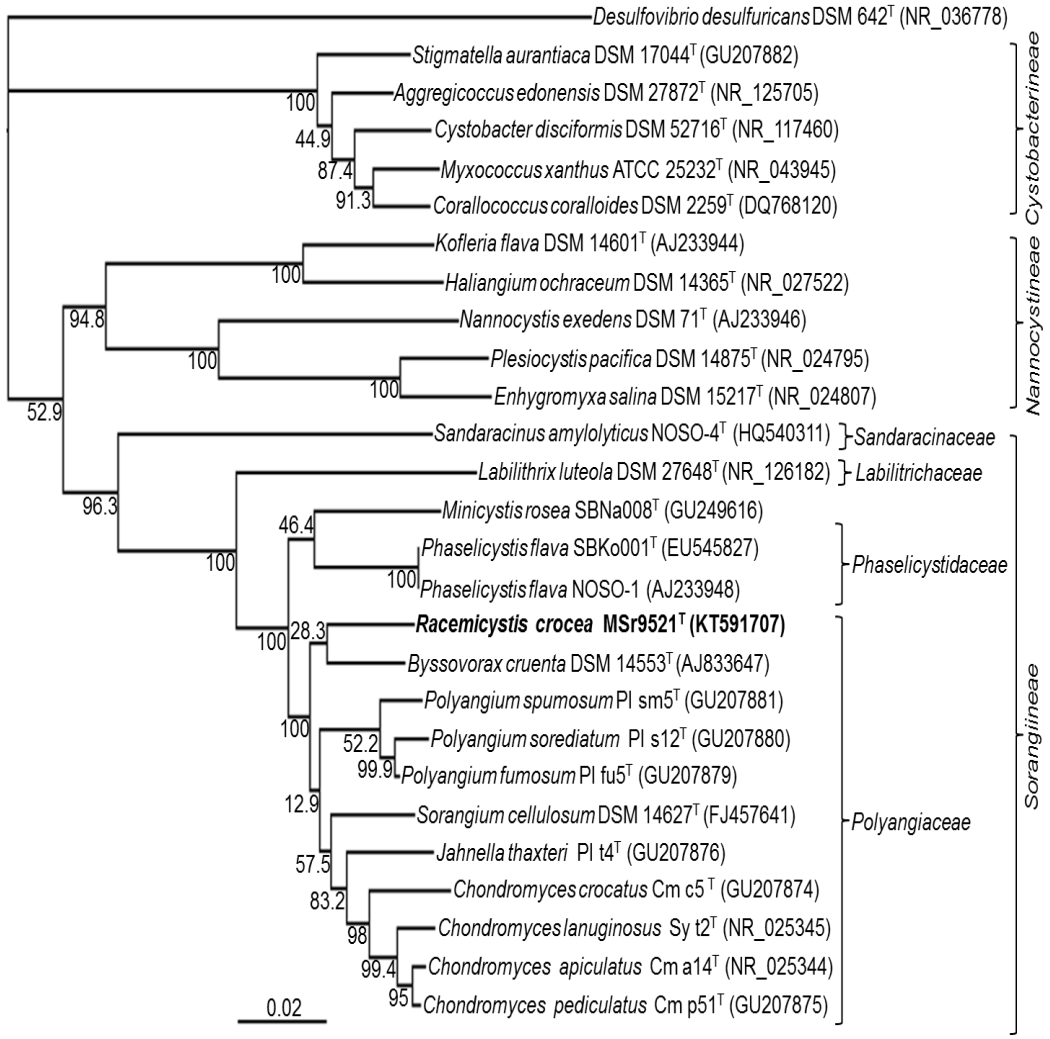

Fig. 2. Neighbour-joining tree of myxobacteria based on 16S rRNA gene sequences showing the position of the novel isolate MSr9521^T in family *Polyangiaceae*, suborder *Sorangiineae*. GenBank accession numbers are shown in parentheses. Values at branch points indicate bootstrap support as percentages based on 1000 resamplings. *Desulfovibrio desulfuricans* DSM 642^T (NR_036778) was used as an out-group to root the tree. Bar, 0.02 substitutions per nucleotide position.

3.5. Description of *Racemicystis* gen. nov.

Racemicystis (Ra.ce.mi.cys′tis. L. masc. n. *racemus* cluster, a bunch of grapes; Gr. fem. n. *kystis* bladder; N.L. fem. n. *Racemicystis* cluster of bladders, referring to the cluster of sporangioles).

Vegetative cells are phase-dark, long and almost cylindrical rods with blunted ends. Movement occurs by gliding on agar surface and as fan-shaped pseudoplasmodial swarm within the agar. Long and tough veins may also appear on the agar surface. Colonies are Congo-red negative. Myxospores are rod-shaped resembling the vegetative cells in shape but shorter slightly refractile, and desiccation resistant. Fruiting bodies are commonly arranged as cluster of sporangioles. Selectively lyses bacteria and incompletely lyses yeast cells. Lack chitinolytic and cellulolytic activities. Degrades agar. Catalase- and oxidase-positive. Major cellular fatty acids are $C_{18:1}$, $C_{17:1}$ 2-OH and iso-$C_{15:0}$. Clusters with *Polyangiaceae,* suborder *Sorangiineae* based on the 16S rRNA gene phylogeny.
The type species is *Racemicystis crocea*.

3.6. Description of *Racemicystis crocea* sp. nov.

Racemicystis crocea (cro′ce.a. L. fem. adj. saffron, golden-yellow colour)

Exhibits all the characteristics of the genus and displays the following additional characteristics. Vegetative cells measure 1.2–1.5 × 3.0–12.0 µm. Swarm colonies vary from pale orange to golden colour. Fruiting bodies are composed of sporangioles that measure 20.0–95.0 µm and are packed in sorus (140–450 µm × 100–250 µm). Myxospores measure 0.8–1.0 × 2.0–5.0 µm. Mesophilic, aerobic, neutrophilic with an optimum growth at pH 7.0–7.5. Selectively lyse *E. coli*, *P. putida* and partially lyse *S. cerevisiae* and *Y. lypolytica*. NaCl is growth inhibitory above 1 %. Hydrolyse casein, Tween 80, soluble starch, xylan, esculin and gelatin. Exhibits good growth in casitone and soytone. Soluble starch represents the best carbohydrate source while cellobiose, sorbitol, fructose, galactose, glucose, arabinose, mannitol, maltose and xylose provide moderate growth. Ammonium sulphate, potassium nitrate and urea are not utilized. Shows API ZYM kit positive reactions with alkaline phosphatase, C4 esterase, C8 esterase lipase, C14 lipase, leucine arylamidase, valine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase and β-glucosidase, and

negative reactions for cysteine arylamidase, trypsin, *α*-galactosidase, *β*-glucuronidase, *α*-glucosidase, N-acetyl-*β*-glucosaminidase, *α*-mannosidase and α-fucosidase. Resistant to bacitracin, hygromycin, polymyxin and tobramycin. Sensitive to ampicillin, apramycin, carbenicillin, cephalosporin, fusidic acid, gentamycin, kanamycin, kasugamycin, neomycin, oxytetracycline, spectinomycin, streptomycin and tetracycline. Contains a moderate amount of iso-$C_{17:1}$ 2-OH and with trace polyunsaturated fatty acids EPA, ETA, ALA and AA.

The type strain is MSr9521^T (=DSM 100773^T =NCCB 100574^T), isolated in 2014 from a Philippine soil sample collected in 1986. The DNA G + C content of the type strain is 70.4 mol %.

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3.8. Supplementary information

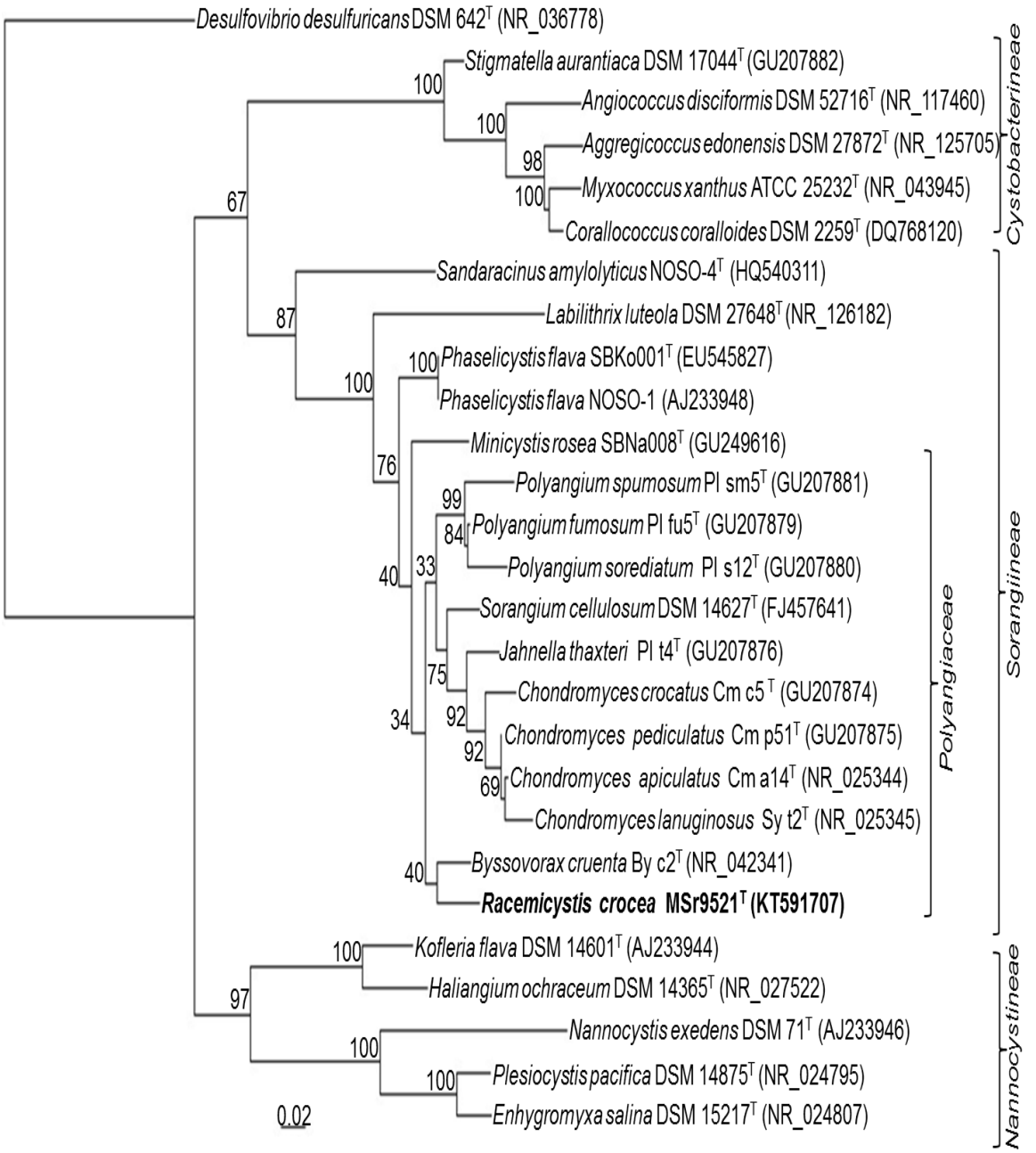

Fig. S1. Maximum-likelihood phylogenetic tree (PHYML) based on 16S rRNA gene sequences of the novel strain MSr9521^T and related taxa. Numbers at branch points indicate bootstrap support as a percentage based on 1000 resamplings. GenBank accession numbers are shown in parentheses. *Desulfovibrio desulfuricans* DSM 642^T (NR_036778) was used as an out-group. Bar, 0.02 substitutions per nucleotide position.

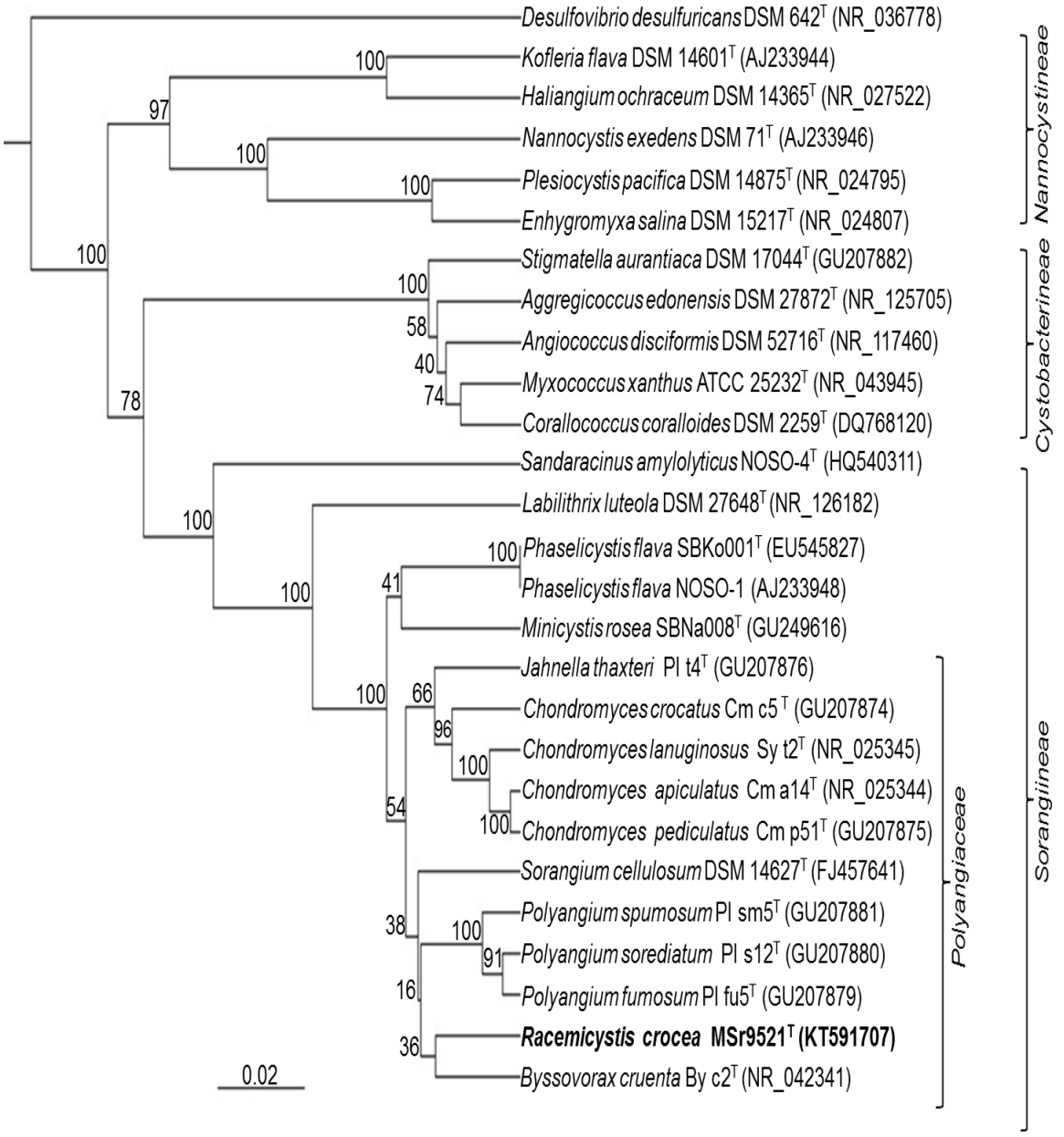

Fig. S2. UPGMA-generated phylogenetic dendrogram showing the relationships of the novel strain MSr9521^T from the related taxa, based on 16S rRNA gene sequences. Values at branch points indicate bootstrap support as a percentage based on 1000 resamplings. GenBank accession numbers are shown in parentheses. *Desulfovibrio desulfuricans* DSM 642^T (NR_036778) was used as an out-group. Bar, 0.02 substitutions per nucleotide position.

Chapter 4

Paucitangium cumulatus gen. nov., sp. nov. and *Paucitangium subalbus* sp. nov., novel soil myxobacteria from Nepalese soil samples

Ram Prasad Awal, [1, 2] Ronald Garcia [1, 2, 3], Katja Gemperlein[1, 2], Joachim Wink[4], Bikram Kunwar[5], Niranjan Parajuli[5] and Rolf Müller[1, 2, 3 *]

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Affiliations

[1]Department of Pharmaceutical Biotechnology, Saarland University, Building E8 1, D-66123 Saarbrücken, Germany

[2]Department of Microbial Natural Products (MINS), Helmholtz Institute for Pharmaceutical Research Saarland (HIPS) – Helmholtz Centre for Infection Research (HZI), Saarland University Campus Building E 8 1, 66123 Saarbrücken, Germany

[3]German Center for Infection Research (DZIF), Partner site Hannover-Braunschweig, D-38124 Braunschweig, Germany

[4]Department of Microbial Drugs, Helmholtz Centre for Infection Research (HZI), Inhoffenstraße 7, D-38124 Braunschweig, Germany

[5]Department of Biotechnology, National College, Tribhuvan University, Naya Bazar, 44600 Kathmandu, Nepal

Contributions

Author's efforts

The author isolated the novel strains and the isolates in *Cystobacterineae* were intensively studied by morphological, physio-chemical, molecular and phylogenetic characteristic. The author performed all the experiments described and analyzed the obtained result. The author conceived and wrote the paper.

