Iso-fatty acids (FAs) are the dominant FA family in all myxobacteria analyzed. Furthermore, it was postulated that iso-FAs or compounds derived thereof are involved in fruiting body formation in *Myxococcus xanthus*, since mutants with a reduced level of iso-FA due to a reduced level of the precursor isovaleryl-CoA, are delayed in aggregation and produce only few myxospores. In order to elucidate the function of iso-FAs and their corresponding lipids we have analyzed the developmental phenotype of mutants having different levels of iso-FAs resulting in a clear correlation between the amount of iso-FAs and the delay of aggregation and reduction in spore yield. Addition of either isovalerate or 13-methyltetradecanoic acid resulted in restoration of the wild-type FA profile and normal development. Detailed analysis of the fatty acid (FA) profile during fruiting body formation in *Myxococcus xanthus* wild-type revealed the specific accumulation of 13-methyltetradecanoic acid resulted in restoration of the wild-type FA profile and normal development. Detailed analysis of the fatty acid (FA) profile during fruiting body formation in *Myxococcus xanthus* wild-type revealed the specific accumulation of 13-methyltetradecanoyl-2-O-(13-methyltetradecanol)-glycero-3-phosphatidylethanolamine (VEPE) and 1,2-di-(13-methyltetradecanoyl)-3-(13-methyltetradecyl)glycerol (TG-1), respectively. The structures of these unusual ether lipids have been determined by spectrometric methods and synthesis (for TG-1). Analysis of several mutants blocked at different stages of development indicated that the biosynthesis of TG-1 is developmentally regulated and that VEPE might be an intermediate in the TG-1 biosynthesis. Finally, addition of TG-1 to mutants blocked in the biosynthesis of isovaleryl-CoA could restore aggregation and sporulation emphasizing the important role of iso-branched lipids for myxobacterial development.

Myxobacteria are Gram-negative soil bacteria that can form fruiting bodies under starvation conditions (1). These fruiting bodies are visible to the naked eye and can reach a tree-like complexity in some species. During the developmental process, a certain amount of vegetative cells differentiates into heat and desiccation resistant myxospores enabling the survival of the colony. The model organism to study this highly complex process is *Myxococcus xanthus* from which different extracellular signals and regulators have already been described which can be combined in a model likely reflecting the essential parts of the underlying regulatory network (2,3). However, the biochemical changes that are involved in this process or appear as a result of it have been barely identified. That biochemical changes occur is evident from change in cell shape from vegetative rods to round myxospores with thick spore coats (4,5) and high amounts of trehalose (6) which is proposed to act as a compatible solute.

Furthermore, the pioneering work of White and co-workers showed that several enzymes involved in increase of carbohydrate biosynthesis are induced during sporulation (7-9).
Ten years ago, Downard and coworkers identified a mutant that was disrupted in the branched chain keto acid dehydrogenase (Bkd) complex which resulted in a developmental phenotype forming almost no aggregates or spores under starvation conditions (10). The Bkd complex is involved in the degradation of the branched chain amino acids leucine, valine and isoleucine, with all three being essential amino acids for *M. xanthus* (11). The degradation products of these amino acids are isovaleryl-CoA (IV-CoA), isobutyryl-CoA (IB-CoA) and 2-methylbutyryl-CoA, respectively (12) which are used as starting units for iso- and anteiso- fatty acids and also for several secondary metabolites from different bacteria (e.g. avermectin from *Streptomyces avermitilis* (13) or myxothiazol (14), myxalamides (15) and aurafuron (16) from *Stigmatella aurantiaca*). In all myxobacteria analyzed so far, iso-FAs are the dominant fatty acid family (17-21). Consequently, disruptions of the *bkd* genes (also termed *esg* from *E*-signal (22)) result in a dramatic decrease in the amount of iso-FAs due to a severe reduction of isovaleryl-CoA levels. Since the wild type fatty acid profile and the normal life cycle can be restored in the *bkd* mutant by adding isovalerate (IVA), it was speculated that an iso-FA or a corresponding lipid is involved in the developmental process (10,23).

In contrast to this, a phosphatidylethanolamine (PE) containing the straight-chain fatty acid (SCFA) hexadecenoic acid 16:1ω5c, has been identified by Shimkets and coworkers to act as a chemotaxis signal with a very narrow activity profile (24). The *bkd* mutant showed a high excess of this and other SCFAs which would result in an excess of the required signal and therefore in a loss of the chemotaxis. However, recent analysis of a mutant which produces only trace amounts of SCFAs revealed that this FA or the corresponding PE is not required for fruiting body formation or spore formation as both occurred indistinguishable to the wild-type (25).

Interestingly, inactivation of the *bkd* locus does not lead to a complete loss of iso-FA production which is due to the presence of a novel alternative pathway to IV-CoA (Figure 1), branching from the well-known mevalonate-dependent isoprenoid biosynthesis (18,26). This pathway is so far unique to myxobacteria and is highly induced in *bkd* mutants. Recently, we were able to show that this pathway is also induced during fruiting body formation and might function as an alternative source for IV-CoA under these leucine limiting conditions (27). Furthermore, we could show that the decrease in iso-FA formation is even more severe in mutants where both pathways to IV-CoA are disrupted (27).

