An iso-15:0 O-alkylglycerol moiety is the key structure of the E-signal in *Myxococcus xanthus*

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The E-signal is one of five intercellular signals (named A- to E-signal) guiding fruiting body development in *Myxococcus xanthus*, and it has been shown to be a combination of the branched-chain fatty acid (FA) iso-15:0 and the diacylmonoalkyl ether lipid TG1. Developmental mutants HB015 (∆elbD MXAN_4265::kan) and elbD (MXAN_1528::kan) are blocked at different stages of fruiting body and spore formation as they cannot form the required iso-FA or the actual ether lipid, respectively. In order to define the structural basis of the E-signal, different mono- and triglycerides containing ether or ester bonds were synthesized and used for complementation of these mutants. Here, the monoalkylglyceride dl-1-O-(13-methyltetradecyl)glycerol exhibited comparably high levels of complementation in both mutants, restoring fruiting body and spore formation, identifying iso-15:0 O-alkylglycerol, part of the natural lipid TG1, as the ‘signalophore’ of E-signalling.

INTRODUCTION

The myxobacterium *Myxococcus xanthus* is well known for forming spore-containing fruiting bodies as a response to amino acid starvation (Zusman et al., 2007). Genetic regulation during this development (Müller et al., 2010), as well as the morphology of fruiting bodies (Kaiser & Welch, 2004), has been studied intensively in the past. However, much less is known about the signalling processes required for the formation of these fruiting bodies. The development is guided by extracellular signals subdivided into complementation groups (A–E) and the actual signalling molecules are only known for the A-signal, being a mixture of amino acids and small peptides (Kuspa & Kaiser, 1989; Garza et al., 2000). E-signal mutants are defective in the branched-chain-2-keto-acid dehydrogenase (Bkd) complex, resulting in incomplete aggregation and decreased spore yield (Downward & Toal, 1995). The enzyme complex catalyses deamination and decarboxylation of branched-chain amino acids and by this means provides isovaleryl-CoA, needed as starter units for the biosynthesis of iso-branched fatty acids (FAs) and secondary metabolites (Ring et al., 2006). However, *M. xanthus* also produces isovaleryl-CoA using a second pathway via 3-hydroxy-3-methylglutaryl-CoA (Bode et al., 2006; Mahmud et al., 2005; Li et al., 2013). Insertion of mutations into both pathways results in the double mutant HB015, which hardly produces any isovaleryl-CoA and is therefore incapable of forming aggregates, not to mention myxospores (Bode et al., 2009). This defect can be complemented by isovalerate providing the starting unit for branched-chain FA biosynthesis or directly by adding iso-15:0 FA during development (Bode et al., 2009). Beyond that, the monoalkyl diacylglycerol TG1 (1-iso-15:0-alkyl-2,3-di-iso-15:0 acyl glycerol), which accumulates during development, is also capable of rescuing fruiting body formation and sporulation (Bhat et al., 2014). In *M. xanthus*, ether lipids are synthesized by the elb biosynthesis gene cluster, coding for the multidomain enzyme ElbD with similarity to polyketide synthases (Lorenzen et al., 2014). A knockout of elbD results in a mutant with delayed fruiting body formation and a defect in sporulation, indicating the importance of the ether lipids to complete development (Lorenzen et al., 2014). Both iso-15:0 FA and TG1 have already been referred to as signals, or at least precursors, for a molecule functioning in E-signalling (Bhat et al., 2014). However, it remains unclear which structural moieties of both molecules are actually forming the signalophore for aggregation, fruiting body formation and sporulation.

In this study various lipids were tested for their potential to complement HB015 or an elbD mutant, deficient in producing iso-FAs (Bode et al., 2009) or ether lipids (Lorenzen et al., 2014), respectively. Thus, glycerolipids with iso-branched- or straight-chain FAs or with alcohols linked were synthesized as esters or ethers. As HB015 and elbD...
METHODS

Bacterial strains and culture conditions. M. xanthus DK1622 and its mutants HB015 (Δakbd MXAN_4265::kan; Bode et al., 2009) and elbD (MXAN_1528::kan; Lorenzen et al., 2014) were grown in CTT medium (Kroos et al., 1986), supplemented with 40 µg ml\(^{-1}\) kanamycin (Carl Roth) if necessary. Fruiting body and spor development was performed on TPM agar plates (Bretscher & Kaiser, 1978). For the isolation of myxospores, fruiting bodies were harvested after 72 h and washed twice with water. Fruiting bodies were incubated for 2 h at 60 °C to inactivate remaining vegetative cells before sonification. Germination experiments were performed on CTTYE agar plates prepared by adding 0.2 % yeast extract to CTT. Sporulation and germination experiments were performed in triplicates.

Chemical complementation, sporulation and germination assays. The complementation with lipids was performed according to Bhat et al. (2014) with modifications. Bacteria were harvested at OD\(_{600}\) 1–1.2 and concentrated to a density of 5 × 10\(^7\) cells ml\(^{-1}\). Five and forty microlitres of cell suspension were mixed with lipids of different concentrations from 1 to 200 nmol per 10\(^8\) cells and put on TPM agar. The plates were incubated at 30 °C. Developing spots were photographed at different time points at × 25 magnification. In order to determine number and size of fruiting bodies, ImageJ 1.48v (Rasband, 1997–2014) was used. Pictures were transformed into a binary format. The size of the fruiting bodies was measured in square pixels and density was determined by the mean grey value of the fruiting bodies. The auto threshold method was used to reduce the background and foreground to the fruiting bodies. Automated particle analysis was performed using the following parameters: size, 2500 to infinity; circularity, 0.75 to 1.00; ‘show outline’ activated. Number, mean size and mean density of the fruiting bodies were set to 100 for WT, and the level of complementation was calculated as the product of all three factors and set to 100 for WT for each time point. Complementation levels for mutants were calculated accordingly and were set with reference to the WT values for each time point. Harvesting of fruiting bodies and germination assay were performed as described previously (Lorenzen et al., 2014). The spore number was determined using a Neubauer counting chamber. Sporulation and germination experiments were performed in triplicates.