Contributions by others

Katja Gemperlein measured and analyzed GC-MS of total cellular fatty acid. Joachim Wink contributed in gyrase B sequence study. Bikram Kunwar and Niranjan Parajuli are the collaborator of the study and provided the soil samples for isolation. The project was supervised by Ronald Garcia and Rolf Müller, who also contributed to conceiving and writing of the manuscript

Chapter: 4 *Paucitangium cumulatus* gen. nov., sp. nov. and *Paucitangium subalbus* sp. nov., novel soil myxobacteria from Nepalese soil samples

4.1. Abstract

Bacterial strains designated MCy10943^T and MCy10944^T were isolated in 2014 from dried Nepalese soil samples collected in 2013 from Phukot, Kalikot, Western Nepal and Godawari, Lalitpur, Central Nepal. The novel organisms showed typical myxobacterial growth characteristics which include swarming colony and fruiting body formation on solid surfaces, and a predatory ability to lyse microorganisms. The strains were aerobic, mesophilic, chemoheterotrophic and showed resistance to various antibiotics. The major cellular fatty acids common to both organisms were $C_{17:0}$ 2-OH, iso-$C_{15:0,}$ $C_{16:1}$ and iso-$C_{17:0.}$ The mol % G + C content of the genomic DNA was 72-75. Phylogenetic analysis showed that the strains belong to the family *Cystobacteraceae*, suborder *Cystobacterineae*, order *Myxococcales*. The 16S rRNA gene sequences of both strains showed 97-98 % similarity to *Archangium gephyra* DSM 2261^T, *Cystobacter violaceus* DSM 14727^T, and 96-97 % to *Angiococcus disciformis* DSM 52716^T and *Cystobacter fuscus* DSM 2262^T. Polyphasic taxonomic characterisation suggested that strains MCy10943^T and MCy10944^T represent two distinct species of a novel genus, for which the names *Paucitangium cumulatus* and *Paucitangium subalbus* are proposed. The type strain of *Paucitangium cumulatus* is MCy10943^T (=DSM 102952^T =NCCB 100600^T) while for *Paucitangium subalbus* is MCy10944^T (=DSM 102953^T =NCCB 100601^T).

4.2. Introduction

Myxobacteria are regarded as social bacteria exhibiting a unique multicellular lifestyle, and producing hydrolytic enzymes and antimicrobials which seem important for predation and survival (Reichenbach, 1999). Many of these organisms were described to produce novel secondary metabolites with therapeutic potential (Wenzel & Müller, 2009; Weissman & Müller, 2010). Myxobacterial diversity appears to be correlated with geography (Dawid, 2000) and with under-explored environments (Garcia *et al.*, 2009). In the past decades, the systematics of myxobacteria was primarily based on phenotypic characteristics such as the description of growth stages. The morphological characteristics

of fruiting bodies, however, may change or be lost after sub-cultivations under laboratory conditions (Garcia *et al.*, 2010). (Shimkets & Woese, 1992) published the first 16S rRNA gene-based phylogenetic analysis of myxobacteria including 12 strains and 11 species of 10 genera. More comprehensive phylogenetic analysis of myxobacteria covering 101 strains (including type strains) representing 3 suborders, 6 families, 20 genera, 46 species, and 12 other novel taxa were later published and validated the correlations with morphology (Garcia *et al.*, 2010), and more recent with fatty acids related phylogeny (Garcia *et al.*, 2011). Besides, 16S rRNA gene sequences, other housekeeping genes including *rpoB, fusA, lepA* and *gyrB* have been successfully used for myxobacteria, specifically with *Corallococcus coralloides* (Stackebrandt & Pauker, 2005; Stackebrandt *et al.*, 2007). Type II DNA topoisomerase, gyrase B, which encodes the *β*-subunit of DNA gyrase B, is known to exhibit a relatively high genetic variation and can thus be used for classification of closely related taxa (Yamamoto & Harayama, 1995; Das *et al.*, 2014). Consequently, a polyphasic taxonomic characterization is to be performed to delineate a novel taxon from its closest relatives. This paper deals with the taxonomy of two strains which were isolated from different geographical locations in Nepal.

4.3. Material and Methods

Strain MCy10943^T and MCy10944^T were isolated from Nepalese soil samples. Both strains were discovered using a novel cultivation approach which is described in the following. Isolation agar medium was prepared by adding 1 % (v/v) soil extract and supplemented with levamisole (25 µg ml^{-1}) and cycloheximide (25 µg ml^{-1}). The soil extract was prepared from the same soil sample used for isolation by suspending 2 g of air-dried sample in 10 ml distilled water, autoclaved and cooled down to room temperature. Soil residue was allowed to settle, and the supernatant was used to prepare the medium containing (w/v) 1.5 % Bacto agar, 25 mM HEPES; pH adjusted to 7.0 with KOH before autoclaving. The standard baiting technique, i.e. autoclaved *Escherichia coli*, was spotted on agar as bait in the isolation. The novel isolates were recognized by their fruiting bodies on agar. Pure isolates were obtained through repeated transfers of the swarm edge on a mineral salt agar (Shimkets *et al.*, 2006). The strains were routinely cultivated and maintained temporarily on buffered VY/2 agar medium at room temperature for 2-3 weeks. Phenotypic growth stages were studied and documented after cultivation of the strains on buffered VY/2 agar (Garcia *et al.*, 2009). For

cryopreservation, pieces of agar taken from actively growing swarm colony were placed in cryotube vials and placed in - 80 °C. The novel strains are reactivated by placing the preserved agar pieces onto a fresh agar medium. For back-up preservation, actively growing vegetative cells taken from liquid culture were cryopreserved in a - 80 °C freezer after the addition of 20% (v/v) glycerol as cryoprotectant which was followed by a slow freezing process in a Cryo-chamber (Mr. Frosty®, Nalgene) using cold isopropanol (Garcia *et al.*, 2014). Morphology stages of the strains were documented and determined according to the previous study (Awal *et al.*, 2016).

Gram-staining (Gerhardt *et al.*, 1981) of vegetative cells and Congo red staining of swarm colony (McCurdy, 1969) were performed according to established protocols. Catalase, oxidase, and API ZYM kit (bioMérieux) reactions were performed as described in a previous study (Awal *et al.*, 2016). The strains were cultivated in PTM medium (w/v: 0.2 % soluble starch, 0.2 % Bacto casitone, 0.005 % KNO_3, 0.1 % $MgSO_4.7H_2O$, 0.0008 % Fe-EDTA, 25 mM HEPES; adjusted to pH 7.2 with KOH before autoclaving), and washed with sterile water before inoculation. The microbial predation using live microorganisms (*Escherichia coli*, *Micrococcus luteus* and *Hansenula anomala*), and degradation assays using cellulose, chitin and agar, and hydrolysis of milk casein, soluble starch and gelatin were done according to the methods described previously (Awal *et al.*, 2016). Hydrolysis of Tweens 20 and 80 (Sigma) was tested in buffered VY/2 and Casitone-based agar at 0.1 % (w/v). Hydrolysis of xylan (Roth) and aesculin (Sigma) was performed according to (Lang *et al.*, 2008).

Growth responses to different temperatures and tolerance to anaerobic growth condition were analysed according to Awal *et al.* (2016) but with the inclusion of PTM agar in the latter test. Antibiotic resistance test was done according to Garcia *et al.* (2014) but with the exclusion of rifampicin, apramycin, carbenicillin, bacitracin, tetracycline, sodium cephalothin, streptomycin and with the addition of cephalosporin C (Sigma) and chloramphenicol (Sigma). Tolerance to sodium chloride, pH and the ability of the strains to utilize peptone and carbohydrate sources were performed as described in (Awal *et al.*, 2016). Cultures were assessed after cultivation in a rotary shaker at 180 r.p.m. Sodium chloride tolerance was done using a 0.5 % (w/v) interval and additionally at a 0.1 % (w/v) interval to narrow down the tolerance limit. Except for temperature tolerance, all tests were incubated for one week at 30 °C.

Cellular fatty acid extraction by fatty acid methyl ester (FAME) and identification by GC-MS analysis was performed according to the standard methods for myxobacteria (Garcia *et al.*, 2011; Gemperlein *et al.*, 2014). Strains used for the analysis were cultivated in 50 ml PTM medium at 180 r.p.m., 5 days. The DNA mol % G + C content of the novel strains including their closest relative species were determined based on standard HPLC-MS method, performed after nuclease P1 digestion of the genomic DNA (Li *et al.*, 2003; Shimelis & Giese, 2006).

Genomic DNA extraction and 16S rRNA gene amplification were described previously (Awal *et al.*, 2016). The isolates were grown in PTM medium for genomic DNA isolation. Specifically designed primers: MCy10944f (5´-CGGTCTCAGTTCAGATTGGAG-3´), MCy10944r (5´-CTCAGACCAGCTACCCGTCG-3´) for strain MCy10944^T and MCy10943f (5´-ATGACCAGGGCTACACACG-3´), MCy10943r (5´-GTCCTCTCAGACCAGCTACC-3´) for strain MCy10943^T were also used for sequencing. The partial sequences of the gyrase B gene (*gyrB*) of strains belonging to selected *Cystobacteraceae* were amplified with specially designed degenerate primers Myxo-gyrB768F (5´–CCNGGNATGTAYATHGGNGAYAC-3´) and Myxo-gyrB512R (5´-RTCNTCRTCNGCRTCNGTGAT -3´). These primers were also used for the sequencing. The 25 µl PCR mixture consisted of 2.5 µl 10 x Qiagen buffer, 0.5 µl dNTP (10 mM), 0.75 µl DMSO, 1.5 µl $MgCl_2$ (25 mM), 0.1 µl Taq polymerase (5 U/µl) (Thermo Scientific), 1.25 µl of each primers gyrB768F (10 pM) and gyrB512R (10 pM), 16.15 µl dH_2O and 1 µl of DNA template (150 ng). The PCR conditions were as follows: 95 °C for 3 min followed by initial 5 cycles of 96 °C for 15 s, 37 °C for 20 s, 72 °C for 70 s; followed by 30 cycles of 96 °C for 15 s, 52.8 °C for 20 s, 72 °C for 70 s, and final elongation at 72 °C for 10 min. The 16S rRNA gene sequence of the novel isolates was compared with those sequences available in public databases (GenBank/EMBL/DDBJ) using the nucleotide Basic Local Alignment Search Tool – BLASTN version 2.2.29+ (Zhang *et al.*, 2000; Morgulis *et al.*, 2008). The 16S rRNA gene sequences of myxobacteria, mostly representing the type strains in the suborder *Cystobacterineae*, and selected members of *Sorangiineae* and *Nannocystineae*, were retrieved from the GenBank. In the case of gyrase B, the partial gene sequence of *Corallococcus exiguus* DSM 14696^T (GenBank accession number AJ811706) and *Corallococcus coralloides* DSM 2259^T (AJ811731) were retrieved from the GenBank. Sequence alignments of the

16S rRNA and gyrase B gene sequence of selected strains of the *Cystobacteraceae* were performed using the MUSCLE software (Edgar, 2004) while the distance matrices between sequences were calculated using the Jukes-Cantor model (Jukes & Cantor, 1969). The phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987), UPGMA (Sneath & Sokal, 1973) and PHYML (Guindon & Gascuel, 2003) methods applying 1000 bootstrap resampling (Felsenstein, 1985). The 16S rRNA gene sequence of *Desulfovibrio desulfuricans* DSM 642^T (GenBank accession number: NR_036778) was chosen as an outgroup to root the trees. All these phylogenetic programs are packed in the Geneious Pro 8.1.3 software suite (Drummond *et al.*, 2010).

4.4. Result and Discussion

The swarm colonies of the novel isolates produced a slime sheath (Fig 1a, 2a) which differed in colour. Strain MCy10943^T showed transparent to yellowish-orange colony while MCy10944^T appeared as a transparent to a smoky whitish colony. The colonies are soft and moved coherently. The colony edges of the novel isolates were composed of flares with wavy surface appearance (Fig. 1b, 2b). Vegetative cells were long, slender, phase dark rods with tapering ends measuring 0.8 – 0.9 x 6 – 16 µm (Fig. 1c, 2c), and were yellowish-orange to whitish colour for MCy10943^T and MCy10944^T, respectively. The two novel isolates differed in fruiting body morphology. Strain MCy10943^T formed oval to bean shaped fruiting bodies (50 – 100 µm) without sporangiole and were often arranged as clusters or heaped on agar (Fig 1d, e). The absence of an enclosing wall in the fruiting body is reminiscent of *Corallococcus* species, *Archangium gephyra* and *Aggregicoccus*. Strain MCy10944^T formed beige to pale orange, globular, glistening solitary fruiting-body-like mounds (20 – 500 µm) which were commonly held in slime (Fig. 2d, e). Both novel isolates contain slightly refractive ovoid to short rod myxospores which were desiccation resistant. Strain MCy10943^T myxospores measure 1.0 x 1.5-3.0 µm (Fig 1f) while MCy10944^T measures 1.0 x 2.0 µm (Fig. 2f). The size and shape of the myxospores produced by the novel isolates appear typical for members of the *Cystobacteraceae* (Reichenbach, 2005), thus supporting their phenotypic clustering within the family.

The novel strains were Gram-negative, Congo-red, catalase, and oxidase positive. The two novel isolates shared positive API ZYM enzymatic reactions with alkaline

phosphatase, C4 esterase, C8 esterase lipase, leucine arylamidase, valine arylamidase, cysteine arylamidase, *α*-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *β*-galactosidase, *β*-glucosidase, and shared negative reactions in C14 lipase, *α*-galactosidase, *β*-glucuronidase, *α*-mannosidase, and *α*-fucosidase. Strain MCy10944^T differed with MCy10943^T as it showed a negative reaction in trypsin and N-acetyl- *β*-glucosaminidase, and a positive reaction with *α*-glucosidase.

Lysis of live microorganisms including *E. coli*, *M. luteus* and *H. anomala* were observed with the novel isolates. Filter paper, chitin and agar were not degraded. Milk casein, soluble starch, gelatin, Tweens 20 and 80, and xylan were all hydrolysed by the novel bacteria. The two novel isolates differ in aesculin test which MCy10943^{T}shows a positive reaction. Table 1 summarizes the differential characteristics of the novel isolates and their closely related species.

Table 1. Differential characteristics of the novel isolates and closely related species

	Cystobacter violaceus DSM14727^T	*Cystobacter minus* DSM14751^T	*Angiococcus disciformis* DSM 52716^T	*Archangium gephyra* DSM 2261^T	MCy10943^T	MCy10944^T	*Cystobacter fuscus* DSM 2262^T
Xylan	w +	-	-	-	+	+	+
Aesculin	-	-	-	-	+	-	w +
Chitin	+	+	-	-	-	-	+
G+C % Mol	73.0	71.1	73.6	58.4	72.5	74.6	68.6
Temperature tolerance (°C)	22-37	16-39	16-37	22-37	22-40	18-37	18-37

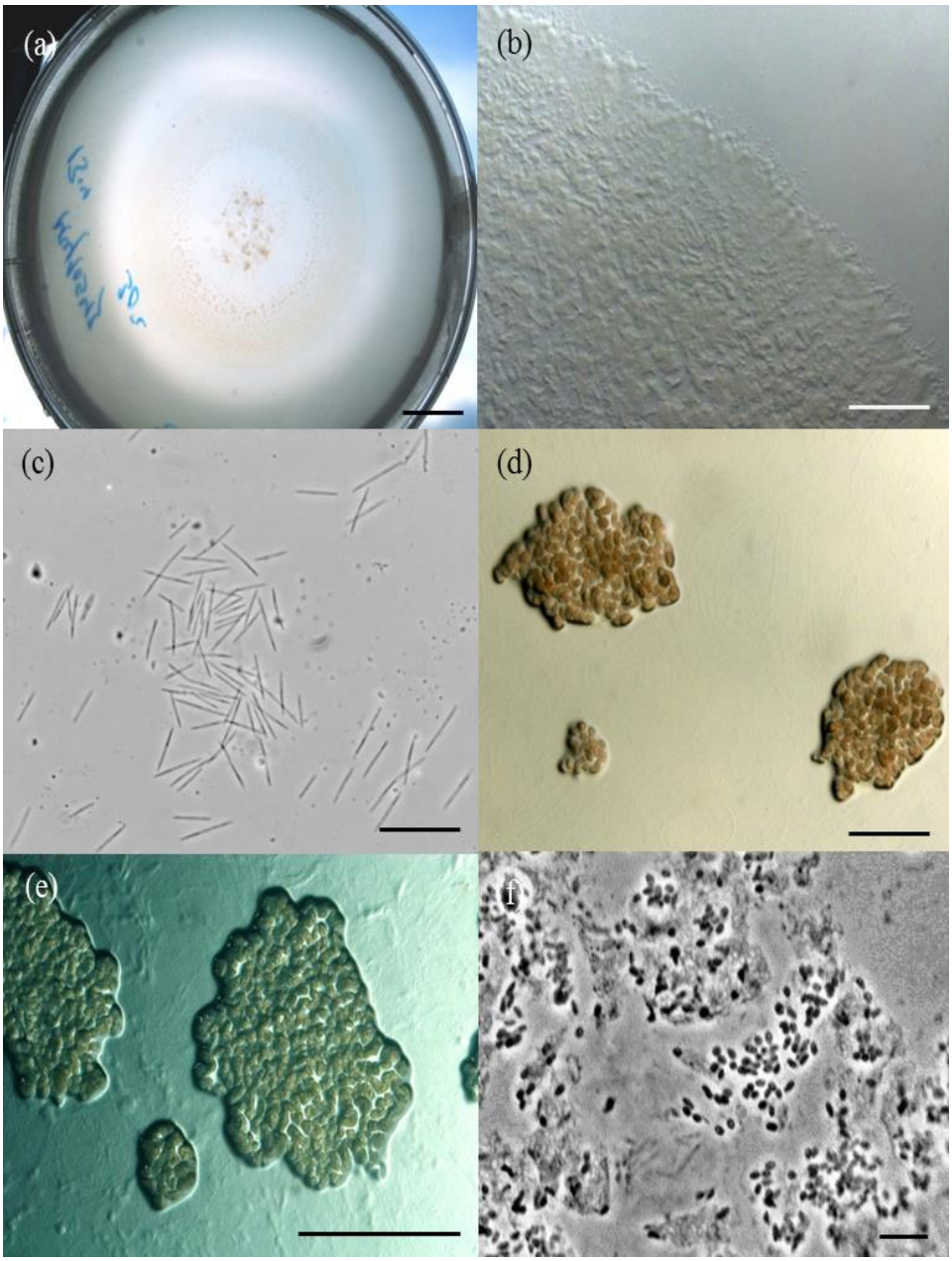

Fig. 1. Photographs of growth stages of MCy10943^{T}, obtained by stereomicroscopy (b, d, e) and phase-contrast microscopy (c, f). (a) The swarming colony on buffered VY/2 agar medium cultivated at 30 °C for two weeks. (b) Coherent swarm with scattered ripples, and flares at the colony edge. (c) Slide-mount of vegetative cells taken from the colony edge on buffered VY2 agar. (d) Fruiting bodies on a VY/2 agar surface. (e) Fruiting bodies with irregular huddles without a distinct wall (f) Optically refractile myxospores released from pressed fruiting bodies. Bars, 1 cm (a), 500 µm (b), 20 µm (c) 500 µm (d , e) and 10 µm (f).