Here, we describe the developmental phenotype of this double mutant and present evidence that the developmental defect might be due to the loss of unusual iso-branched ether lipids which were identified as novel biochemical biomarkers of myxobacterial development since they are produced mainly in fruiting body-derived myxospores.

**Figure 1**

**EXPERIMENTAL PROCEDURES**

**General** - CTT medium (28), TPM agar (11), glycerol- (29) and starvation buffer induced sporulation (SBS) (30), large scale fruiting body formation (27), and GC-MS based fatty acid analysis of total cellular lipids (27) have all been published. *M. xanthus* strains used in this study are listed in Table 1. For complementation experiments isovalerate (IVA), 13-methyldodecanoic acid (iso15:0), 13-methyldodecanol, rac-1,2-di-(13-methyldodecanoyl)glycerol (TG-1), rac-1,2-di-(hexadecanoyl)-3-(13-methyldodecyl)glycerol (TG-2) and glycerol tripalmitate (TPG) were dissolved in water (IVA) or methanol to a final concentration of 100 mM and diluted into TPM agar to a final concentration of 1 mM directly before plating. The triglycerides (which did not dissolve well in methanol) were mixed vigorously and added as suspension. Developmental aggregation was followed visually using an Olympus SZH or a Zeiss Axiovert 200 inverted microscope. Cells were photographed using a Zeiss Axiocam MRC camera. Levels of sporulation were determined after development for 72h as the number of sonication and heat-resistant colony forming units (31). Separation of peripheral rods (PR) and myxospores from developing cultures at 72h (32) and feeding experiments with L-[5,5,5-D3]leucine (Deutero GmbH, Kastellaun, Germany) (27) were performed as described previously.
Extraction and fractionation of lipids - Extraction of lipids from bacterial cells was carried out using the method of Bligh and Dyer (37), modified by Lewis et al. (38). The lipid extract was evaporated to dryness under a gentle stream of nitrogen and dissolved in chloroform to give a concentration of 10 mg total lipids per ml. One ml of this solution was fractionated by solid-phase extraction on a column of 300 mg of unmodified silica gel 60 (Merck, Darmstadt, Germany) in chloroform. Neutral lipids were eluted from the column with 15 ml of chloroform, medium polar lipids were eluted with 15 ml of an acetone – methanol mixture (9:1 v/v), and phospholipids were eluted with 10 ml of methanol. For higher lipid quantities, amounts of silica gel and solvents were scaled up appropriately.

Separation of lipids by HPLC - Phosphatidylethanolamine molecular species were separated similar to a published procedure (39) with UV detection at 206 nm and a Nucleosil C18 column (250 × 10 mm, Macherey-Nagel, Düren, Germany). The mobile phase was acetonitrile – methanol – water (30:68:2 v/v) containing 5 µM ethanolamine at a flow rate of 4 ml/min. For further purification, the mobile phase composition was changed to acetonitrile – methanol – water (30:65:5 v/v). Neutral lipid classes were fractionated by NP-HPLC on a LiChrospher silica gel column (250 × 4 mm, Merck, Darmstadt, Germany) with hexane – methyl tert-butylether (MTBE) (98:2 v/v) as a mobile phase at a flow rate of 1 ml/min and UV detection at 215 nm.

Structural elucidation of lipids - Samples were directly injected into a Bruker HCTplus ESI ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Phosphatidylethanolamine and plasmeylenethanolamine molecular species were analyzed in negative ionization mode, and MS/MS fragmentation patterns were interpreted according to the literature (40,41). In positive mode, triacylglycerols and their ether analogues could be analyzed in the presence of 10 mM of LiCl, favorizing the formation of [M+Li]⁺ ions, whose structural elucidation was carried out according to the literature as well (42,43). For high resolution mass spectrometry, samples were injected into a Bruker micrOTOF ESI-TOF mass spectrometer, and the sum formula was calculated from the obtained exact ion mass. NMR spectra were recorded on Bruker DRX 500 or Bruker Avance 500 spectrometers with CDCl₃ as solvent and internal reference.

Analysis of phospholipids via HPLC-MS - For relative quantitation of phospholipid molecular species, 10 µl of the phospholipid fraction dissolved in acetonitrile – methanol (1:3 v/v) were separated by analytical HPLC (Agilent 1100 HPLC system (Agilent, Waldbronn, Germany)) on a Nucleodur Gravity RP18 column (125 × 2 mm, Macherey-Nagel) coupled to a Bruker HCTplus ion trap mass spectrometer. The mobile phase consisted of methanol – water (9:1 v/v; solvent A) and acetonitrile (solvent B) with both solvents containing 5 µM ethanolamine. The gradient started at 50 % B for 2 min, then decreased to 30 % B over a time of 48 min, increased to 95 % B over 5 min and held at this percentage for 4 min, followed by a decrease to 50 % over 5 min and a reequilibration period of 4 min. The flow rate was maintained at 0.4 ml/min. Quantitation was carried out using Bruker QuantAnalysis software, calculating the areas under the [M-H]⁻ ions from appropriate extracted ion chromatograms.