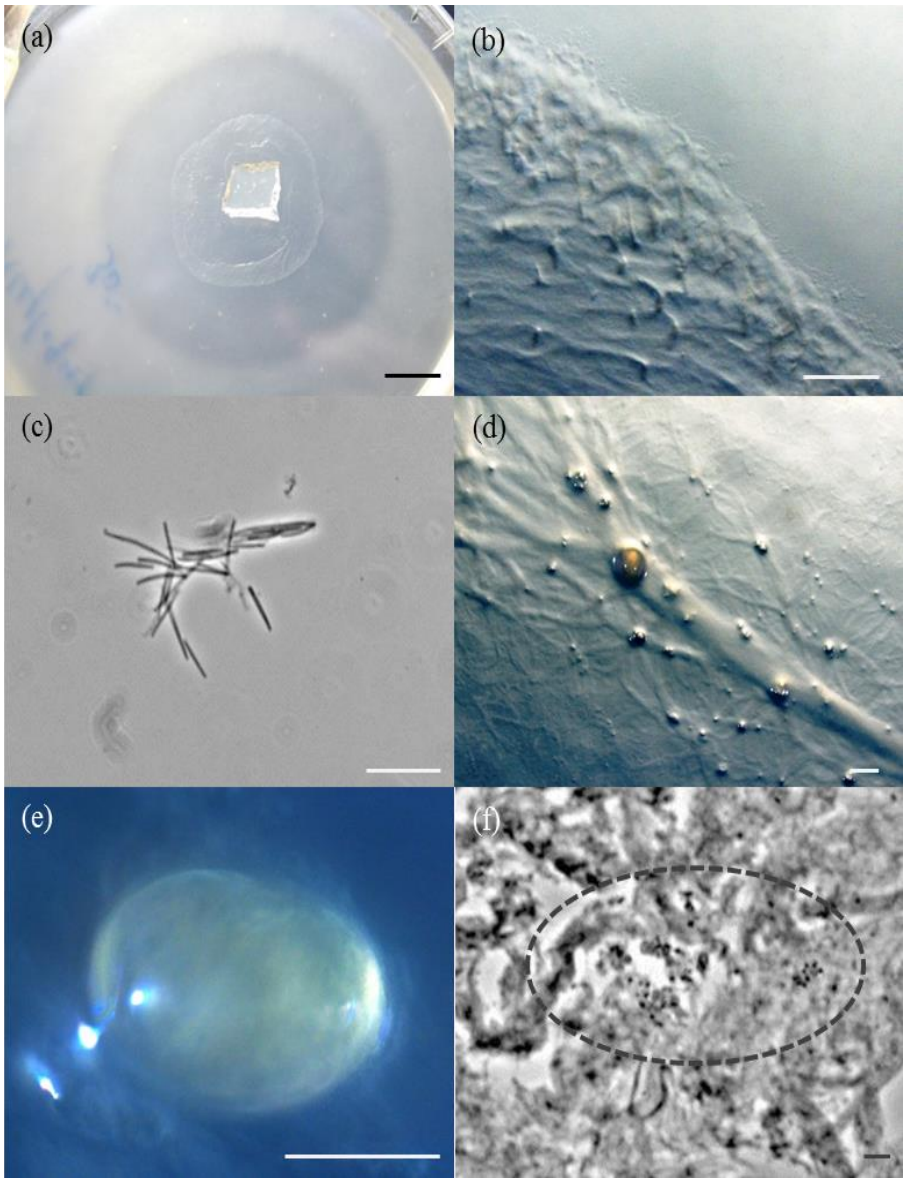

Fig. 2. Photographs of growth stages of MCy10944^{T}, obtained by stereomicroscopy (b, d, e) and phase-contrast microscopy (c, f). (a) Swarming colony formed around inoculated agar block on buffered VY/2 agar cultivated at 30 °C for one week. (b) Swarm pattern showing waves of veins and flares at the edge. (c) Slide-mount of vegetative cells taken from the colony edge on buffered VY2 agar. (d) Scattered Fruiting bodies like aggregates on an agar surface. (e) Fruiting bodies like aggregate on the agar-medium surface. (f) Optically refractile presumably myxospores released from pressed fruiting bodies like aggregate (encircled). Bars, 1cm (a), 500 µm (b), 20 µm (c), 200 µm (d, e) and 10 µm (f).

Growth response to temperatures differed between the two isolates. The largest colony diameter on buffered VY/2 was seen at 37 °C for MCy10943^T while best growth was observed at 30 °C for MCy10944^T. Both strains were characterised aerobic as they failed to grow in an anaerobic jar. The novel isolates shared similar antibiotic susceptibility patterns by showing resistance to ampicillin, kanamycin, gentamicin, hygromycin B, trimethoprim, polymyxin, and neomycin, and showing sensitivity to cephalosporin C, oxytetracycline, fusidic acid, thiostrepton, and chloramphenicol. Strain MCy10944^T differed from MCy10943^T in its sensitivity to spectinomycin and kasugamycin. The novel isolates were non-halophilic with maximum NaCl tolerance of 0.6% and 0.5% for MCy10943^T and MCy10944^T, respectively. Growth was best at neutral to slightly alkaline pH for the novel bacteria. Strain MCy10943^T exhibited growth at pH 6.0 – 9.5 with an optimum at pH 7.0–8.0 while MCy10944^T showed spreading colony at pH 5.5 – 9 with an optimum at pH 6.5–7.5.

The novel strains differed distinctly in their nutritional requirement. MCy10943^T exhibited high cell yield in the presence of xylose, galactose, and cellobiose while MCy10944^T exhibited the best growth in the presence of potato starch and arabinose. Both isolates exhibited good growth with supplementation of soluble starch. MCy10943^T grew poorly in the presence of sorbitol, sucrose, raffinose, arabinose and rhamnose. Poor growth was observed in MCy10944^T after supplementation with sucrose, raffinose, maltose, galactose and fructose. In peptone supplementation, MCy10943^T showed optimal growth in casitone, tryptone, and soytone; moderate growth in polypeptone, peptone, and proteose peptone, and no growth was observed with neopeptone and casamino acids. MCy10944^T exhibited optimal growth in tryptone, peptone, and polypeptone, moderate in soytone, proteose peptone, casitone, and neopeptone, and poor in casamino acids. Complex organic sources such as meat extract enhanced the growth while yeast and beef extracts supported only little growth of novel isolates. All inorganic nitrogen sources tested did not induce the growth of the novel strains. Taken together the nutritional requirements, the novel isolates can be regarded as chemoheterotrophic bacteria.

The novel isolates shared nearly identical fatty acids profiles with major cellular fatty acids iso-$C_{15:0}$ (12.8 % in MCy10943^T, 20.3 % in MCy10944^T), iso-$C_{17:0}$ 2-OH (20.9 % in MCy10943^T, 26.6 % in MCy10944^T) and $C_{16:1}$ (14.8 % in MCy10943^T, 11.3 % in MCy10944^T). The closest fatty acid pattern was determined with the *Archangium*-

Cystobacter cluster where branched-chain fatty acids dominate over the straight-chained type (Table 2) (Garcia *et al.*, 2011). The novel isolates are distinct from their closely related species *Cystobacter violaceus* DSM 14727^T in as much as a three-fold higher content of iso-$C_{17:0}$ and three-times less of iso-$C_{15:0}$ DMA. The two novel myxobacteria differ in $C_{16:0}$ and iso-$C_{15:0.}$ Strain MCy10943^T contained twice the amount of $C_{16:0}$ and much less iso-$C_{15:0}$ compared with MCy10944^T (Table 2).

Table 2. Cellular fatty acid profile of MCy10943^T, MCy10944^T and *Cystobacter violaceus* DSM 14727^T after cultivation in PTM medium. Fatty acids with nearly or more than 10 % of the total are marked in bold. -, Not detected; DMA, dimethylacetal; OAG, *O*-alkylglycerol.

Fatty acids	**MCy10943^T**	**MCy10944^T**	***C. violaceus* DSM 14727^T**
Straight chain			
$C_{10:0}$	0.2	0.2	-
$C_{12:0}$	0.1	-	-
$C_{14:0}$	3.9	1	2.7
$C_{14:1}$	0.8	0.5	0.9
$C_{15:0}$	0.3	0.4	0.1
$C_{16:0}$	**11.4**	5.5	2.1
$C_{16:1}$	**14.8**	**11.3**	**14.1**
$C_{18:0}$	0.4	0.6	0.5
$C_{14:0}$ 3-OH	0.2	0.2	0.8
$C_{16:0}$ 3-OH	0.1	0.1	0.1
$C_{16:0}$ 2-OH	0.6	1.6	0.9
$C_{12:0}$ DMA	-	-	0.1
$C_{14:0}$ DMA	-	-	1.8
$C_{16:0}$ DMA	-	-	0.4
$C_{16:1}$ DMA	-	0.2	0.4

$C_{14:0}$ OAG	-	-	0.2
$C_{16:0}$ OAG	0.2	0.1	0.1
$C_{16:1}$ OAG	-	-	0.1
PUFAs			
$C_{16:2}$	0.4	0.1	0.2
$C_{18:1}$ ω 9 cis	-	0.9	-
Total SCFA	**33.4**	**22.7**	**25.5**
Branched chain			
iso-$C_{11:0}$	0.1	0.2	-
iso-$C_{13:0}$	0.4	0.5	0.1
iso-$C_{14:0}$	0.2	0.5	0.1
iso-$C_{15:0}$	**12.8**	**20.3**	**19.7**
iso-$C_{15:1}$	0.3	0.1	0.2
iso-$C_{16:0}$	7.2	5.1	5.0
iso-$C_{17:0}$	**10.4**	**8.3**	2.6
iso-$C_{17:1}$	0.4	2.4	0.5
iso-$C_{15:0}$ 3-OH	1.4	2.7	2.3
iso-$C_{16:0}$ 2-OH	1.2	0.1	0.1
iso-$C_{17:0}$ 2-OH	**20.9**	**26.6**	**20.0**
iso-$C_{18:0}$ 2-OH	0.1	0.1	-
iso-$C_{15:0}$ DMA	3.7	5.8	**18.0**
iso-$C_{15:0}$ OAG	7.1	4.4	5.6
iso-$C_{16:0}$ OAG	-	0.1	0.1
iso-$C_{17:0}$ OAG	0.1	0.1	0.1
Total BCFA	**66.3**	**77.8**	**74.4**

The novel isolates show a high DNA G + C content (72.5-74.6 mol %) with strain MCy10944^T showing a slightly higher content (Table 1). The BLASTn analysis of the 16S rRNA gene sequence of MCy10943^T showed 98 % closest similarity to *Cystobacter violaceus* Cb vi29 (GenBank accession number AJ233905) and *Cystobacter* sp. 94032 (DQ520899). The strain MCy10944^T showed the closest similarity (99 %) to a clone of uncultured bacteria (GenBank accession number EU193069) derived from a soil sample, (98 %) to *Cystobacter* sp. 94032 (DQ520899) and *Cystobacter violaceus* Cb vi34 (AJ233906). Strain MCy10943^T and MCy10944^T showed 98 % 16S rRNA gene sequence similarity. The BLASTn analysis of partial gyrase B gene sequence (ca. 1300 nucleotides) revealed closest similarity to myxobacterial type strains: 95-94 % to *Cystobacter minus* DSM 14751^T (KX444680), 94 % to *Archangium gephyra* DSM 2261^T (KX444679), *Cystobacter violaceus* DSM 14727^T (KX444678) and *Angiococcus disciformis* DSM 52716^T (KX444675). Phylogenetic analysis based on 16S rRNA gene sequence and partial gyrase B gene sequence showed that the two novel strains cluster together in a unique new clade within the *Cystobacteraceae* (Figs 3, 4, and Figs S1 and S2, available in the Supplementary Material). A divergence of 97 – 98 % 16S rRNA gene sequence from myxobacterial closest relatives has been observed and described among genera within *Cystobacteraceae* (Garcia *et al.*, 2011). A distinct and novel phylogenetic position together with their typical *Cystobacter-like* phenotypic characteristics supports the proposal to erect a novel genus and species for these two novel isolates in *Cystobacteraceae*; for which the name *Paucitangium* gen. nov. is proposed.

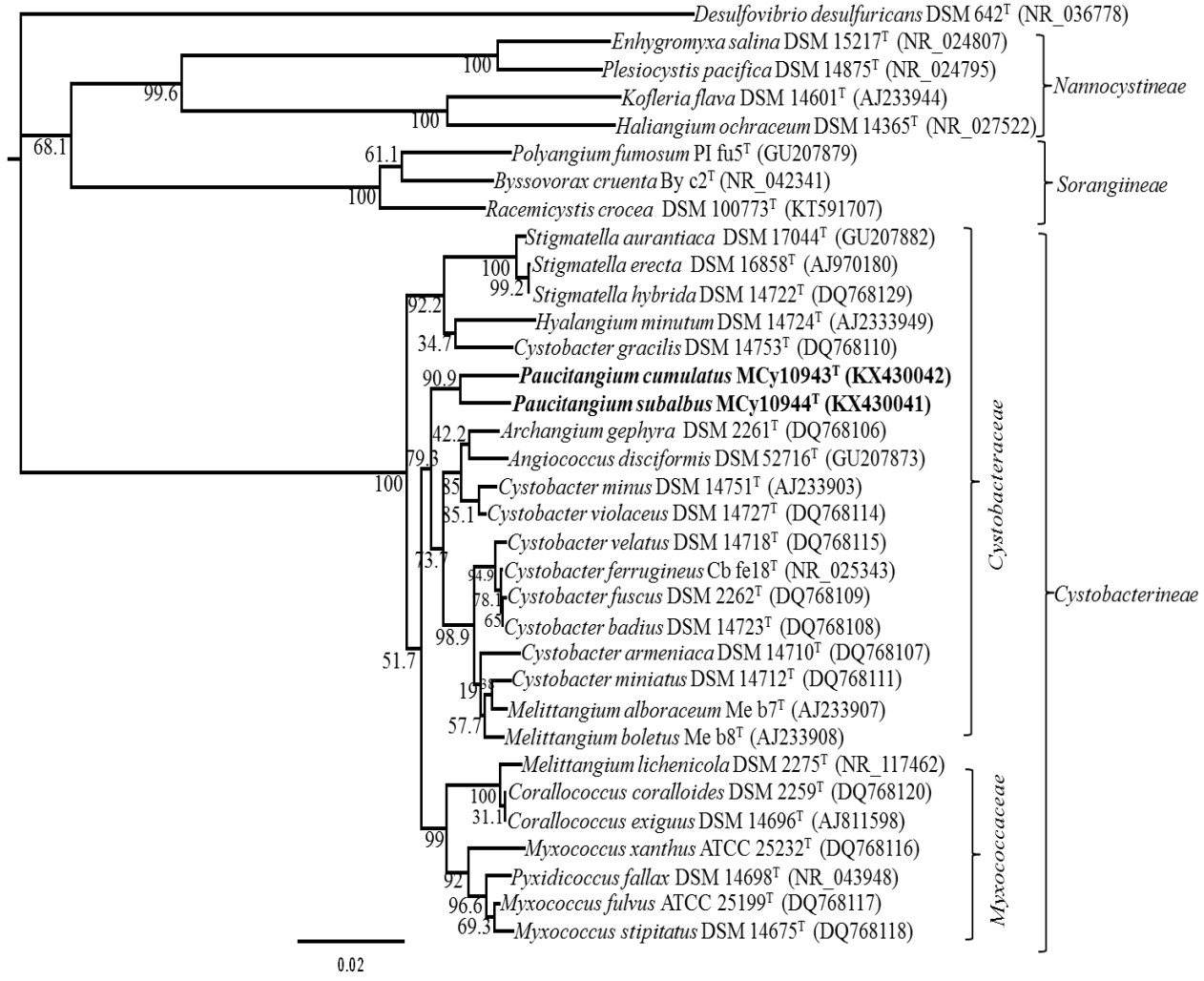

Fig. 3. Neighbour-joining tree based on 16S rRNA gene sequences showing the position of the novel isolates MCy10943^T and MCy10944^T in the suborder *Cystobacterineae*. GenBank accession numbers are shown in parentheses. Values at branch points indicate bootstrap support as percentages based on 1000 resamplings. *Desulfovibrio desulfuricans* DSM 642^T was used as an out-group to root the tree. Bar, 0.02 substitutions per nucleotide position.

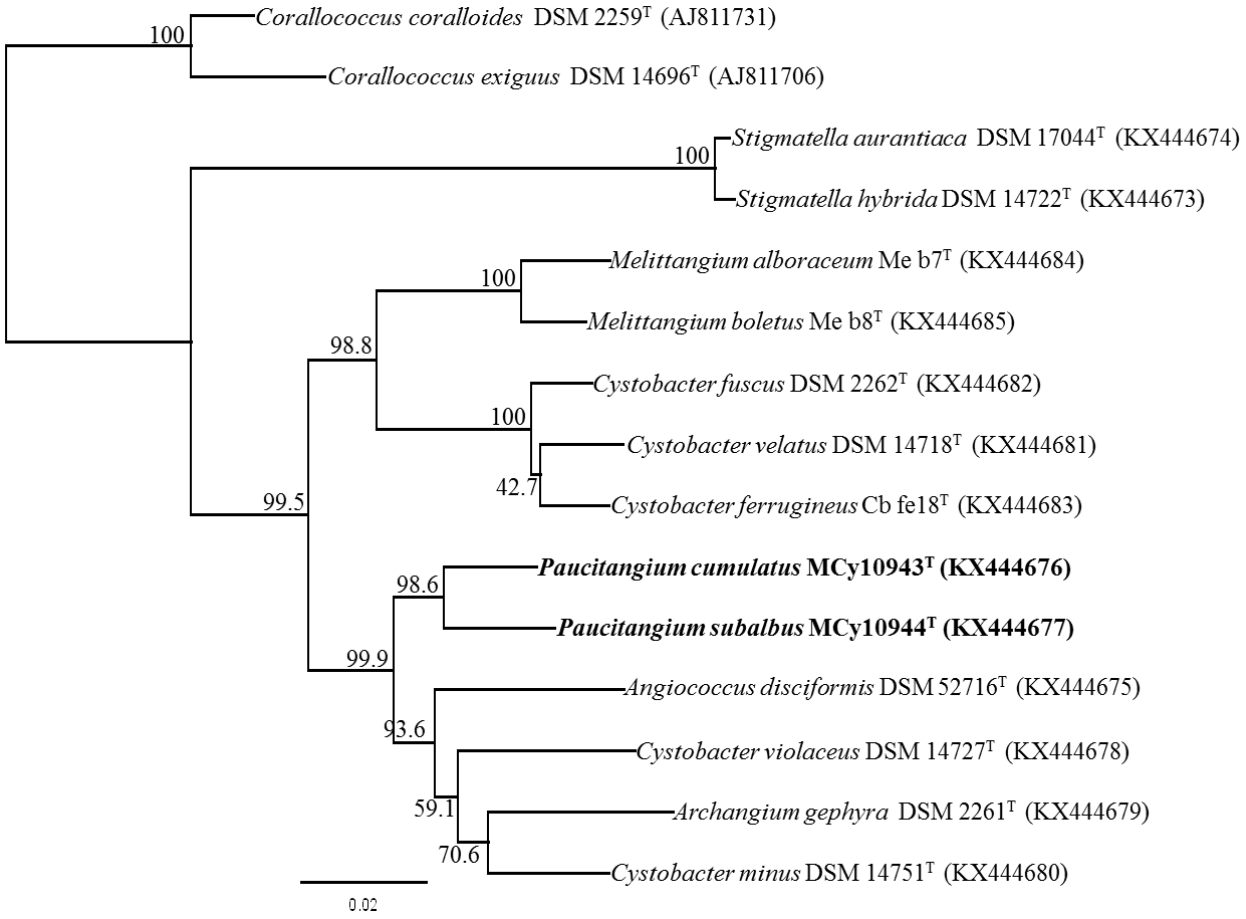

Fig. 4. Neighbour-joining tree based on gyrase B gene sequences of selected members of *Cystobacteraceae* showing the position of the novel isolates MCy10943^T and MCy10944^T in the suborder *Cystobacterineae*. GenBank accession numbers are shown in parentheses. Values at branch points indicate bootstrap support as percentages based on 1000 resamplings. The tree is rooted with *Corallococcus exiguus* DSM 14696^T (AJ811706) and *Corallococcus coralloides* DSM 2259^T (AJ811731). Bar, 0.02 substitutions per nucleotide position.

4.5. Description of *Paucitangium* gen. nov.

Paucitangium (Pau.ci.tan´gi.um. L. fem. n. *paucitas* lack, scarcity; Gr. neut. n. *angion* vessel, container; N.L. neut. n. *Paucitangium* lacking vessel, referring to the scarcity of sporangioles in fruiting bodies and fruiting bodies like aggregates.

Vegetative cells are phase dark, long and flexuous rods with tapered ends. Mesophilic, chemoheterotrophic and aerobic. Swarm forms halo around the colony in buffered yeast agar and move by gliding. The colony appears as film, thin and transparent with flare colony edges, and stains with Congo-red. Myxospores are slightly refractile, short rods to oval, and desiccation resistant. Fruiting bodies or fruiting body-like aggregates lack sporangioles. Lyse bacteria and yeast. Lack cellulolytic, chitinolytic, and agarolytic activities. Vegetative cells are catalase- and oxidase-positive. Contain a high amount of iso-$C_{17:0}$ 2-OH, iso-$C_{15:0,}$ $C_{16:1}$ and iso-$C_{17:0.}$ Clusters within the *Cystobacteraceae* family, suborder *Cystobacterineae*. The G + C content ranges 72.0 – 75.0 mol %. The type species is *Paucitangium cumulatus.*

4.6. Description of *Paucitangium cumulatus* sp. nov.

Paucitangium cumulatus (cu.mu.la′tus L. masc. adj. heaped, referring to the arrangement of fruiting bodies.

Exhibits all the characteristics of the genus. Vegetative cells measure 0.8–0.9 x 7.0–16.0 µm. Swarm colonies vary from transparent to yellowish-orange colour on buffered VY/2 agar. Fruiting bodies are not enclosed in sporangioles, and the shape varies from oval to bean shaped (50 – 100 µm), often appearing as clusters or heap. Myxospores measure 1.2–1.5 x 1.5–3.0 µm. Mesophilic, aerobic, and tolerates a wide pH range (6.0-9.5) with optimal growth at pH 7.0-8.0. NaCl is inhibitory at above 0.6 %. Hydrolyse milk casein, soluble starch, gelatin, Tweens 20 and 80, xylan and aesculin. Exhibits good growth in casitone, tryptone, soytone and meat extract. Grows best with xylose, soluble starch, galactose and cellobiose. Shows positive reactions in the API ZYM kit for alkaline phosphatase, C4 esterase, C8 esterase lipase, leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, *α*-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *β*-galactosidase, *β*-glucosidase, and *N*-acetyl-*β*-glucosaminidase and negative reactions for C14 lipase, *α*-galactosidase, *β*-glucuronidase, *α*-glucosidase, *α*-

mannosidase and *α*-fucosidase. Resistant to ampicillin, cephalosporin C, kanamycin, kasugamycin, gentamicin, hygromycin B, trimethoprim, polymyxin, neomycin and spectinomycin. Sensitive to chloramphenicol, oxytetracycline and thiostrepton. Major cellular fatty acids are iso-$C_{17:1}$ 2-OH, $C_{16:1}$, iso-$C_{15:0}$ and iso- $C_{17:0}$.

The type strain is MCy10943^T (=DSM 102952^T =NCCB 100600^T), isolated in 2014 from a Nepalese soil sample collected in 2013. The DNA G + C content of the type strain is 72.5 mol %.

4.7. Description of *Paucitangium subalbus* sp. nov.

Paucitangium subalbus (sub′al.bus. L. n. adj. whitish).

Exhibits all the characteristics of the genus. Vegetative cells measure 0.8–1.0 x 6.0 –16.0 µm. Swarm colonies vary from transparent to smoky whitish colour on buffered VY/2 agar. Fruiting body-like aggregates are beige to pale orange colour and appearing as solitary mounds (20–200 µm). Myxospore-like cells appear oval to small rod measuring 1.0 x 2.0 µm. Tolerates a wide pH range (5.5-9.0) with optimal growth at the neutral value (pH 7.0). Lyse *E. coli*, *M. luteus* and *H. anomala*. NaCl inhibits growth at above 0.5 %. Hydrolyse milk casein, soluble starch, xylan, Tween 20 and 80. Exhibits good growth in tryptone, peptone, polypeptone, meat extracts and yeast extracts. Grows best with soluble starch and arabinose. Shows API ZYM kit positive reactions with alkaline phosphatase, C4 esterase, C8 esterase lipase, leucine arylamidase, valine arylamidase, cysteine arylamidase, *α*-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *β*-galactosidase, *α*-glucosidase and *β*-glucosidase and negative reactions for C14 lipase, trypsin, *α*-galactosidase, *β*-glucuronidase, N-acetyl-*β*-glucosaminidase, *α*-mannosidase, and *α*-fucosidase. Resistant to ampicillin, kanamycin, gentamicin, hygromycin B, trimethoprim, polymyxin, and neomycin. Sensitive to cephalosporin C, chloramphenicol, fusidic acid, kasugamycin, oxytetracycline, spectinomycin and thiostrepton. Major cellular fatty acids are iso-$C_{17:0}$ 2-OH, $C_{16:1}$ and iso-$C_{15:0.}$

The type strain is MCy10944^T (=DSM 102953^T =NCCB 100601^T), isolated in 2014 from a Nepalese soil sample collected in 2013. The DNA G + C content of the type strain is 74.6 mol %.

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4.9. Supplementary Information

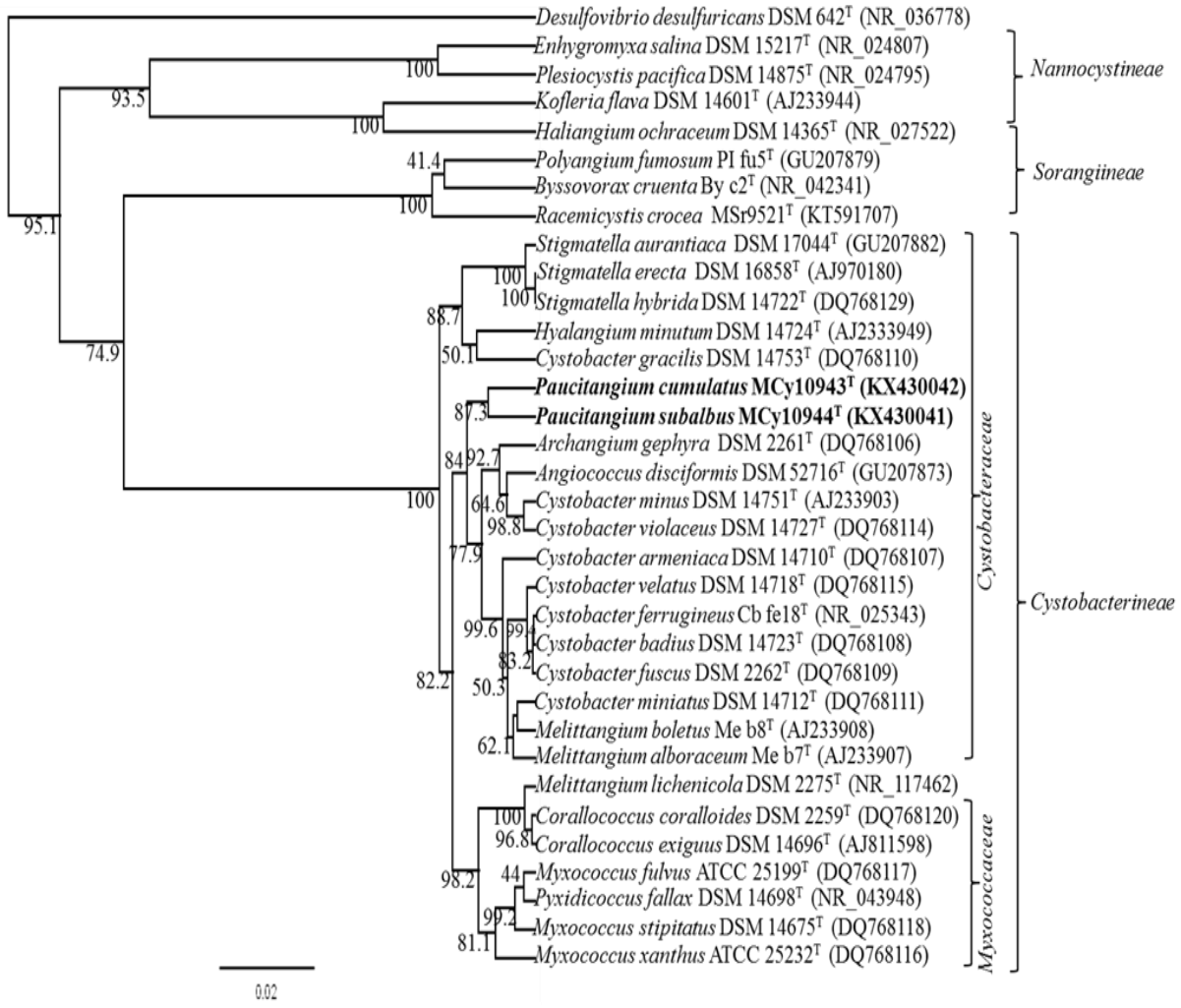

Fig. S1. UPGMA-generated phylogenetic dendrogram showing the relationships of the novel strains MCy10943^T and MCy10944^T from the related taxa, based on 16S rRNA gene sequences. Values at branch points indicate bootstrap support as a percentage based on 1000 resamplings. GenBank accession numbers are shown in parentheses. *Desulfovibrio desulfuricans* DSM 642^T (NR_036778) was used as an outgroup. Bar, 0.02 substitutions per nucleotide position

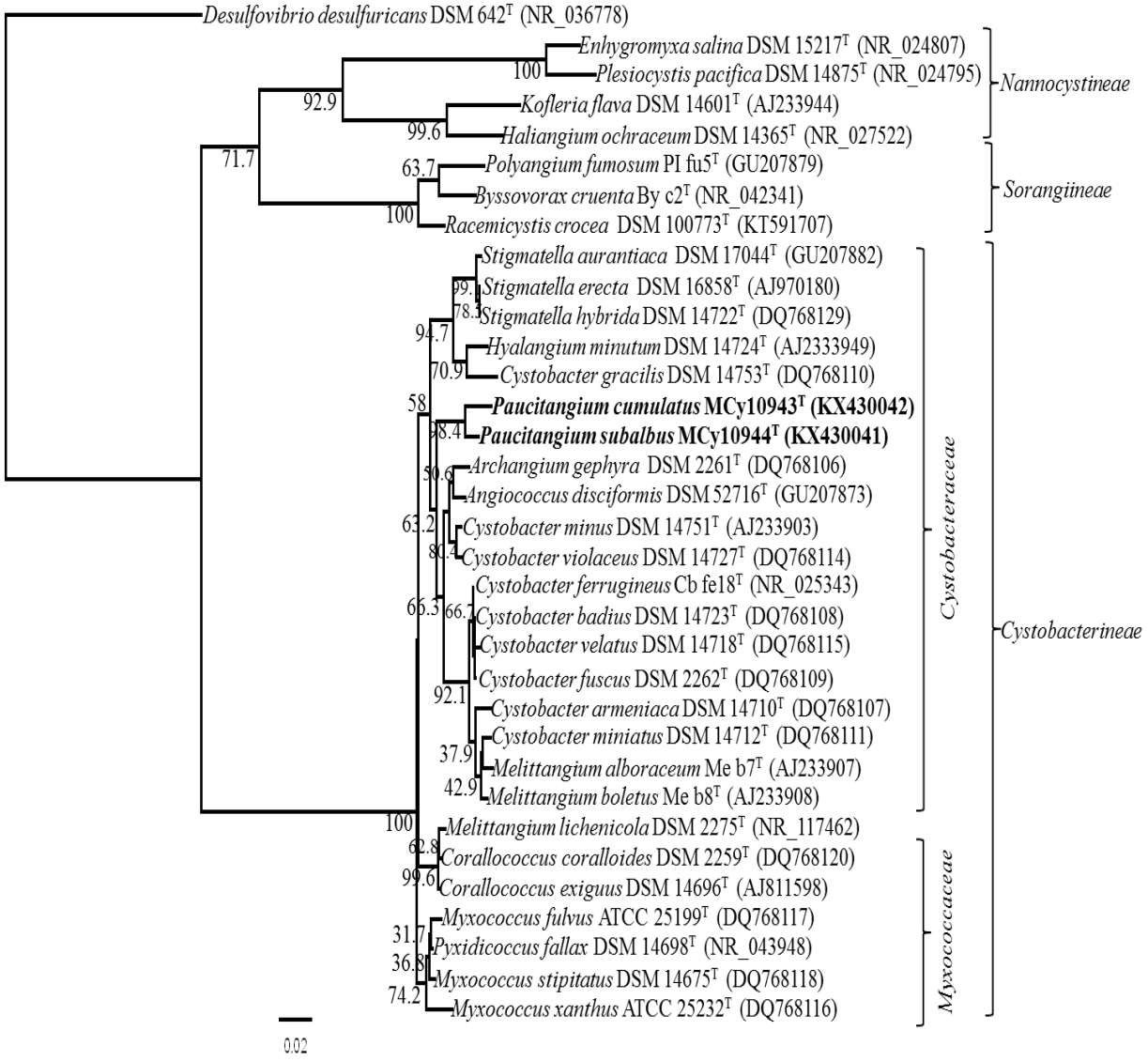

Fig. S2. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences showing the relationships of the novel strains MCy10943^T and MCy10944^T from the related taxa. Values at branch points indicate bootstrap support as a percentage based on 1000 resamplings. GenBank accession numbers are shown in parentheses. *Desulfovibrio desulfuricans* DSM 642^T (NR_036778) was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

Chapter 5

Co-culture studies of myxobacteria reveal an inter-species signal for the induction of fruiting body formation

Ram Prasad Awal, [1, 2] Carsten Volz[1] and Rolf Müller[1, 2, 3 *]

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Affiliations

[1]Department of Pharmaceutical Biotechnology, Saarland University, Building E8 1, D-66123 Saarbrücken, Germany

[2]Department of Microbial Natural Products (MINS), Helmholtz Institute for Pharmaceutical Research Saarland (HIPS) – Helmholtz Centre for Infection Research (HZI), Saarland University Campus Building E 8 1, 66123 Saarbrücken, Germany

[3]German Center for Infection Research (DZIF), Partner site Hannover-Braunschweig, D-38124 Braunschweig, Germany

Contributions

Author’s efforts

The author performed co-cultivation studies of different myxobacterial strains. The author observed for the first time the phenomenon described in this paper. The author designed, conducted all the experiments described and interpreted the resulting data. The author conceived and wrote the manuscript.