Analysis of whole lipids by GC-MS - The lipid sample was evaporated to dryness and dissolved in a mixture of hexane and MTBE (1:1 v/v). To 80 µl of this solution, 20 µl of N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA, Macherey-Nagel) were added, and the sample was incubated at 37°C for 30 min. The gas chromatograph was an Agilent model 6890 with a 7683N mass selective detector and a high-temperature 50%-phenyl-dimethylpolysiloxane capillary column (OPTIMA-17TG 25 m × 0.25 mm × 0.1 µm, Macherey-Nagel). 2 µl of sample solution were injected using a pulsed splitless injection technique. The helium flow rate was set to 1.5 ml/min, inlet and GC-MS transferline temperatures were held at 300°C. Column oven temperature was initially set to 200°C and held for 5 min, then increased to 340°C at a rate of 4°C/min followed by 15 min at 340°C.

Synthetic procedures - 13-methyltetradecanal and 13-methyltetradecanoic acid were synthesized by stepwise oxidation of 13-methyltetradecanol (44) similar to published procedures (45,46). Additionally, 13-methyltetradecanol was
transformed to 1-iodo-13-methyltetradecane which then was reacted with DL-1,2-O-isopropylideneglycerol resulting in the formation of DL-1-O-13-methyltetradecylglycerol after hydrolysis of the protecting group following standard synthesis methods. Acylation of the free hydroxy groups of DL-1-O-13-methyltetradecylglycerol by either 13-methyltetradecanoic acid or palmitic acid similar to published procedures (47) resulted in the final dialkyl ether lipids rac-1,2-di-(13-methyltetradecanoyl)-3-(13-methyltetradecyl)glycerol (TG-1) and rac-1,2-di-(hexadecanoyl)-3-(13-methyltetradecyl)glycerol (TG-2), respectively. Detailed descriptions and spectroscopic data of all synthesis products and intermediates can be found as supplemental information on the JBC website.

Table 1

RESULTS

Fruiting body formation and developmental sporulation in Myxococcus xanthus is dependent on the amount of iso-branched fatty acids - Analysis of fruiting body formation of the wild-type (DK1622), a mutant impaired in leucine degradation (DK5643/Δbkd) and a strain with an additional disruption of the 3-hydroxy-3-methylglutaryl synthase encoding gene (DK5624/Δbkd, mvaS::kan) showed that aggregation was delayed most severely in DK5624 which produces only trace amounts of iso-FAs (27). Similarly, the number of spores decreased 100-fold from 4% for DK5643 to 0.04% for DK5624 compared to the wild-type (100%). As expected, the aggregation and sporulation (to 40% of wild-type level) of both mutants could be restored by the addition of IVA (1 mM). In contrast to previous observations based on IVA addition to the growth medium and into the starvation medium (10), IVA supplementation of the starvation medium alone was sufficient for complementation (Figure 2a). Furthermore, complementation to 30% of the wild-type level was achieved by supplementation of the starvation medium with the most abundant iso-FA 13-methyltetradecanoic acid (iso15:0, final concentration 1 mM). Microscopy employing higher magnification of the colonies after 72h of development showed that the fruiting bodies of DK5624 (Figure 3b) were less compact and hardly produced any spores surrounding the aggregates as it could be frequently observed for DK1622 fruiting bodies (Figure 3a).