Contributions by others

The project was designed and supervised by Carsten Volz and Rolf Müller. Both were involved in data interpretation, as well as in conceiving, writing and proof-reading of the manuscript.

Chapter: 5 Co-culture studies of myxobacteria reveal an inter-species signal for the induction of fruiting body formation

5.1. Abstract

Myxobacteria exhibit a complex social behavior and upon starvation undergo dramatic morphological changes including cell differentiation. At this, the interaction and communication between cells is essential. In the best studied myxobacterium *Myxococcus xanthus*, a contact-dependent morphogen called CsgA plays a key role in the induction of this cellular differentiation resulting in fruiting body and spore formation under nutrient-limiting conditions. A debate is ongoing whether CsgA functions as a morphogen in its unprocessed p25-form or processed p17-form. In our laboratory, besides other approaches, co-cultivation studies of different myxobacterial species are performed in order to obtain a deeper understanding of myxobacterial communication. In this approach, an unexpected effect on fruiting body formation of two species (*Chondromyces crocatus* DSM 14714^{T} and *Myxococcus xanthus* DK1622) attracted our attention. Our investigations support the finding of p25 being an active enzyme and show that the simultaneous presence of both, p25 and one of its substrates not only generates a signal for fruiting body formation in *Myxococcus xanthus*: We here present an interesting example of inter-species communication and are able to report for the first time how one myxobacterial species is able to activate development in another myxobacterial species most probably involving the activity of the p25 form of CsgA. Additional experiments need to be carried out to decipher the real morphogen which is likely a chemical and not a protein.

5.2. Introduction

Soil living social myxobacteria live in a complex environment being in permanent contact with miscellaneous bacterial species. Myxobacteria are supposed to possess a variety of antibacterial and antifungal agents or signaling molecules that allow them to proliferate in this competitive ecological niche (Weissman & Müller, 2009). Up to date, a full exploitation of these secondary metabolites under laboratory conditions could not be

obtained. For example, the products of roughly only one third of the biosynthetic gene clusters present in *Myxococcus xanthus* have been identified (Wenzel & Müller, 2009).

In general, bacteria are known to communicate through some diffusible signaling molecules such as acyl-homoserine, lactones, oligopeptides, unsaturated fatty acids, and quinolones, to name only a few (Lowery *et al.*, 2009; Lowery *et al.*, 2010). Myxobacteria seem mainly to communicate in a contact-dependent manner also seen in a phenomenon called development leading to the formation of multicellular spore containing fruiting bodies upon starvation (Shimkets, 1999). However, the discrepancy in the existing models of signaling mechanisms related to fruiting body formation in myxobacteria makes them an interesting topic for investigation, but is also confusing (Shimkets, 1999; Lobedanz & Sogaard-Andersen, 2003; Simunovic *et al.*, 2003; Konovalova *et al.*, 2010).

Myxobacteria show a coordinated behavior during the formation of fruiting bodies. The mechanism of fruiting body formation is well studied in the model organisms *M. xanthus* DK1622. At this, the so called A- and S- motility play a crucial role. A- signaling is known to be involved in quorum sensing leading to the clustering of the cells under nutrient-limiting conditions (Kuspa *et al.*, 1992a, 1992b). Once the cells come in contact, a mechanism called the C- signal induces the concerted cellular development by activating the transcription of necessary genes. C-signaling depends on CsgA (C-factor), a membrane-associated protein initializing this gene expression (Kroos *et al.*, 1988; Shimkets & Rafiee, 1990). CsgA is found to be expressed at a maximum level during the sporulation stage which marks the end of the developmental phase (Kim & Kaiser, 1991). CsgA is a 25 kDa protein (full length) that shows homology to short chain alcohol dehydrogenases (SCAD) (Lee *et al.*, 1995). SCADs are known as intracellular enzymes that catalyze reactions using NAD^+ or $NADP^+$ as co-factors. CsgA features a N-terminal Rossmann fold forming the $NAD(P)^+$ binding pocket and a C-terminal catalytic domain which are both reported to be essential for development in vivo (Lee *et al.*, 1995). Mutants lacking the *csgA* gene do not enter the developmental phase whereas increased levels of CsgA induce cell aggregation leading to fruiting body formation (Lee *et al.*, 1995).

C-signaling is a well elaborated transmission mechanism in *M. xanthus* DK1622, albeit not to full extent. Immunoblot assays proved the presence of two CsgA forms: p25 and p17 (Lobedanz & Sogaard-Andersen, 2003). A debate is ongoing about how and in which

form (p25 and / or p17) the C-factor is initiating development. p17 is similar in size to the C-signal protein purified earlier (Kim & Kaiser, 1990a, 1990b). p17 lacks the N-terminal coenzyme binding pocket. It has been proposed to be the active form of CsgA after a cleavage of p25 by a protease PopC to produce p17. Subsequently, p17 binds to some receptor on the cell surface of adjacent cells (Lobedanz & Sogaard-Andersen, 2003; Rolbetzki *et al.*, 2008; Konovalova *et al.*, 2012) However, no receptor could be identified up to date. These results are not in agreement with other observations: Conserved catalytic residues typical of short-chain alcohol dehydrogenases have been shown to be essential for development in vivo (Lee *et al.*, 1995). Another study revealed that only p25 oxidizes cardiolipin and phosphatidylglycerol to produce diacylglycerol (DAG), dihydroxyacetone, and orthophosphate, but p17 showed no activity with these substrates. The same study reports that the products of this reaction are the cause for initiation of development in *M. xanthus* DK1622 (Boynton & Shimkets, 2015).

We here present data supporting the finding of p25 being an active enzyme. We are able to show that p25 is more than a signal necessary for induction of development in *M. xanthus*: There is evidence that p25 of *M. xanthus*, together with one of its two substrates is an inter-species signal for the induction of fruiting body formation of *C. crocatus*.

5.3. Materials and methods

5.3.1. Strains and growth conditions

M. xanthus DK1622 was grown in CTT liquid medium (Hodgkin & Kaiser, 1977) or on TPM agar (10 mM Tris-HCl at pH 7.6, 1 mM KPO_4 at pH 7.6, 8 mM $MgSO_4$, 1.5 % Bacto agar, BD) or on MVY/2S agar (w/v: 0.2 % soluble starch (Roth), 0.05 % $CaCl_2.2H_2O$, 0.1 % $MgSO_4 \cdot 7H_2O$, 0.5 % Baker's yeast, 25 mM HEPES; adjusted to pH 7.0 with KOH before autoclaving). *Chondromyces crocatus* DSM 14714^T was cultivated in Pol-medium (Kunze *et al.*, 2004) or on MVY/2S agar plates. *Aetherobacter rufus* was grown in PTM medium (w/v: 0.2 % soluble starch, 0.2 % Bacto casitone, 0.005 % KNO3, 0.1 % $MgSO_4 \cdot 7H_2O$, 0.0008 % Fe-EDTA, 25 mM HEPES; adjusted to pH 7.2 with KOH before autoclaving) as well as in TPM agar (1.5 % Bacto agar). *Archangium sp.* Ar3548 was grown in MD1G liquid medium (w/v: 0.3 % Bacto casitone, 0.05 % $CaCl_2.2H_2O$, 0.1 % $MgSO_4 \cdot 7H_2O$, 0.35 % glucose, 5 mM HEPES; adjusted to pH 7.0 before autoclaving with KOH) or on MVY/2S agar plate. The strains were cultivated at 30 °C.

Escherichia coli DH10B and *E. coli* BL21(DE3) were grown in Luria-Bertani medium (Sambrook & Russell, 2001).

5.3.2. Co-cultivation on solid media

Co-cultures of *M. xanthus* with *C. crocatus* or *Archangium sp.* Ar3548 were carried out on MVY/2S agar. Co- culture of *M. xanthus* with *Aetherobacter rufus* was carried out on PTM agar medium. Cell pellets were harvested, washed with sterile water and resuspended in sterile water and adjusted to 5 x 10^9 cells ml^{-1} except for *C. crocatus* as this strain does not grow homogeneously in Pol-medium. Cell clumps of *C. crocatus* were washed with sterile water before using these clumps as the inoculum. 30 µl of resuspended cells were used as inoculum for the co-cultivations. Two myxobacterial strains were inoculated opposite to each other on an agar plate. Incubation was carried out at 30 °C.

5.3.3. Preparation and purification of myxospores

Myxospores were purified with an adaptation of a previous protocol (Inouye *et al.*, 1981). *M. xanthus* grown on starvation agar (TPM agar) was harvested and resuspended in TM buffer (10 mM Tris-HCl, pH 7.6, 8 mM $MgSO_4.7H_2O$). Vegetative cells in liquid CTT medium were stimulated to form myxospores within a few hours as described previously (Dworkin & Gibson, 1964). Chemically induced myxospores were harvested together with the rest of the vegetative cells by centrifugation. The pellet resuspended in TM buffer was sonicated to disrupt the vegetative cells. The obtained cell lysates were layered on a sucrose step gradient: 7.5 %, 15 %, 30 % and 60 %, sucrose prepared in TM buffer. Spores were collected by centrifugation at 1975 rpm for 6 min in Eppendorf centrifuge 5810R centrifuge. The spores formed a band at the interface of the 30 % and 60 % layer of the gradient. The gradient above the spore band was removed, and the rest was collected by centrifugation at 8000 rpm, 20 min at 4 ºC. The collected spores were again subjected to sonication. The procedure was repeated until pure spores were obtained. The purity of spores was verified under a microscope.

5.3.4. Extraction of myxobacterial metabolites from solid/liquid media

Agar (TPM and MVY/2S) containing 8 d old cultures of *M. xanthus* was collected and freeze-dried. The dried material was treated with a solvent mixture of acetone: methanol

(1:1). Extraction was performed in an ultrasonic bath at room temperature for 20 min. The extracts were filtered using sterile filter paper, dried in vacuum and finally resuspended in methanol.

Liquid cultures of *M. xanthus* were cultivated in 300 ml non-baffled flasks with 100 ml of CTT medium at 200 r.p.m , 30 °C for a week. A total of 2 ml of XAD-16 was added to the culture to bind secondary metabolites. Metabolites bound to XAD were extracted with a solvent mixture of acetone: methanol (1:1), evaporated in vacuum, and resuspended in methanol. The obtained crude extract was placed next to the growing swarm of *C. crocatus*.

5.3.5. Preparation of Cell Lysates

M. xanthus cultivated on different agar media (MVY/2S and TPM) was scrapped off the plates at different time points (5 d and 8 d). Collected cells were resuspended in sterile water and lysed by sonication. The cell lysate was centrifuged to get soluble and insoluble fractions. The insoluble fraction was resuspended in sterile water. The resulting sub-fractions were spotted next to a growing swarm of *C. crocatus*.

5.3.6. Enzymatic digest of insoluble fractions of cell lysates

The insoluble fraction of cell lysate was resuspended in a buffer containing (Tris-HCl pH 8.0, Ethylenediaminetetraacetic acid (EDTA) 1mM, 1mM $CaCl_2$). 25 µl of the insoluble fraction was treated with DNase I (Sigma), RNase A (Sigma), lipase from *Pseudomonas cepacia* (Sigma) and proteinase K (Sigma). 2.5µl of DNase (2 mg/ml) was added to the mixture and incubated on ice for 15min. 2.5µl of RNase (10 mg/ml) was added and incubated for 1 hour at 37 °C. Similarly, 30µl of lipase (2 mg/ml) was added to the mixture and incubated at 40 °C for an hour. 5 µl of proteinase k (10 mg/ml) was added to the mixture and incubated at 37 °C for an hour. These pre-treated cell fractions were placed next to the growing swarm of *C. crocatus*.

5.3.7. Cell membrane fractionations of *M. xanthus* DK1622

The membrane separation was performed like described previously (Simunovic *et al.*, 2003). Homogenized spheroplasts were layered on a sucrose gradient and subsequently

divided into different sub-fractions of the cell envelope namely light membrane LM, medium membrane MM and heavy membrane HM. These membrane fractions were placed next to the growing swarm of *C. crocatus*. The HM fraction was further separated into two fractions (peak I and II) according to the same protocol. The obtained fractions were spotted next to the swarm of *C. crocatus*. The plates were incubated at 30 °C.

5.3.8. Treatment of heavy membrane fractions with protease

To 100µl heavy membrane sub-fractions (peak I and II), 25µl a buffer containing (Tris-HCl pH 8.0, EDTA 1mM, 1mM $CaCl_2$), 5µl (20% glucose), 5µl Proteinase K (20 mg/ml), 1µl (10% SDS) were added. The reaction mixture was incubated at 37 °C for an hour. After the incubation, the reaction mixture was cooled down at room temperature and spotted next to the growing swarm of *C. crocatus*.

5.3.9. Cloning, expression and purification of p25-CsgA

The gene encoding *csgA* was amplified from genomic DNA of *M. xanthus* using the primers sumop25_F (5'-GAGGGATCCATGCGCTACGTCATCACC-3') and sumop25_R (5'-CCGGTACCCTACCAGGGCACTTCGG-3'). Restriction sites are underlined. The PCR-product was digested with *Bam*HI and *Kpn*I and ligated with pE-SUMO3kan (LifeSensors) digested with the same pair of enzymes. The construct (pSUMO-CsgA-p25) was transformed into *E.coli* BL21 (DE3) via electroporation. CsgA (p25) was expressed as an N-terminal 6xHIS-SUMO-tag fusion protein in *E. coli* BL21 (DE3).

E. coli carrying the expression construct was grown in LB medium at 37°C with kanamycin (50 µg ml^{-1}) and chloramphenicol (35 µg ml^{-1}) until an OD_{600} of 0.6 was reached. The protein overexpression was performed for 20 h at 16 °C after induction with 0.5 mM Isopropyl *β*-D-1-thioglactopyranoside (IPTG). Cells were harvested and resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM imidazole). The cells were disrupted using Microfluidics Homogenizer at 10,000 psi. Protein purification was performed on an FPLC Äkta Avant (GE Healthcare). About 50 ml of cell lysate was used as a starting material and loaded on a Ni-NTA Histrap FF column pre-equilibrated with 5 column volumes (CVs) of lysis buffer. After sample loading, the column was washed with 5 CVs washing buffer. The protein was eluted with 3 CVs

elution buffer (20 mM Tris-Cl pH 8.0, 500 mM NaCl, 250 mM Imidazole). The eluted protein was further purified on an FPLC Äkta Pure using size exclusion chromatography SEC (16/600 Hiload superdex 200 pg). The flow rate of the running buffer (25 mM Tris-HCl, 500 mM NaCl) was maintained at 1 ml/min. Protein was desalted using PD-10 columns and digested with an SENP2 protease. After enzymatic removal of the SUMO-tag, the protein lost its solubility and precipitated. The precipitated protein was dissolved in TE buffer containing 1.5 % sodium lauryl sulfate and dialyzed overnight in refolding buffer (50 mM Tris-buffered MOPS, pH 8.0, 100 mM KCl, 0.5 % Sodium deoxycholate). The purified recombinant CsgA (p25) was analyzed on a 15 % SDS-PAGE. The protein was confirmed by tryptic in-gel digestion and MALDI-TOF analysis.

5.3.10. Enzymatic reaction of endogenous protein with substrate (cardiolipin and phosphatidylglycerol)

Cardiolipin (CL 18:1) and phosphatidylglycerol (PG 16:0-18:1), both from Avanti polar lipids, were dissolved in 1 % Triton X-100 aqueous solution. CL and PG were mixed with buffer (50 mM Tris-buffered MOPS, pH 8.0, 100 mM KCl, 0.5 % Sodium deoxycholate) containing either CsgA protein (p25) and NAD^+ separately or both together. The reaction mixture was incubated at 30 °C for 15 min. After incubation, the reaction mixture was spotted next to the growing swarm of *C. crocatus*.

5.4. Results

5.4.1. *C. crocatus* DSM 14714^T forms fruiting bodies on top of fruiting bodies of *M. xanthus* DK1622

Under nutrient-limiting conditions, *C. crocatus* DSM 14714^T (*C. crocatus*) forms spectacular fruiting bodies that are ≥ 1000 µm high and consist of orange-branched stalks bearing clusters of small sporangioles at the tips of the branches (Fig. 1a) (Reichenbach, 2005). *C. crocatus* forms its fruiting bodies exclusively on the inoculated cell clump and at the edge of the agar plate. Upon co-cultivation of *C. crocatus* and *M. xanthus* DK1622 (*M. xanthus*) on MVY/2S agar-medium, an interesting example of bacterial interaction and communication could be observed: In contrast to the single cultivation, *C. crocatus*

was able to form fruiting bodies not only on the inoculated cell clump and on the edge of the agar plate but also on top of fruiting bodies of *M. xanthus* (Fig. 1b).