Reduced fatty acid derivatives accumulate during fruiting body formation - The complementation of the developmental defect of DK5624 by addition of either IVA or iso15:0 indicated the involvement of iso-FAs in fruiting body formation which was postulated previously. However, gas chromatographic (GC) analysis of the wild-type FA profile during fruiting body formation revealed no significant change in the level of major iso-FAs. Nevertheless, two compounds increased during development (Figure 4). The first compound increased during the first 24 h of development and was predicted to be iso14:0 3-OH by the microbial identification system used but GC-coupled mass spectroscopy (MS) of this compound led to its identification as dimethyl acetal of 13-methyltetradecanal, the aldehyde derived by reduction of iso15:0. The second compound increased constantly during the whole experiment (72h) and eluted very late from the GC column. Furthermore, it could only be detected reproducibly after trimethylsilylation of the extract generated by the fatty acid methyl ester (FAME) method (27), thus indicating the presence of acidic protons. Subsequent GC-MS analysis of this compound led to the identification of 1-O-13-methyltetradecylglycerol bearing an ether bond between 13-methyltetradecanol, the alcohol of iso15:0, and glycerol at the 1-position. The structure of both compounds was confirmed by synthesis and subsequent comparison of natural and synthetic compounds with respect to their fragmentation pattern and retention time. During fruiting body formation only ~1% of all cells involved transform into myxospores, a process which is known to be dependent on the culture conditions (48,49). The remaining cells are thought to be used as nutrient resource to support fruiting body formation and sporulation or retain their morphology as vegetative-like cells called peripheral rods (PRs). Moreover, despite their rod-shaped appearance PRs are clearly distinguishable from vegetative cells by several characteristics (50-52). In order to analyze whether the two
unusual compounds were present in all cells during fruiting body formation. PRs and myxospores were separated by sucrose density centrifugation (32) and the FA profile of both cell types was analyzed. Surprisingly, both new compounds are highly enriched in the myxospores with up to 20% and 40% of the total FAME extract consisting of the derivatized aldehyde and the glycerol ether, respectively. Contrary, PRs show no such increase and look much more like vegetative cells with respect to their fatty acid profile (Figure 5). Furthermore, a third compound was detected in the spores by GC-MS that was identified as the vinyl ether analogue of the already described alkyl ether. Additional analyses showed that the ratio of vinyl ether to dimethyl acetal is strongly dependent on the workup procedure. Prolonged acidic conditions and the absence of water led to the exclusive formation of the dimethyl acetal whereas only vinyl ether could be detected after alkaline hydrolysis, careful neutralization and silylation of the resulting mixture (data not shown). This readily indicated that the dimethyl acetal might be derived from acidic vinyl ether cleavage and that the alkyl and the vinyl ethers are derived from larger lipids. No accumulation of the aldehyde or the glycerol ether were observed in spores obtained by glycerol induction which are already known to be different from fruiting body spores by several other features (51). Moreover, SBS spores show an increase of the aldehyde. This indicates that the morphological differences of the three spore types (30,51) are associated with specific biochemical differences.

**Figure 4**

**Figure 5**

13-methyltetradecanal and 1-O-13-methyltetradecylglycerol originate from two lipids accumulating during fruiting body formation - During the derivatization cellular lipids decompose and their acyl residues are transformed into the corresponding fatty acid methyl esters. Similarly, vinyl ethers (e. g. plasmenyl lipids, also known as plasmalogens) are not stable under these acidic conditions and give rise to aldehydes which are converted into the corresponding dimethyl acetals whereas alkyl ethers (e. g. plasmanyl lipids) are stable but loose the acyl groups at the sn-2 and sn-3 positions (53). In order to identify the true nature of the lipids yielding 13-methyltetradecanal and 1-O-13-methyltetradecylglycerol we isolated the different lipid classes from developing cells. The main membrane lipid family of Gram-negative bacteria is thought to be phosphatidylethanolamine (PE) as already described for *M. xanthus* (54). Eleven major PE species have been identified by HPLC-MS following described methods reflecting derivatives of the most abundant fatty acid moieties (Table 2). Acyl residues have been identified by their corresponding carboxylate fragments (R\(^{-}\)COO\(^{-}\) and R\(^{-}\)COO\(^{-}\)), which are the most abundant ions in the spectra (Figure 6A). Furthermore, the acyl residue at the sn-2 position always gives a more abundant carboxylate fragment ion than the one at the sn-1 position (40,41), enabling the identification of positional isomers. Additionally, the size of the acyl residues was confirmed by the neutral loss of the corresponding ketenes, giving [M-RCO] ions with a rather low abundance in comparison to the carboxylate fragments.

It was previously shown that in *M. xanthus* DK1622 iso-FA are derived from incorporation of leucine-derived isovaleryl-CoA (17,21). Therefore, feeding experiments with L-[5,5,5-D\(_3\)]leucine (1 mM) were performed during vegetative growth and development resulting in an incorporation rate of 60-80 % into iso-FA derived compounds, leading to the unambiguous positional assignment of iso-moieties.

**Table 2**

As expected from the FA profile with approximately 40% representing iso15:0 (27), the most abundant PE species was 1,2-O-di-(13-methyltetradecanoyl)-glycerol-3-phosphatidylethanolamine (di-iso15:0-PE) under vegetative conditions as determined by HPLC coupled to MS. During development the amount of this compound decreased whereas the corresponding vinyl ether 1-O-(13-methyl-1-zetadecenyl)-2-O-(13-methyltetradecanoyl)-glycerol-3-phosphatidylethanolamine increased strongly and was the major PE species after 72h of development (Figure 7). Unexpectedly, only trace amounts of the corresponding alkyl ether 1-O-(13-methyltetradecyl)-2-O-(13-methyltetradecanoyl)-glycerol-3-phosphatidylethanolamine could be
detected which is in contrast to the data from the FAME analysis (Figure 4). Methanolation followed by GC-MS analysis of the remaining lipid fractions obtained during the PE purification revealed that most of the alkyl ether fragment was found in the neutral lipids. Normal-phase HPLC separation followed by a similar analysis led to the isolation of a single peak containing the alkyl ether fragment. Both fractions containing the vinyl and the alkyl ether, respectively, were subjected to ESI ion trap MS analysis, resulting in characteristic spectra for plasmeneylethanolamine and triacylglycerol, with the parent ion masses corresponding to the theoretical values for 1-O-(13-methyl-1-Z-tetradecenyl)-2-O-(13-methyltetradecanoyl)-glycerol-3-phosphatidylethanolamine (VEPE) and 1,2-di-(13-methyltetradecanoyl)-3-(13-methyltetradecyl)glycerol (TG-1). TG-1 showed typical fragments indicating that iso15:0 is present as an acyl residue, namely m/z 510 for [M+Li-RCOOLi]+ and m/z 516 for [M+Li-RCOOH]+ (Figure 6) (43). Both VEPE and TG-1 were also subjected to FAME analysis which confirmed the presence of iso15:0. For final confirmation, HRESI-TOF-MS was performed leading to the sum formula C35H69NO7P for VEPE (m/z 646.4803 for [M-H]−) and C48H94LiO5 for TG-1 (m/z 757.7257 for [M+Li]+) which is in good accordance with the theoretical values of 646.4811 and 757.7261, respectively. Additionally, the vinyl moiety of the plasmalogen was confirmed by 1H-NMR analysis of the enriched fraction representing the typical signals at 5.92 ppm (H-1) and 4.34 ppm (H-2) as observed previously (55).

Moreover, TG-1 was identified directly by GC/MS of total lipids isolated from developing cultures whereas no TG-1 could be detected in vegetative cells (data not shown). The identity of this peak as TG-1 was furthermore confirmed by total synthesis similar to published procedures and comparison of synthetic and natural material with respect to retention time and fragmentation pattern in the GC/MS.

Figure 6
Figure 7

The biosynthesis of TG-1 is developmentally regulated - Because it was not clear how the formation of the unusual etherlipids was regulated, we analyzed the FA profile of selected mutants of specific marker genes during development by GC-MS of FAME extracts.

As expected, no ether lipids could be detected for DK5643 and DK5624 after 72h of development, because both mutants are impaired in the biosynthesis of iso-FAs (27), whereas normal ether lipid accumulation was observed in DK5614, a mutant having almost exclusively iso-FAs (25). DK5057 and DK5208 are defective in the production of A- and C-signal, respectively. These mutants show an arrest in development after 2h and 6h, respectively (28,35). DK11063 is defective in the production of FruA, a histidine kinase that is expressed after 6h of development (56). FruA regulates developmental gene expression and itself is regulated by a complex regulatory network (56-59). For A- and C-signal as well as FruA several additional genes are known which are regulated either directly or indirectly by them (2,3,60). DK7536 is blocked in spore formation and expression of the Ω7536 locus appears at 17h of development in a wild-type background (36).

Unexpectedly, all mutants blocked at certain time points during fruiting body formation show a strong accumulation of vinyl ether but only vegetative amounts of alkyl ether (compare Figure 5), respectively. Although, these mutants are blocked between 2h and 17h of development, the FA profile is very similar (Figure 8) and follows a similar kinetic during development (data not shown). Furthermore, no other difference in the FA pattern was observed for these mutants as confirmed by FAME analysis (data not shown).

Figure 8

TG-1 rescues fruiting body formation and sporulation in iso-fatty acid depleted mutants - TG-1, 13-methyltetradecanal and 13-methyltetradecanol were tested for their ability to complement the developmental defect as shown for iso15:0. As a control, the effects of glycerol tripalmitate (TPG) and rac-1,2-di-(hexadecanoyl)-3-(13-methyltetradecyl)glycerol (TG-2) were also investigated. TG-2 was synthesized as TG-1 analogue in order to clarify the role of the ether moiety. Interestingly, all triglycerides were able to rescue fruiting body formation with TG-1 being the most effective compound (Figure 2b). Addition of palmitate served as control, but no fruiting bodies could be observed. In fact,
palmiate (1 mM) inhibited swarming and aggregation in the mutant and the wild-type (data not shown). Addition of TG-1 resulted in the formation of spore-filled dark fruiting bodies (Figure 3c) similar to the wild-type (Figure 3a) with several spores detected as light-breaking round cells surrounding the fruiting bodies (Figure 3d). Although TG-2 and TPG seemed to restore fruiting body formation to a similar extent than TG-1 (Figure 2b), the resulting fruiting bodies were less compact and dark and showed no surrounding myxospores. Because all tested compounds were applied to the agar as suspension or emulsion in methanol, in some cases lipid droplets occurred on the agar surface. Interestingly, fruiting bodies form preferentially around the TG-1 lipid droplets (Figure 3e-h) whereas no such effect was observed for any of the other lipids tested. Sporulation was partially restored by addition of the tested triglycerides. Here again TG-1 had the most significant effect (leading to a restoration of 25% of the number of wild-type spores), whereas TG-2 (1%) and TPG (1%) were only weakly active.