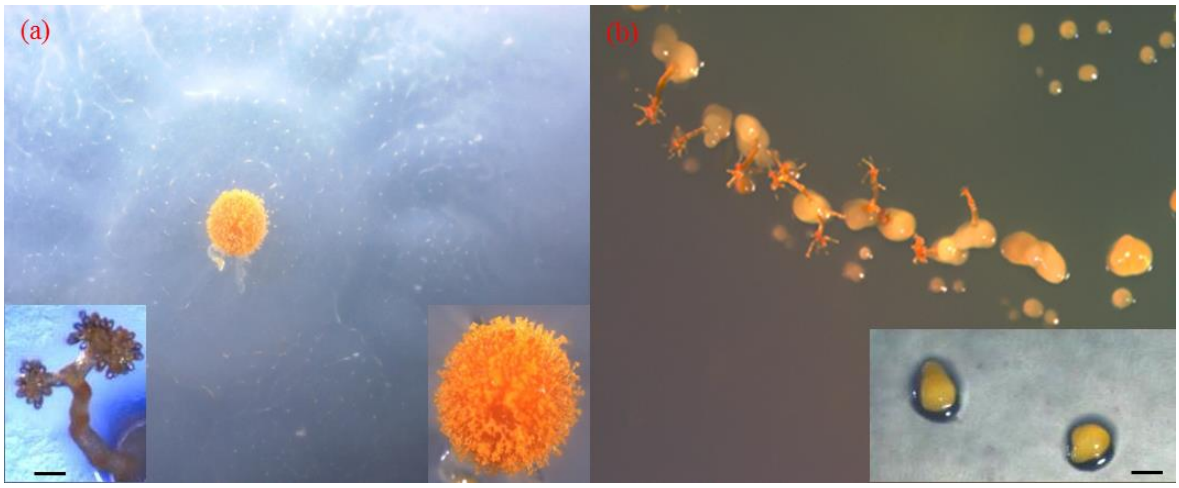

Fig. 1. A) Cell clump of *C. crocatus* on MVY/2S agar medium. Bottom right: Fruiting bodies on the cell clumps (zoom). Bottom left: Single fruiting body of *C. crocatus* (zoom). B) Fruiting bodies of *C. crocatus* on top of fruiting bodies of *M. xanthus* (top). Fruiting bodies of *M. xanthus* in absence of *C. crocatus* (bottom right). Bars: 200 µm.

5.4.2. Myxospores and secondary metabolites of *M. xanthus* are not able to induce fruiting body formation of *C. crocatus*

As *C. crocatus* formed its fruiting bodies on top of fruiting bodies of *M. xanthus*, we aimed to identify the mechanism of the phenomenon. Fruiting bodies of *M. xanthus* contain large numbers of myxospores. In addition to this, the production of secondary metabolites of *M. xanthus* may differ significantly during formation of fruiting bodies when compared to the profile of secondary metabolites under full nutrient supply. Consequently, we first aimed to elucidate if one of both, myxospores or secondary metabolites produced by *M. xanthus* are involved in induction of fruiting body formation of *C. crocatus* on top of fruiting bodies of *M. xanthus*. Purified myxospores spotted on an agar plate beside a swarming colony of *C. crocatus* were not able to induce fruiting body formation of *C. crocatus* (not shown). This was true for both, myxospores harvested from TPM or MVY/2S agar plates and chemically induced spores in liquid CTT medium. We concluded that myxococcal spores are not responsible for the observed phenotype.

To elucidate the involvement of secondary metabolites of *M. xanthus*, secondary metabolites of a culture grown for 8 d on TPM or MVY/2S agar as well as from liquid

CTT medium were extracted. None of the extracts spotted close to a growing swarm of *C. crocatus* induced fruiting body formation (not shown). Consequently, we ruled out the involvement of secondary metabolites in this phenomenon which are extractable with our procedure.

5.4.3. A subfraction of cell lysates from *M. xanthus* is able to induce fruiting body formation of *C. crocatus*

As neither myxospores nor secondary metabolites of *M. xanthus* seemed to be involved in this phenomenon, we tried to systematically eliminate possible inducing components present in *M. xanthus* cultures of different origin. At this, we also tried to elucidate if such a component is only produced in cells of *M. xanthus* under starvation conditions. Cell lysates of *M. xanthus* from cultures grown for 8 d on TPM or MVY/2S agar (starvation conditions for *M. xanthus*) as well as from liquid CTT medium (full nutrient supply) were prepared. The lysates were separated into an insoluble and a soluble fraction by centrifugation. When the insoluble fraction of all lysates was spotted next to the growing swarm of *C. crocatus* in a fruiting body assay, *C. crocatus* formed fruiting bodies within the area where the insoluble material was spotted like observed in presence of intact fruiting bodies of *M. xanthus* (Fig. 2a, b, c). In contrast, the soluble fraction of the lysates did not induce fruiting body formation (Fig. 2d, e, f). As this was true for both, lysates originating from starving and non-starving cultures, we concluded that the inducing component has to be present under both conditions most probably in viable cells and most probably not in myxospores.

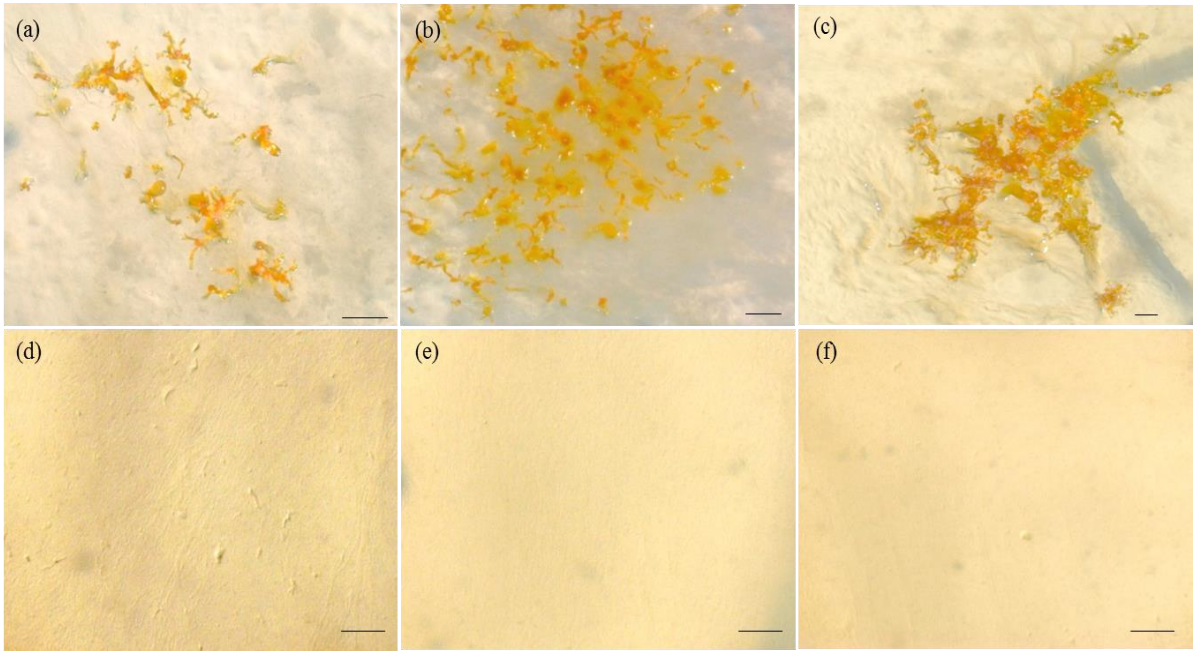

Fig. 2. Result of the fruiting body assay using soluble and insoluble subfractions of cell lysates. Effect of the insoluble fraction of cell lysates prepared from *M. xanthus* cells grown on TPM agar (a), MVY/2S (b) and CTT liquid medium (c). Effect of the soluble fraction of cell lysates prepared from *M. xanthus* cells grown on TPM agar (d), MVY/2S agar (e) and CTT liquid medium (f). Bars: 500 µm.

5.4.4. Proteins and lipids of *M. xanthus* might be involved in induction of fruiting bodies of *C. crocatus*

In order to further narrow down the number of possible candidates for an inducer of the fruiting body formation by *C. crocatus*, we treated the insoluble fraction of the *M. xanthus* lysates with different enzymes prior to the testing for fruiting body induction on agar plates. The insoluble fraction of a cell lysate prepared from *M. xanthus* cells grown in liquid CTT medium was treated in different attempts with DNase I, RNase A, lipase and proteinase K assuming that compounds like DNA, RNA, lipids or proteins might be playing a role in this phenomenon. The pretreated fractions were spotted next to the growing swarm of *C. crocatus*. Insoluble fractions pretreated with RNase A or DNase I induced fruiting body formation of *C. crocatus*. In contrast, no fruiting bodies of *C. crocatus* were observed using a proteinase K pretreated sample and only a few fruiting bodies were observed using a sample treated with lipase prior to spotting (Fig. 3). We concluded that both, membrane proteins and lipids might be involved in induction of fruiting body formation of *C. crocatus*.

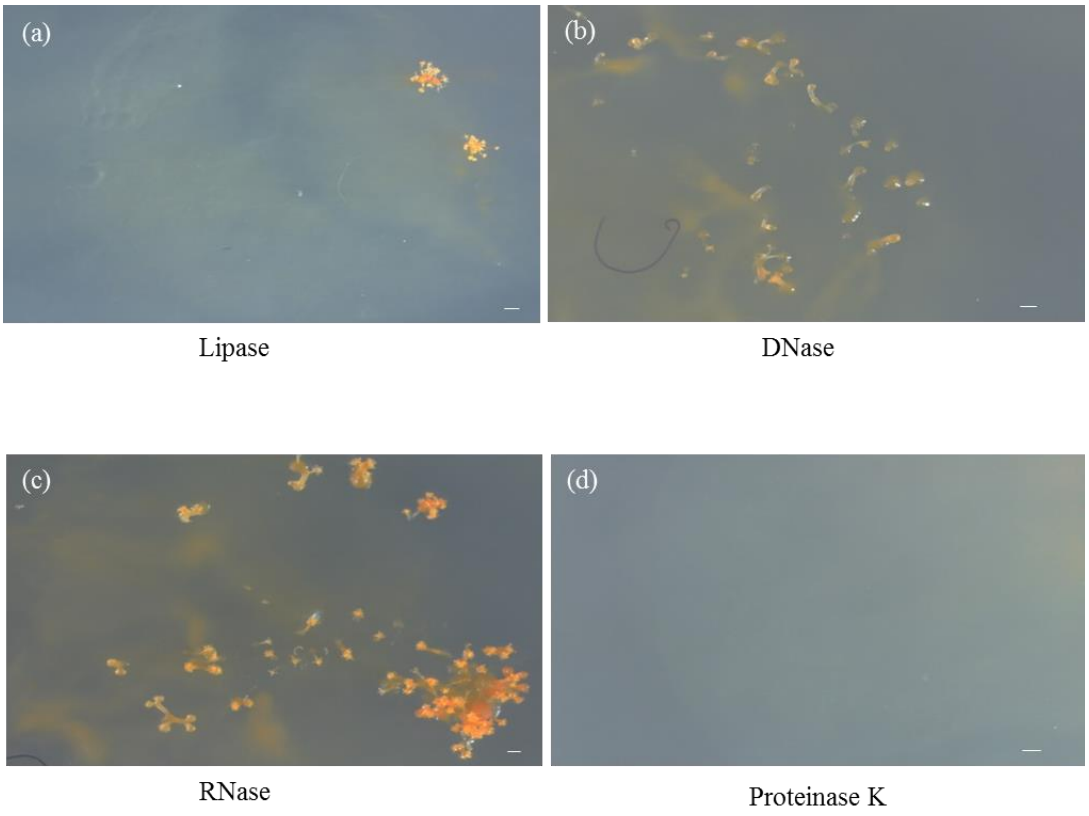

Fig. 3. Result of the fruiting body assay using enzymatically pretreated insoluble fractions of cell lysates prepared from cells grown in CTT liquid medium. (a) Effect of a lysate pretreated with lipase. (b, c) Effect of a lysate pretreated with DNase I and RNase A, respectively. (d) Effect of a lysate pretreated with proteinase K. Bars: 500 µm.

5.4.5. Compounds of the inner membrane of *M. xanthus* are responsible for the stimulation of *C. crocatus* fruiting body formation

The insoluble fraction of the cell lysates should contain material originating from the cell envelope (e.g. hydrophobic lipid membranes and related components like membrane proteins). A pretreatment of this fraction with lipase and protease dramatically reduced or abolished the ability of the mixture to induce fruiting body formation of *C. crocatus*. Based on this we hypothesized that the inducer of fruiting body formation could be either a membrane protein or a lipid-like compound resulting of an enzymatic conversion of a catalytically active membrane protein. To answer this question we aimed to verify if the unknown inducer of fruiting body formation is located in the cell envelope (e.g. inner or outer membrane) of *M. xanthus*. Therefore, we decided to isolate spheroplasts of *M. xanthus* containing all membrane material and subsequently divided this starting material

into subfractions of the cell envelope (light membrane LM; medium membrane MM; heavy membrane HM; HM peak I and peak II) like described previously (Simunovic *et al.*, 2003). Using the homogenized spheroplasts of *M. xanthus* in a fruiting body assay we were able to induce fruiting body formation of *C. crocatus* on agar plates like seen before when using the insoluble fraction of whole cell lysates (Fig. 4a). Using fractions LM, MM, and HM, we could observe the formation of fruiting bodies only in case of fraction HM (Fig. 4b). Fraction HM has been shown to consist predominantly of material originating from the inner membrane (IM) of *M. xanthus* (Simunovic *et al.*, 2003). Fraction HM was further divided into the subfractions peak I and peak II as described by Simunovic and coworkers. These two sub-fractions were spotted again next to *C. crocatus* on agar plates. Fruiting bodies were observed only in presence of one fraction (peak I) (Fig. 4c, i). Interestingly, it has been shown that peak I contains both, the metalloprotease FibA and the p25 form of CsgA, a protein that plays a significant role in C-signaling in *M. xanthus* (Simunovic *et al.*, 2003). No fruiting bodies were observed when these fractions were treated with proteinase K (Fig. 4c, ii and 4d, ii), indicating that one of these proteins might play a crucial role in the induction of fruiting body formation of *C. crocatus*.

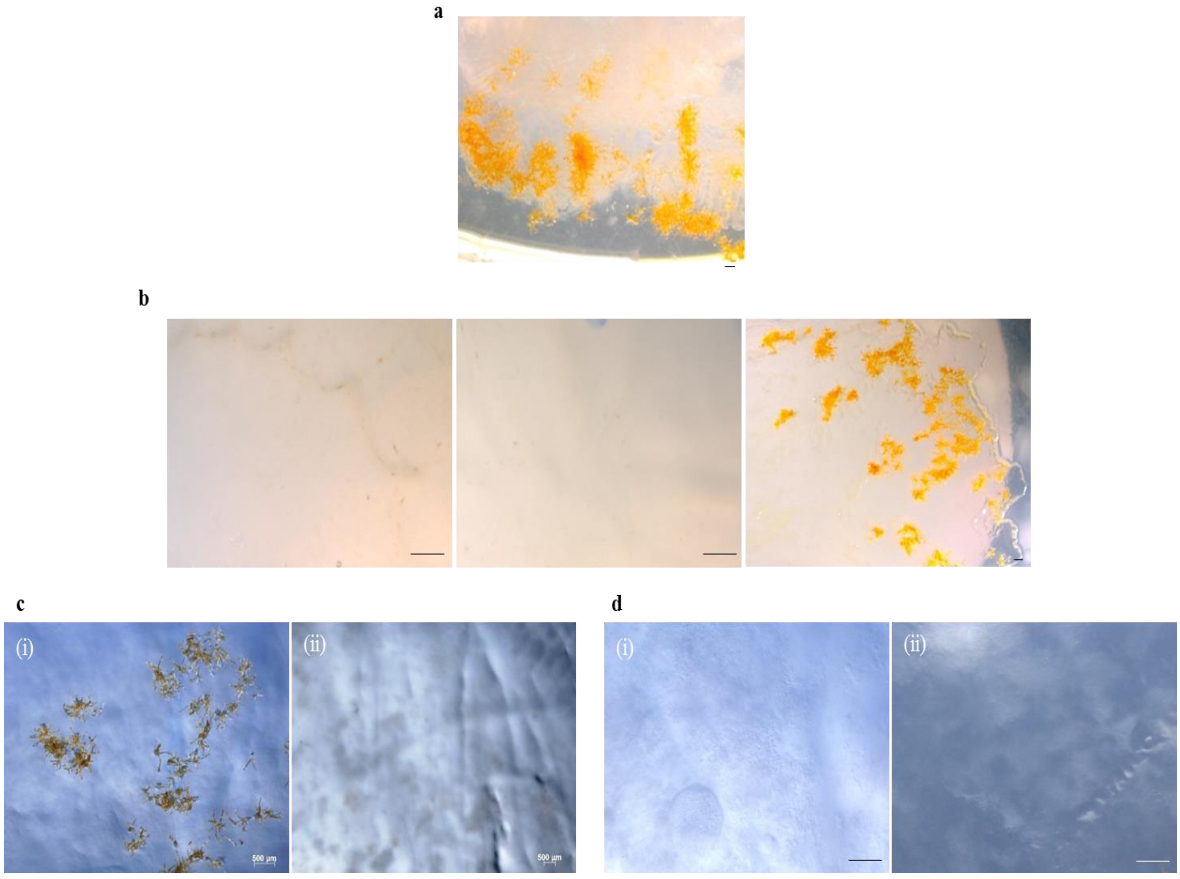

Fig. 4 Effect of various membrane fractions of *M. xanthus* on fruiting body formation of *C. crocatus*. a) Effect of total homogenized spheroplasts. b) Effect of membrane fractions LM, MM, and HM (from left to right), c) Effect of untreated (left) and enzymatically treated (right) subfraction peak I of fraction HM. d) Effect of untreated (left) and enzymatically treated (right) subfraction peak II of fraction HM. Bars: 500 µm.