**DISCUSSION**

The two unusual ether lipids 1-O-(13-methyl-1-Z-tetradecenyl)-2-O-(13-methyltetradecanoyl)-glycerol-3-phosphatidylethanolamine (VEPE) and 1,2-di-(13-methyltetradecanoyl)-3-(13-methyltetradecyl)glycerol (TG-1) have been shown to accumulate during fruiting body formation in *M. xanthus*. VEPE has been isolated from vegetative cells of *Myxococcus stipitatus* (55) and even dialkyl ether phospholipids have been identified in the myxobacterium *Stigmatella aurantiaca* (61). Furthermore, 1-O-13-methyltetradecylglycerol has been found in cell methanolysates of *S. aurantiaca* DW4/3-1 whereas VEPE has been identified in its phospholipid fraction (M.W.R. and H.B.B., unpublished results), indicating a more general occurrence of these ether lipids in myxobacteria. In contrast to ether phospholipids, TG-1 represents a new example of a diacylglycerol ether which are rarely found in nature (e.g. in the dogfish *Squalus acanthias*) (62). Triglycerides in general are well-known storage compounds of carbon and energy which is used by several euukaryotes like mammals and plants (12) but have only rarely been found in prokaryotes which in some cases seem to use polyhydroxyalkanoates instead (63-68). Complementation of DK5624 with different triglycerides (Figure 2b) showed that all compounds were effective with respect to complementation of aggregation but only TG-1 restored sporulation as well (Figure 3). As iso15:0 itself can restore fruiting body formation and sporulation, this effect might in fact be the result of iso15:0 derived from TG-1 hydrolysis by one of the several lipases encoded in the *M. xanthus* genome (unpublished results). Interestingly, high concentrations of TG-1 (1 mM final concentration) were required to complement DK5624 as application of TG-1 solutions (5 µl of different concentrations up to 1 mM) on the agar surface and application of the cells on top of this spot as described previously (24) did not result in any difference to the non-treated control (data not shown). This is surprising since there should be almost no diffusion of these very lipophilic compounds into the agar resulting in the same local concentration independent from the mode of application. Moreover, fruiting bodies formed preferentially around lipid droplets of TG-1 on the agar surface (Figure 3e-h) when TG-1 was not emulsified properly whereas this formation of fruiting body rings could not been observed for TG-2 or TPG. The partial complementation of aggregation by all triglycerides might indicate a non-specific role of these compounds which are good carbon and energy sources. Supply of additional nutrients during the fruiting body formation might partially complement the defect in aggregation but might not restore sporulation. Similar results were obtained for different fatty acids and lipid classes isolated from *M. xanthus* which could speed up and increase aggregation and sporulation of some developmental deficient mutants (69,70). Here, the authors postulated that this effect is due to an increased autolysis in the treated cultures. Autolysis which is the specific lysis of cells by their siblings is postulated to play an important role in the development of *M. xanthus* (48,49,71) and has also been shown to occur in *Bacillus subtilis* in order to delay sporulation (72). Interestingly, TPG showed complementation of aggregation whereas its free fatty acid inhibited fruiting body formation even in the wild-type. Analysis of the FA profile of selected mutants blocked at specific time points between 2-17h of development showed that the production of the alkyl ether is developmentally regulated (Figure
All analyzed mutant strains show no formation of 1-O-13-methyltetradecylglycerol but accumulate 13-methyltetradecananal. Furthermore, a higher amount of iso-FAs does not result in an increase in ether lipid production as shown for DK5614 which produces almost exclusively iso-FAs. The data might indicate that VEPE is a precursor of TG-1 (Figure 1, pathway A) that cannot be processed further due to missing enzymatic activities not available in the developmentally defective mutants. Additionally, the biosynthesis of TG-1 might be regulated by the amount of TG-1 itself, similar to well-known feedback inhibitions (12) and might not be dependent on the amount of substrate. The postulated pathway would be in contrast to the ether lipid biosynthesis in mammals (Figure 1, pathway B) where the first product of the ether lipid biosynthesis is the alkyl ether which is then desaturated to give the corresponding vinyl ether as shown in the biosynthesis of the platelet-activating factor (73-75), a plasmamylcholine that plays an important role as lipid mediator during inflammation (76). Interestingly, vinyl- and alkyl ether lipids have been identified in anaerobic bacteria belonging to the species Clostridium (77-79), Megasphaera (80-81) and Propionibacterium (53), respectively. A rare example of their occurrence in aerobic bacteria is Mycoplasma fermentans (82). Furthermore, rumen-inhabiting protozoa have also been described as producers of ether lipids (83). It was shown in Megasphaera elsdenii that vinyl ether biosynthesis is different from the mammalian pathway in that it does not start with dihydroxyacetone phosphate but glycerol-3-phosphate (83) resulting in the formation of normal PE which then serves as the vinyl ether precursor as shown for Clostridium (79). Moreover, glycerol acetals of several phospholipid species have been identified in different Clostridium species (77-79,84) and elimination of glycerol or the glycerol ester from these acetals might result in the formation of the vinyl ether moiety as proposed.