5.4.6. p25 is not able to induce fruiting body formation of *C. crocatus*

There is an ongoing debate if CsgA is involved in fruiting body formation in *M. xanthus* either in its unprocessed p25-form as an active enzyme producing a signal for fruiting body formation or in its processed p17-form as a signal itself (see introduction). In this study we were trying to identify a substance in *M. xanthus* being able to induce fruiting body formation *of C. crocatus*. We could now show that the unknown compound in *M. xanthus* might either be of a proteinaceous and / or a lipid-like nature (see above).

In addition, we were able to show that this unknown compound of *M. xanthus* can be co-extracted with a subfraction of the membrane (HM peak I) previously shown to contain p25. Based on this we hypothesized that indeed p25 of *M. xanthus* might be involved in

the induction of fruiting body formation of *C. crocatus*. To answer this, we aimed to repeat the fruiting body assay with *C. crocatus* using purified p25 of *M. xanthus*. Therefore, p25 was cloned and expressed as an N-terminal 6xHIS-SUMO-tag fusion protein like described in the methods section. After removal of the SUMO-tag and followed by a re-solubilization (Fig. 5b), the resulting p25 was spotted directly next to a growing swarm of *C. crocatus* in a fruiting body assay. Interestingly, no fruiting body formation of *C. crocatus* could be observed (Fig. 5c).

5.4.7. p25 requires the presence of cardiolipin or phosphatidylglycerol to induce fruiting body formation in *C. crocatus*

It has been reported recently that p25 has a novel phospholipase C like activity that oxidizes the 2'-OH in the head group of cardiolipin (CL) and phosphatidylglycerol (PG). After a subsequent spontaneous conversion this reaction finally leads to the formation of diacylglycerol (DAG), dihydroxyacetone (DHA) and orthophosphate (*P*i) ((Boynton & Shimkets, 2015). In the presence of NAD^+, p25 displays activity on these lipids whereas no such activity was noted for p17-CsgA and CsgA mutant K155R. As we could not observe fruiting body formation in our assays using p25 alone, we concluded that p25 itself might not be the inducer of fruiting body formation of *C. crocatus*, but possibly one of the described products of the enzymatic conversion of CL or PG. To verify this, we included CL and PG in our assays. p25 was incubated with CL and PG in the presence and absence of NAD^+ prior to the fruiting body assays. Both reactions were spotted beside a swarming culture of *C. crocatus* like before and investigated for fruiting body formation. As negative controls CL or PG, as well as p25 alone were used. Indeed, *C. crocatus* formed fruiting bodies only upon pre-incubation of p25 with either CL or PG (Fig. 5d and 5e). No induction of fruiting body formation could be observed on spots containing either p25 or one of the substrates alone. The observed fruiting body formation was independent of the addition of NAD^+, although less pronounced in absence of NAD^+ during pre-incubation. Nevertheless, at this point it remains elusive if the underlying signal of fruiting body formation is one of the reaction products of p25 activity or a complex of CsgA and either CL or PG.

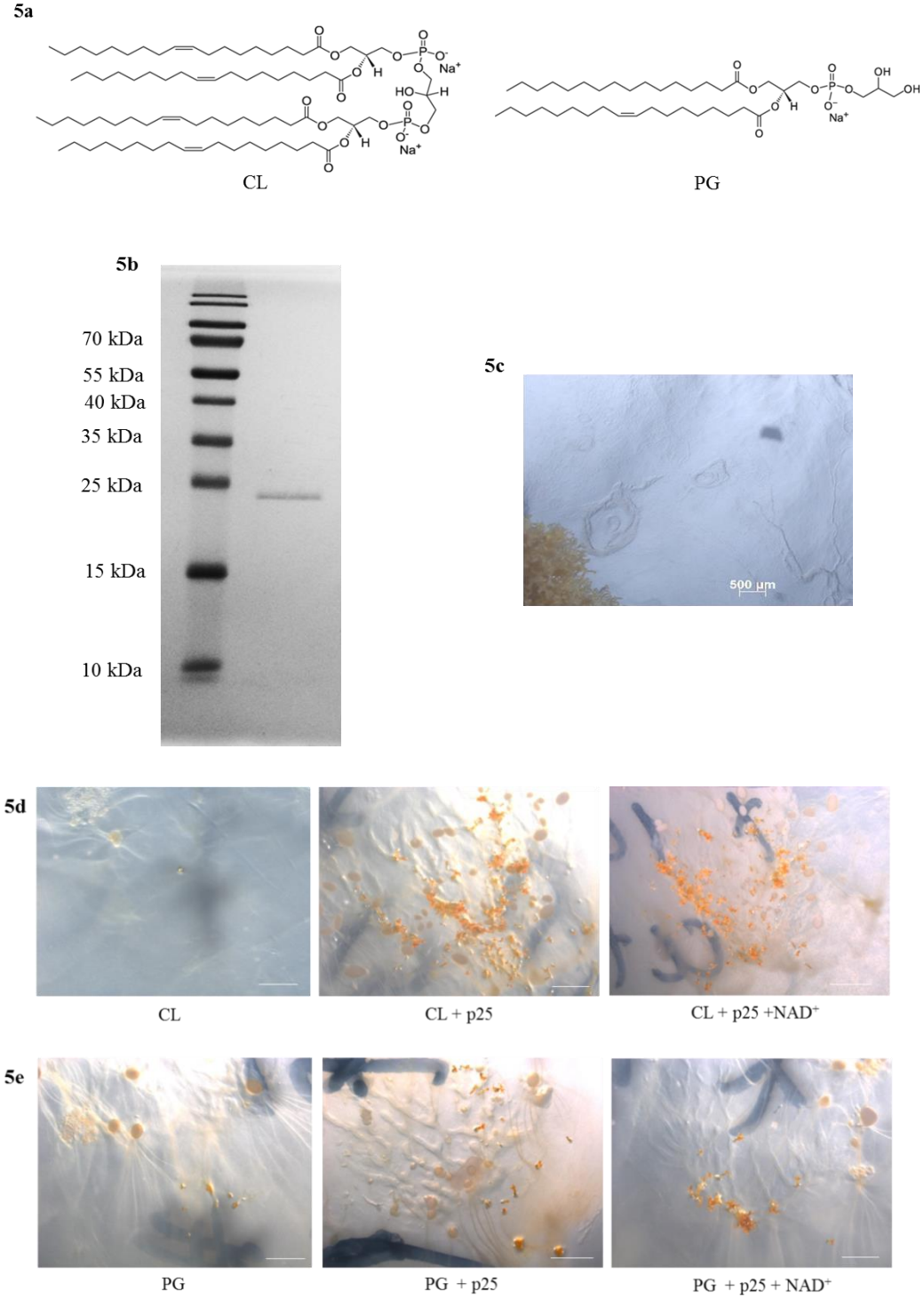

Fig. 5. Effect of p25 and its substrates on fruiting body formation of *C. crocatus*. a) Structure of used cardiolipin (CL) and phosphatidylglycerol (PG) species (from left to right). b) SDS-PAGE analysis of purified recombinant CsgA (p25). Molecular weight markers are shown next to the bands. c) No effect in the presence of p25 alone. *C. crocatus* on the inoculated cell clump (left edge) d) Effect of p25 in presence of CL and CL+NAD^+. e) Effect of p25 in presence of PG and PG+NAD^+. Bars: 500 µm.

5.4.8. Effects upon co-cultivation of various myxobacterial species may highlight the importance of the signal generated by p25-CL-PG

As we could show that p25 together with one of its substrates CL or PG is able to induce fruiting body formation of *C. crocatus* we wondered if this phenomenon is restricted to this special pairing of myxobacterial species. In an initial screen to elucidate this we co-cultured *M. xanthus* with ten different myxobacterial strains. Two of these co-culture attempts resulted in similar interesting observations like in case of *M. xanthus* and *C. crocatus*. Co-cultivation of *M. xanthus* with *Aetherobacter rufus* resulted in an excessive fruiting body formation of *M. xanthus* on top of the *Aetherobacter* culture (Fig. 6a). Co-cultivation of *M. xanthus* and an *Archangium* species resulted in the formation of not yet identified structures on top of fruiting bodies of *M. xanthus* (Fig. 6b). The nature of these observations led us to the hypothesis that also here p25 and its substrates might be involved what is currently under examination.

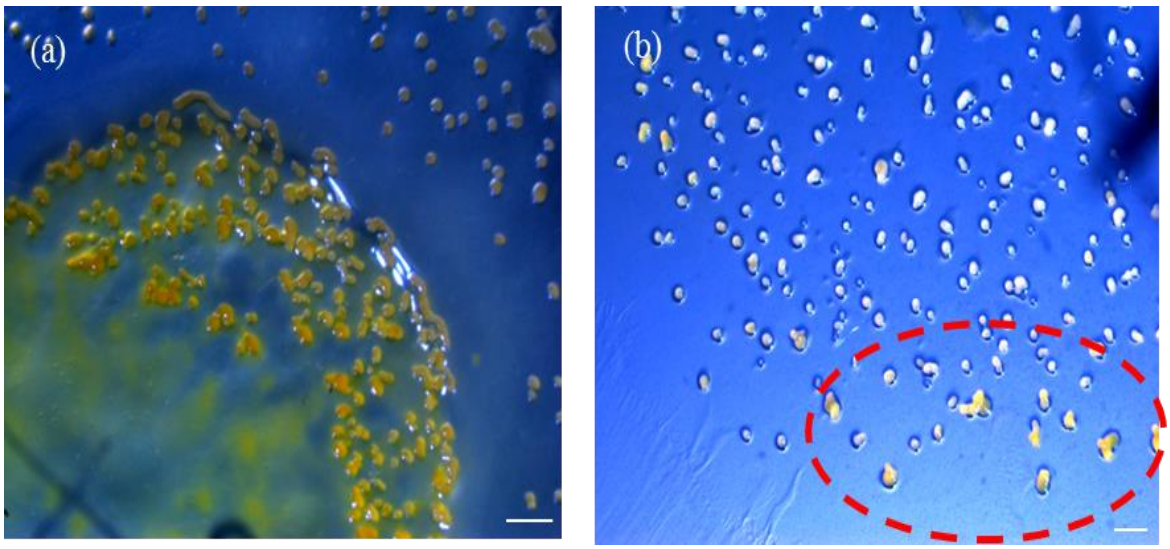

Fig. 6. Phenotypical responses and morphological changes of *M. xanthus* in contact with other myxobacterial species. A) Formation of fruiting bodies of *M. xanthus* on *A. rufus*. B) Formation of unidentified structures on top of fruiting bodies of *M. xanthus* in contact with *Archangium sp.* Ar3548 (encircled). Bars: 500 µm.

5.5. Discussion

We described the identification of a signal being responsible for the induction of fruiting body formation of *Chondromyces crocatus* DSM 14714^T exclusively on top of fruiting bodies of *Myxococcus xanthus* DK1622. We could finally prove by our observation that the presence of purified p25 with either CL or PG is sufficient to induce the formation of fruiting bodies of *C. crocatus*. Consequently, a C-factor generated by one myxococcal species can also be recognized by another myxobacterium triggering a similar developmental programme like in the producer of the C-factor.

To the best of our knowledge, no such signal being able to induce these morphological changes in another myxobacterial species has been observed up to date. In addition, these findings support the recently reported activity of p25 which could be proven to be an SCAD-like enzyme being able to oxidize cardiolipin and phosphatidylglycerol to produce diacylglycerol (DAG), dihydroxyacetone, and orthophosphate (Boynton & Shimkets, 2015). However, at this time it remains elusive if the products of the conversion of CL or PG, or a complex of p25 with one of the two substrates is the signal for fruiting body formation of *C. crocatus*. Nevertheless, it would be astonishing if the latter would be the case. To clarify this, additional fruiting body assays have to be performed using the described reaction products of p25 activity (e.g. DAG). We propose that one of the described products of the reaction is recognized by some unknown receptor on the cell surface of *C. crocatus* to activate the signaling gene cascade like proposed for *M. xanthus* (Boynton & Shimkets, 2015).

Formation of fruiting bodies is well studied in *M. xanthus* DK1622. A similar mechanism occurs in many other myxobacteria and the genomes of these fruiting body forming myxobacteria also encode the genes necessary for development. *C. crocatus* normally forms fruiting bodies either on the inoculum (initial clumps of cells) or at the edge of an agar plate (unpublished data). It is not clear why *C. crocatus* shows this behavior, but it is assumed that these two regions (area of inoculum and the edge of an agar plate) exhibit the necessary features leading to initiation of fruiting body formation: Enough cells gather in an area leading to a rapid local depletion of nutrients and based on both leading to an accumulation of a C-factor like signal to induce fruiting body formation.

We observed fruiting body formation of *C. crocatus* on the top of fruiting bodies of *M. xanthus*. This phenomenon indicated that either the lack of nutrients on the spot where *M. xanthus* formed its fruiting bodies induced the developmental phase in *C. crocatus* or that some unknown compound from *M. xanthus* induced fruiting body formation of *C. crocatus*. As this was initially observed on top of fruiting bodies of DK1622, myxospores were primarily thought to be a possible reason for the formation of fruiting bodies of *C. crocatus*. The coat of myxospores consists of proteins S, C, and U (Inouye *et al.*, 1979; McCleary *et al.*, 1991). Chemically induced spores lack S and C proteins. Protein C was identified to be a 31 kDa C-terminal fragment of the extracellular metalloprotease FibA (Lee *et al.*, 2011). We aimed to verify an involvement of these proteins and other compounds related to myxospores. Therefore, myxospores (formed under starvation conditions and glycerol-induced spores) were harvested, purified and spotted next to *C. crocatus*. Formation of fruiting bodies was not observed on spores ruling out the role of myxospores and its surface proteins in this phenomenon. Secondary metabolites of DK1622 extracted from cells grown on the same agar medium or from liquid culture could also not induce the phenomenon. However, at this point we could not completely rule out their involvement because unknown secondary metabolites might not be extracted sufficiently by the extraction procedure and might need alternative extraction methods using different solvents.

During the formation of fruiting bodies, only 10 to 20 % cells undergo sporulation. Remaining cells either lyse or remain as peripheral rods (Higgs *et al.*, 2014). Components released from lysed cells or even cells in the peripheral rods could have induced the developmental phase in *C. crocatus* and to form fruiting bodies on top of fruiting bodies of *M. xanthus*. The insoluble fraction of cell lysates prepared from 8 d old cultures of *M. xanthus* grown on TMP and MVY/2S agar induced *C. crocatus* to form fruiting bodies. This observation, taken together with the finding that myxospores or secondary metabolites alone were not able to cause this, led us to the conclusion that components of either lysed cells or of cells from peripheral rods were responsible for this activity. The activity could result from any compound present in a viable or lysed cell including DNA, RNA, and protein or lipid species.

To narrow down the possible candidates, the insoluble fraction of the cell lysates was treated with different enzymes. Cell lysates treated with lipase or proteinase K caused

significantly reduced or no fruiting body formation of *C. crocatus*. This result clearly indicated that either a lipid-like compound or protein was responsible for the fruiting body formation of *C. crocatus*. We hypothesized that most probably a membrane-related factor is involved in the induction. Nevertheless, neither the proteinase K nor the lipase in the pretreated solutions prepared from the insoluble fractions could be inactivated without simultaneous inactivation of an unknown inducer. At this point an uncertainty remained about a possible direct effect of residual lipase or proteinase K on growing *C. crocatus* inhibiting its fruiting body formation. Despite this uncertainty and based on our hypothesis of a membrane compound being responsible for the phenomenon, we decided to continue and to focus on membrane-related material.

C. crocatus formed fruiting bodies in presence of total homogenized spheroplasts, heavy membrane fraction (HM) and peak I generated from HM. As it is known that fraction HM largely consists of proteins and lipids of the inner membrane of *M. xanthus*, we could be quite sure that the exogenous inducer of fruiting body formation of *C. crocatus* was located in the inner membrane of *M. xanthus*: As the method and organism used for the fractionation of the membrane material were the same as mentioned by Simunovic and coworkers (Simunovic *et al.*, 2003), the contents of the fractions should be similar to their results: Interestingly, this study reported fraction HM to contain the CsgA protein using immunoblot assays. We assumed that the CsgA present in fraction HM could be involved in induction of fruiting body formation of *C. crocatus*. Nevertheless, CsgA was reported to be present in both fractions derived from fraction HM, in peak I and peak II. We could observe fruiting body formation of *C. crocatus* only on spotted fraction peak I. As we did not perform a similar immunoblot to identify CsgA in the samples we could only hypothesize that in our preparation, CsgA was predominantly present in fraction peak I and absent or present only in a small amount in fraction peak II. Retrospectively, it has to be stated that there was also the possibility that fractions peak I and peak II also differ significantly in CL or PG content (either CL or PG together with CsgA is necessary for induction of fruiting body formation as could be seen in our fruiting body assays).

After the treatment with proteinase K, both, fraction HM and fraction peak I lost their ability to induce fruiting body formation indicating that indeed a membrane protein of the inner membrane has to be involved. At this and because of the observed phenotype of an induction of fruiting body formation we focused on CsgA and its possible role. When

recombinant CsgA (p25) was placed directly next to the growing swarm of *C. crocatus*, no fruiting body formation could be observed. However, it has been shown that supplementation of exogenous, purified CsgA can rescue aggregation and sporulation (Kim & Kaiser, 1991). In this study, pure CsgA protein could not induce *C. crocatus* to form fruiting bodies. This result could have been due to the usage of inactive purified p25 as an outcome of the purification and re-solubilization procedure. (Boynton & Shimkets, 2015) reported that the addition of a lipid extract from *Myxococcus* wild type can restore lipid body production (which is essential for development) during the sporulation stage in a *M. xanthus csgA*$^{-}$ mutant and that p25 degrades CL and PG. We could observe no induction of fruiting bodies on a lipase-treated lysate. Taken together, this led us to the assumption that not inactive CsgA (p25) is the reason for the absence of fruiting body formation on purified p25, but possibly the absence of the substrates of p25, namely CL or PG. This study leads to the enzyme model where CsgA reacts with some cellular component most probably lipid to induce signaling.

CL is reported to be abundant in *M. xanthus* and exclusively present in the inner membrane (Orndorff & Dworkin, 1980). This is in agreement with the outcome of our study as the inner membrane fraction HM contained both, p25 and its substrate CL. The products of the enzymatic reaction may have induced *C. crocatus* to form fruiting bodies. Consequently, the p25 substrates CL and PG were incubated with purified p25 in the presence or absence of NAD^{+}. *C. crocatus* formed a significant amount of fruiting bodies in presence of either p25 and CL or p25 and PG. No fruiting bodies were observed on CL, PG, or p25 alone. Although, this did not finally prove that the enzymatic products of a conversion of CL and PG are the inducers, this at least revealed that both is necessary: enzyme p25 and one of its substrates. Nevertheless, this result fits perfectly to our described results: We were finally searching for an unknown inducer which had to be present in the inner membrane consisting of both, a proteinaceous and a lipid-like nature. p25 together with CL/PG is such an inducer.

As we were not able to use the pure reaction products in a fruiting body assay up to date, we can only hypothesize at this point that (in our experiments) a proteinase K treatment inactivated the p25 enzyme necessary for the formation of DAG and a lipase treatment degraded the reaction product (e.g. DAG). Most likely DAG seems to be the true inducer of fruiting body formation in *C. crocatus*. This has to be shown as soon as DAG is

available in a fruiting body assay. In our assays, the addition of NAD^+ was not necessary to induce fruiting body formation, although the addition resulted in an increase of fruiting body formation. We explain this observation either with the presence of NAD^+ in the growing culture of *C. crocatus* or with NAD^+ originating from heterologous expression being bound to p25.

Taken all together, we here present evidence of the existence of an inter-species signal for fruiting body induction in myxobacteria and that the signal is in general of a double nature: an intra- and inter-species signal.

5.6. References

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Chapter: 6 Discussion and Outlook

6.1. The general scope of this study

This work focused on the discovery of novel myxobacteria. At this, the identification of novel secondary metabolites was of special interest. New strains should be characterized using microbiological, chemical, molecular, and phylogenetic techniques. Numerous strains were isolated and few strains representing new genera and species (*Aggregicoccus edonensis, Racemicystis crocea*, *Paucitangium cumulatus* and *Paucitangium subalbus*) were characterized. Polyphasic taxonomic approaches were applied in the characterization of these novel isolates. In a case of the novel genus *Pautiangium*, not only 16S rRNA gene sequences were analysed; for determining their phylogenetic position, another housekeeping gene, *gyrB*, was amplified and used for phylogenetic analysis.

A second focus of this study was the elucidation of phenotypical effects upon co-incubations of various myxobacterial species. The interaction of different species is of great interest as numerous biosynthetic gene clusters of secondary metabolites seem not to be expressed under routine laboratory cultivation. In addition, very little is known about the inter-species communication of myxobacteria. It was assumed that some of these secondary metabolite gene clusters might be induced when competing swarms of myxobacteria encounter each other. Therefore, co-culture of different myxobacterial strains under laboratory condition were carried out. These co-cultivation studies led to the identification of an interesting phenomenon of cell-cell communication leading to the induction of fruiting body formation induced by an inter-species signal.

6.2. The isolation of novel Myxobacteria and its significance

The isolation methods described in this study not only mimic the natural predatory environment of myxobacteria (Reichenbach, 1999) but also provide its natural nutrient components, as described for *Paucitangium* spp. Myxobacteria are known as the producer of structurally diverse natural products with unique modes of action (Weissman & Müller, 2010). Recently, novel compounds (Edonamides A, B) were isolated from the *Aggregicoccus edonensis* strain (Karwehl *et al.*, 2015). The discovery of novel compounds from *Aggregicoccus edonensis*, described in this study, shows the potential of

novel myxobacteria to produce novel secondary metabolites. The presence of novel compounds in *Aggregicoccus edonensis* revealed that the screening of the novel strains for secondary metabolites under optimum conditions, in combination with their metabolomics analysis, could lead to the detection of novel compounds with potential therapeutic application. The unusual characteristics of the novel strains are discussed below.

6.2.1. *Aggregicoccus edonensis* gen. nov., sp. nov., an unusually aggregating myxobacterium isolated from a soil sample

A novel myxobacterial strain, MCy10622, was isolated in 2013 from a soil sample collected in Irchelpark, Zürich, Switzerland. The bacterium was noted for its aggregate nature of growth in liquid medium. BLASTn analysis of the 16S rRNA gene sequences showed 97 % similarity to *Corallococcus coralloides* (GenBank accession no: AJ811602), *Corallococcus exiguous* (GenBank accession no: AJ233932), and *Stigmatella koreensis* (undescribed strain, GenBank accession no: EF112185). During the characterization of MCy10622, another strain, MCy1366^T, from HZI, Braunschweig attracted our attention. The MCy1366^T strain exhibited similar characteristics to MCy10622. Moreover, their 16S rRNA gene sequences showed 99.9 % similarity indicating that the stains might belong to the same taxon. MCy1366^T was isolated in 1980 from a soil sample collected in Tokyo, Japan. Some morphological features like the fruiting body formation were barely reproducible in MCy1366^T. This has led to the characterization of both strains. MCy10622 strain showed all phenotypic characteristics including the fruiting bodies that were spherical to irregularly shaped, cushion-like, yellow-orange and lacked sporangioles and stalks. A polyphasic approach including phenotypic, genotypic and chemotypic features was applied during the characterization of these novel strains. Many characteristics which include inability to degrade monosaccharides, disaccharides, chitin, cellulose, and Congo- Red stain uptake have strongly suggested their support within the *Myxococcaceae* family.

Fatty acids (FAs) profile of the MCy10622 strain differed from all other strains used in the study due to the presence of significant amounts of iso-$C_{17:1}$ (10.9 %) and the absence of iso-$C_{17:1\omega5cis}$. An extensive comparative study of FAs profiles of MCy10622 and MCy1366^T along with their closely related strains supported the taxonomic placement of the new isolates into a new taxon. DNA-DNA hybridization values for the pairs

MCy1366^T and *C. exiguous* DSM 14696^T (28 %; with 97 % 16S rRNA gene sequence similarity), and MCy1366 and *C. coralloides* DSM 2259^T (35.1 %; with 96.9 % 16S rRNA gene sequence similarity), and 100 % DNA-DNA relatedness of MCy10622 and MCy1366 ^{T} clearly supported their unification as a new species belonging in a novel genus. This finding was further supported by chemotaxonomic studies which include member type species of the *Myxococcaceae* family, and have shown that the two novel myxobacterial isolates were clearly delineated from *Myxococcus, Corallococcus* and *Pyxidicoccus*.

Based on the polyphasic analysis, especially DNA-DNA hybridization, MALDI-TOF data and 16S rRNA gene sequences, it could be determined that strains (MCy1366 ^{T} and MCy10622) belong to a new genus named *Aggregicoccus* and *Aggregicoccus edonensis* species. The *Aggregicoccus* genus represents the most recent genus after *Anaeromyxobacter* (described in 2002) in the family of *Myxococcaceae*. The *Myxococcaceae* now includes the following genera: *Myxococcus, Corallococcus, Pyxidicoccus, Anaeromyxobacter* and *Aggregicoccus*.

The members of the *Myxococcaceae* are known as producers of novel bioactive compounds. Many of these compounds have antimicrobials, cytotoxic and antifungal activities (Garcia & Müller, 2014a). These novel strains might contain compounds with potential activities. Therefore, the discovery of novel strains is clearly correlated with increased changes in finding novel compounds.

6.2.2. *Racemicystis crocea* gen. nov., sp. nov., a soil myxobacterium in the family of *Polyangiaceae*

A bacterial strain designated MSr9521^T was isolated in 2014 from a Philippine soil sample collected in 1986. The bacterium showed a pseudoplasmodial swarm pattern similar to many *Polyangium* strains or *Byssovorax crue*nta By c2^T but differs in shape, colour and size. The peculiar character of the strain was empty looking sporangioles in an older culture which shows similarity with *Hyalangium minutum* in the suborder *Cystobacterineae* and some species of *Polyangium* in the suborder *Sorangiineae*. The vegetative cell size of the novel isolate was significantly longer compared to other members of the *Polyangiaceae*. A polyphasic taxonomic approach was used for the characterization of the strain.

The uniqueness of the isolate was reflected in its fatty acid composition. The novel isolate contained a higher amount of iso-$C_{17:1}$ 2-OH among described species within *Polyangiaceae.* Trace amounts of omega-3/-6 polyunsaturated fatty acids (PUFAs) were found and identified as $C_{20:4}\omega3$, 6, 9, 12 all cis (eicosatetraenoic acid, ETA), $C_{20:5}\omega3$, 6, 9, 12, 15 all cis (eicosapentaenoic acid, EPA), $C_{20:4}\omega6$, 9, 12, 15 all cis (arachidonic acid, AA), and $C_{18:3}\omega3$, 6, 9 all cis (*α*-linolenic acid, ALA). The presence of a significant amount of $C_{17:1}$ 2-OH (determined as a biomarker for *Sorangiineae*) and the predominance of the straight-chain fatty acids over the branched-chain fatty acids confirms that the MSr9521^T strain belongs to the *Polyangiaceae,* suborder *Sorangiineae* (Garcia *et al.*, 2011).

Although the MSr9521^T strain was unable to degrade filter paper, 16S rRNA gene sequence analysis revealed their closest similarity (96 %) to cellulose degrading *Byssovorax cruenta* By c2^T. A distinct branch in the *Polyangiaceae* in the phylogenetic tree suggests that the novel strain represents a novel genus, *Racemicystis,* and a novel species *Racemicystis crocea*.

Studies have shown that 50 % of the myxobacterial compounds are derived from the *Polyangiaceae* (Garcia & Müller, 2014b). The compound diversity in *Polyangiaceae* is astounding. *Racemicystis crocea* being a novel genus in the *Polyangiaceae* is expected to be a promising candidate that most likely harbour novel compounds.

6.2.3. *Paucitangium cumulatus* gen. nov., sp. nov. and *Paucitangium subalbus* sp. nov., novel soil myxobacteria from Nepalese soil samples

The myxobacterial strains designated as MCy10943^T and MCy10944^T were isolated in 2014 form soil samples collected from Phukot, Kalikot, Western Nepal and Godawari, Lalitpur, Central Nepal in 2013. The bacteria share common characteristics for producing transparent, thin, film colonies with flare colony edges. As with other myxobacteria, the novel isolates exhibit a predatory ability to lyse microorganisms and grow under aerobic and mesophilic conditions. The novel isolates share many similarities, but they differ morphologically and physiologically in some developmental stages. *Paucitangium cumulatus* MCy10943^T forms irregular variable oval to bean shaped fruiting bodies whereas *P. subalbus* MCy10944^T exhibits fruiting bodies-like aggregates forming as oval, small to large mounds. Both of them lack sporangioles.

The novel isolates share fatty acids profiles with $C_{17:0}$ 2-OH, iso-$C_{15:0,}$ $C_{16:1}$ and iso-$C_{17:0}$ as the major fatty acids. Both strains differ in the amount of $C_{16:0}$ and iso-$C_{15:0}$. MCy10943^T contains a higher percentage of $C_{16:0}$ but with a lower amount of iso-$C_{15:0}$ compared with MCy10944^T.

The BLASTn analysis of the 16S rRNA gene sequence of MCy10943^T and MCy10944^T showed their closest similarity with (98 %) to *Cystobacter violaceus* Cb vi29 (GenBank accession numbers: AJ233905) and *C. violaceus* Cb vi34 (GenBank accession numbers: AJ233906). Since there was only 2 % difference in the phylogenetic distance from its closely related strains, the housekeeping gyrase B gene was chosen to be amplified using specifically designed degenerate primers. The phylogenetic analysis of 16S rRNA gene sequences and partial gyrase B gene sequence confirmed their separate clade position in the *Cystobacteraceae*. Moreover, a similarity of 97-98 % in the 16S rRNA gene sequence from its closest relatives has been found among genera within the *Cystobacteraceae* (Garcia *et al.*, 2011).

Members of the *Cystobacteraceae* are the best producers of bioactive compounds in addition to *Polyangiaceae*. The closest relatives of *Paucitangium* spp. produce a number of active compounds. For example: argyrin, vioprolide from *Archangium*, althiomycin, myxalamide from *Cystobacter* and angiolam, tubulysin from *Angiococcus.* Therefore, these novel strains are predicted to produce compounds which remained undiscovered till date.

6.3. Interaction between myxobacteria reveal an inter-species signal for induction of fruiting body formation

This study presents an interesting inter-species communication in myxobacteria and shows the role of CsgA in this interaction. We showed that the downstream enzymatic products produced by the full length CsgA protein (p25) with one of its substrates cardiolipin (CL) or phosphatidylglycerol (PG) could act as a C-factor to induce the fruiting body formation in *Chondromyces crocatus* DSM14714^T and other myxobacteria. Till date, no such inter-species nature of C-signal has been elaborated in myxobacteria.

The C-signal has been exclusively studied in the model myxobacterium *Myxococcus xanthus* DK1622. However, the transmission mechanism of C-signal has not been

elaborated to the full extent. Other fruiting body forming myxobacteria also contain genes necessary for development including *csgA*. But, the role of CsgA in inter-species interaction or communication has not been reported so far, although these bacteria live together in their ecological niche. We observed the interesting myxobacterial interaction during the co-cultivation of different myxobacterial strains where *C. crocatus* formed its fruiting bodies on top of the fruiting bodies of *M. xanthus*.

Similarly, morphological changes of *M. xanthus* in contact with other myxobacteria (*Aetherobacter rufus* and *Archangium* sp. Ar3548) were also observed. This phenomenon further consolidates our results presented here. These results presented in this study validate that both p25 and its substrate are necessary for inducing *C. crocatus* to form its fruiting body. However, it could not be demonstrated that the downstream reaction products are the main inducers of the fruiting body formation. Therefore, the downstream reaction products are further needed to be used in this study to determine the inducing signal of the fruiting body formation.

To sum up, this study shows the existence of an inter-species signal for the fruiting body induction in myxobacteria.

6.4. References

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Chapter: 7 Appendix

Curriculum Vitae

Personal information

Name	Ram Prasad Awal
CurrentAddress	Pfaffenkopfstraße 24 66125 Saarbrücken, Germany
Home Address	Bekhal, Bhaktapur 44800, Nepal
Nationality	Nepalese
Date of birth	26.07.1983

Education

Oct 2012 -Sep 2016 **PhD**
Department of Microbial Natural Products,
Helmholtz-Institute for Pharmaceutical Research Saarland (HIPS),
Department of Pharmaceutical Biotechnology, Saarland University, Germany

Publications

Sakshi Sood*, **Ram Prasad Awal***, Joachim Wink, Kathrin I. Mohr, Manfred Rohde, Marc Stadler, Peter Kämpfer, Stefanie P. Glaeser, Peter Schumann, Ronald Garcia and Rolf Müller (2015). *Aggregicoccus edonensis* gen. Nov., sp. nov., an unusually aggregating myxobacterium isolated from a soil sample. *Int J Syst Evol Microbiol* **65**, 745-753. (* equally contributed)

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Oral presentation	*Aggregicoccus edonensis*, a novel genus and species of myxobacterium isolated from soil sample 41st International Conference on the Biology of Myxobacteria, Nice, France. May 25-28, 2014
	Myxobacteria- fascinating bacteria with potential application (2013). Summer symposium of the Interdisciplinary Graduate School of Natural Products Research, Saarbrücken, Germany
Poster presentation	MSr9521- a novel genus and species of myxobacterium in the suborder Sorangiineae. July, 2015. HIPS-Symposium, Saarbrücken, Germany.
	Discovery of three novel genera in Myxobacteria, VAAM Workshop 'Biology of Bacteria Producing Natural Products' University of Freiburg, Freiburg, Germany. September 28-30, 2016.
Nov. 2005- Mar. 2011	**Master of Science (M.Sc.) in Medical Microbiology, Tribhuvan University, Nepal**.
Thesis Title	Method for investigating Toll-Like Receptor 1 Iso602Ser (T1805G) polymorphism associations with tuberculosis in Nepalese patients from Mycobacterial Research Laboratory, Anandaban Hospital, Lele, Nepal.
Lab exposure	Three months in the laboratory of Dr. Neil T. Young in Institute of Medical Sciences, University of Aberdeen, UK.
Publication	Identifying the function of a novel tolerogenic dendritic cell population, Michail Kalogeropoulos, Rahul Chaitanya Khanolkar, Ram Prasad Awal, Neil Young (July 2011). Poster presented at BSI meeting on Dendritic Cell Regulation of immunity at the University of Manchester
2002 –2005	Bachelor in Microbiology, Tribhuvan University, Nepal.

2004 - 2007	Post Advanced course (6 Semesters) in German Language from Campus of International Language, Tribhuvan University, Kathmandu, Nepal.

Research and Teaching Experience

July, 2011-Sep.2012	Lecturer for immunology in Kantipur College of Medical Sciences, Kathmandu, affiliated to Tribhuvan University
July 26-Oct.12 2010	Research Associate in the School of Medicine and Dentistry, College of Life Sciences and Medicine, University of Aberdeen, UK
Sep. 2008-July 17 2010	Research Student in Mycobacterial Research Laboratory, Anandaban Hospital, Lele, Nepal.
Feb. 2008 – Sep.2012	German Language Teacher at Campus of International Language, Tribhuvan University, Kathmandu