The presented data add new insights into the huge complexity of myxobacterial lipid metabolism which is remarkably complex compared to other soil-inhabiting bacteria like bacilli or streptomycetes. This complexity might be the result of their developmental life cycle including the formation of different cell types (vegetative cells, peripheral rods and spores), macroscopic and microscopic changes (fructing body formation and sporulation), as well as a regulatory network with similarities to eukaryotic systems (57,85). Moreover, the developmental defect of the bkd mutants could be correlated with the reduced amount of the identified unusual ether lipids as these strains show only trace amounts of the required precursor FAs. However, the huge amount of alkyl ether lipid and the identification of TG-1 argues against the signaling hypothesis of Downard and co-workers (23) as triglycerides function as neutral storage compounds whereas signaling lipids are usually phospho- or polar lipids located in the membrane (86). Nevertheless it can not be excluded that intermediates in the TG-1 biosynthesis act as signaling compounds during fructing body formation. Furthermore, only iso-FA derived lipids have been shown to increase during fructing body formation emphasizing their important role in myxobacteria which is also reflected by their huge abundance compared to straight-chain FAs (17,20,21,26). This is supported by the independence of fructing body formation from the amount of SCFAs, as mutants producing only trace amounts of these FAs can still develop normally (27). Currently, experiments are in progress in our group to elucidate the biosynthesis and the role of triglycerides and ether lipids in the life cycle of M. xanthus.

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The abbreviations used are: Bkd, branched-chain keto acid dehydrogenase; FA, fatty acid; SCFA, straight-chain fatty acid; FAME, fatty acid methyl ester; IVA, isovalerate; IV-CoA, isovaleryl-CoA; iso15:0, 13-methyltetradecanoate; MvaS, 3-hydroxy-3-methylglutaryl-CoA synthase; kan, Kanamycin; PE, phosphatidylethanolamine; di-iso15:0-PE, 1,2-di-(O-(13-methyltetradecanoyl)-glycero-3-phosphatidylethanolamine; VEPE, 1-O-(13-methyl-1-Z-tetradecenyl)-2-O-(13-methyltetradecanoyl)-glycero-3-phosphatidylethanolamine; AEPE, 1-O-(13-methyltetradecyl)-2-O-(13-methyltetradecanoyl)-glycero-3-phosphatidylethanolamine; TG-1, 1,2-di-(13-methyltetradecanoyl)-3-(13-methyltetradecyl)glycerol; TG-2, 1,2-di-(hexadecanoyl)-3-(13-methyltetradecyl)glycerol; TPG, glycerol tripalmitate; MTBE, methyl tert-butylether; GC, gas chromatography; HPLC, high performance liquid chromatography; ESI, electrospray ionisation; HR, high resolution; NMR, nuclear magnetic resonance.
FIGURE LEGENDS

Figure 1. Biosynthesis of isovaleryl-CoA and hypothetical biosynthesis of TG-1 in *M. xanthus* similar to the ether lipid biosynthesis in anaerobic bacteria (A) or to the mammalian ether lipid biosynthesis (B). See discussion for details. Broken arrows indicate multistep reactions. Bkd (branched-chain keto acid dehydrogenase), MvaS (3-hydroxy-3-methylglutaryl-CoA synthase), di-iso15:0-PE (1,2-di-((13-methyltetradecanoyl)-glycerol-3-phosphatidylethanolamine), VEPE (1-O-(13-methyl-1-Z-tetradecenyl)-2-O-(13-methyltetradecanoyl)-glycerol-3-phosphatidylethanolamine), AEPE (1-O-(13-methyldecany)-2-O-(13-methyltetradecanoyl)-glycerol-3-phosphatidylethanolamine), TG-1 (1,2-di-(13-methyltetradecanoyl)-3-(13-methyltetradecyl)glycerol).

Figure 2. Fruiting body formation in DK1622 (wild-type), DK5643 (∆bkd), and DK5624 (∆bkd, mvaS::kan) with or without the addition of isovalerate (IVA) or iso15:0 (a). Fruiting body formation in DK5624 with the addition of TG-1, TG-2, and tripalmitate (TPG) (b). All compounds tested were applied at a final concentration of 1mM, DK5624 was additionally supplemented with mevalonolactone (0.3 mM). The sporulation rates (upper right corner in the 72h pictures) are given relative to DK1622 (100%). White bar is 100 µm.

Figure 3. Fruiting bodies of *M. xanthus* after 72h of development at high magnification (white bar is 20 µm). DK1622 (wild-type) (a), DK5624 (∆bkd) (b), DK5624 (∆bkd, mvaS::kan) with 1mM TG-1 (c), enlargement of the white box in c (d). Fruiting bodies of DK5624 surrounding TG-1 lipid droplets (*) (e-h).

Figure 4. Relative amount (in % of total fatty acid methyl ester) of 13-methyltetradecanoic acid (circle), 13-methyltetradecanal (triangle) and 1-O-13-methyltetradecylglycerol (square) in *M. xanthus* DK1622 during fruiting body formation.

Figure 5. Relative amount (in % of total fatty acid methyl ester) of 13-methyltetradecanoic acid (open bars), 13-methyltetradecanal (grey bars) and 1-O-13-methyltetradecylglycerol (black bars) in *M. xanthus* DK1622 under different conditions as detected by gas chromatography. In the spores the amount of 13-methyltetradecanoyl and the corresponding vinyl ether is summarized to one column. Analysis of cells at 0h and 72h of starvation, glycerol- (glyc; after 6h) and starvation buffer-induced cultures (SBS, after 72h), peripheral rods (PR) and developmental spores (spores) (both from 72h starvation cultures) are shown.

Figure 6. ESI-mass spectra (MS²) of di-iso15:0-PE (1,2-di-((13-methyltetradecanoyl)-glycerol-3-phosphatidylethanolamine, A), VEPE (1-O-(13-methyl-1-Z-tetradecenyl)-2-O-(13-methyltetradecanoyl)-glycerol-3-phosphatidylethanolamine, B), AEPE (1-O-(13-methyltetradecanoyl)-glycerol-3-phosphatidylethanolamine, C), and TG-1 (1,2-di-(13-methyltetradecanoyl)-3-(13-methyltetradecyl)glycerol, D). The position of the parent ion is indicated by a diamond.

Figure 7. Relative amount of iso15:0 derived phosphatidylethanolamine species during development as deduced by HPLC-MS. 1,2-Di-((13-methyltetradecanoyl)-glycerol-3-phosphatidylethanolamine (open bars), 1-O-(13-methyl-1-Z-tetradecenyl)-2-O-(13-methyltetradecanoyl)-glycerol-3-phosphatidylethanolamine (grey bars), 1-O-(13-methyldecany)-2-O-(13-methyltetradecanoyl)-glycerol-3-phosphatidylethanolamine (black bars).

Figure 8. Relative amount (in % of total fatty acid methyl ester) of 13-methyltetradecanoic acid (open bars), 13-methyltetradecanoyl (grey bars) and 1-O-13-methyltetradecylglycerol (black bars) in selected *M. xanthus* strains after 72h of development as detected by gas chromatography.
Table 1. *Myxococcus xanthus* strains used in this study.

| Strain   | phenotype            | Reference |
|----------|----------------------|-----------|
| DK1622   | wild-type            | (33)      |
| DK5643   | Δbkd                 | (27)      |
| DK5624   | Δbkd, mvaS::kan      | (27)      |
| DK5614   | fabHac::kan          | (25)      |
| DK5057   | asg                  | (28,34)   |
| DK5208   | csg                  | (35)      |
| DK11063  | fruA                 | (31)      |
| DK7536   | Δ7536::Tn3lac        | (36)      |
Table 2. Main phosphatidylethanolamine and plasmenylethanolamine molecular species relative to the most abundant species in *M. xanthus* DK1622 under vegetative conditions, identified by ESI ion trap MS/MS in negative ionization mode. The two PE positional isomers containing 16:1 and 16:2 were quantitated together (*).

| Phospholipid class         | sn-1  | sn-2  | [M-H] | R₁COO⁻ | R₂COO⁻ | amount |
|----------------------------|-------|-------|-------|--------|--------|--------|
| Phosphatidylethanolamine   | 16:2  | 16:2  | 683   | 251    | 251    | 0.09   |
|                            | is17:2| 16:2  | 697   | 265    | 251    | 0.12   |
|                            | 16:2  | 16:1  | 685   | 251    | 253    | 0.73*  |
|                            | 16:1  | 16:2  | 685   | 253    | 251    |        |
|                            | 14:1  | is15:0| 647   | 225    | 241    | 0.24   |
|                            | 16:1  | 16:1  | 687   | 253    | 253    | 0.68   |
|                            | 14:0  | is15:0| 649   | 227    | 241    | 0.54   |
|                            | is15:0| is15:0| 663   | 241    | 241    | 1.00   |
|                            | is17:0| 16:1  | 703   | 269    | 253    | 0.64   |
|                            | is17:0| is15:0| 691   | 269    | 241    | 0.04   |
| Plasmenylethanolamine      | is15:0| is15:0| 647   | 241    |        | 0.45   |
Figure 1

Acetoacetyl-CoA → 2-ketoisocaprate → isovaleryl-CoA → 3-hydroxy-3-methylglutaryl-CoA → acetoacetyl-CoA

Secondary metabolites (e.g., myxothiazol)

Fatty acid biosynthesis

Isoprenoids

13-methyltetradecanoic acid (iso15:0)

Phospholipid biosynthesis

Triglyceride biosynthesis

VEPE desaturase
Figure 2
Figure 4

![Graph showing relative amount (%) over time (h)]
Figure 5
Figure 6
Figure 7
Novel iso-branched etherlipids as specific markers of developmental sporulation in the myxobacterium *Myxococcus xanthus*

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