General methods for chemical synthesis. All reagents and anhydrous solvents were purchased from Sigma-Aldrich and used without further purification. Analytical TLC was performed on precoated silica gel plates (Polygram SIL G/UV254; Macherey-Nagel) using KMnO\(_4\) solution as staining reagent. Flash column chromatography was carried out on a Biotage SP1 Flash Purification System using prepacked silica gel (particle size, 40–65 µm) cartridges from Biotage [Flash 12+M KP-Sil 12 × 150 mm (flow rate, 12 ml min\(^{-1}\)) or Flash 25+M KP-Sil 25 × 150 mm (flow rate, 25 ml min\(^{-1}\))]. \(^1\)H NMR spectra were recorded on either a Bruker Av400 (400 MHz) or a Bruker AV300 (300 MHz) spectrometer. \(^13\)C NMR spectra (Figs S1–S20) were recorded on a Bruker AV300 (75 MHz) spectrometer. Chemical shifts are reported in p.p.m. (δ) with respect to the residual solvent signal of CDCl\(_3\) [δ=7.24 p.p.m. (\(^1\)H); δ=77.0 p.p.m. (\(^13\)C)]. Further information on lipid synthesis can be found in the Supplementary Material.

Fig. 1. Glycerolipids synthesized and tested, featuring 13-methyltetradecanol (iso-15:0 alcohol; 1–6), tetradecanol (14:0 alcohol; 7–9), 13-methyltetradecanoic acid (iso-15:0; 10, 11, 14) or tetradecanoic acid (14:0; 12, 13). See the Supplementary Material for further details on the structure and synthesis of these compounds.
RESULTS AND DISCUSSION

Iso-15:0 is required for complementation of fruiting body development in *M. xanthus* mutant HB015

Glycerolipids with 13-methyltetradecanol (iso-15:0 alcohol; lipids 1–6), tetradecanol (14:0 alcohol; lipids 7–9), 13-methyltetradecanoic acid (iso-15:0; lipids 10, 11, 14) and tetradecanoic acid (14:0; lipids 12 and 13) (Fig. 1) were synthesized according to standard procedures; these lipids exhibited different chemical features in order to define the actual signalophore responsible for E-signalling in fruiting body and spore formation of *M. xanthus*. Lipids were dissolved in DMSO in different concentrations and were added directly to vegetative cells before allowing them to develop on TPM agar plates (Bhat *et al.*, 2014). DMSO alone did not influence the development (Fig. S21). The first complementation assay was performed with the mutant strain HB015, which forms only loose aggregates and neither fruiting bodies nor spores (Fig. 2) owing to its loss of iso-FAs. The cells were allowed to develop on TPM agar for 72 h and the level of complementation was determined as the product of number, size and density of the fruiting bodies determined by ImageJ (Rasband, 1997–2014) in comparison with developing WT cells. The negative control palmitic acid (16:0) indeed showed no complementation (Fig. S22). This is not surprising since C16:0 is only present in minute amounts in the FA profile of *M. xanthus* (Bode *et al.*, 2006; Garcia *et al.*, 2011) and has never been described as exhibiting any signalling function. The positive controls isovalerate and iso-15:0 FA were capable of promoting fruiting body formation, as already described (Fig. S22) (Bode *et al.*, 2009; Bhat *et al.*, 2014). Here, higher amounts of isovalerate also led to a higher level of complementation (Figs 2 and S23). Unexpectedly, iso-15:0 FA concentrations higher than 40 nmol per 10^9 cells led to a total loss of aggregates (Fig. 2). The same observation was made for lipids 1 and 10. Lipids 7 and 12, carrying C14:0 chains, did not complement the mutant at any concentration, indicating that glycerol alone did not promote fruiting body formation at the tested concentrations, although it is well known that the addition of glycerol at concentrations of 0.5 M and higher can induce spore formation in *M. xanthus* from vegetative cells (Dworkin & Gibson, 1964; Komano *et al.*, 1980). This strongly supports the already described signalling character of iso-15:0, required at a specific concentration, with a loss of development being the result of a signal 'overdose'. However, it was not crucial whether iso-15:0 was bound to the glycerol backbone by an ester or an ether linkage since both compounds 1 and 10 led to a similar level of complementation (Fig. 2). The important factor for complementation of HB015 is iso-15:0. Addition of isovalerate did not lead to a reduction in development at high concentrations as it is only the precursor

Fig. 2. Complementation of *M. xanthus* mutant HB015 with various lipids in different concentrations. A 40 µl aliquot of a cell suspension with a density of 5 x 10^9 ml^-1 was allowed to develop on TPM agar plates for 72 h at 30 °C. Data were acquired by graphical analysis of fruiting bodies using ImageJ (Rasband, 1997–2014). The number in the upper right corner of each image indicates the level of complementation, which was calculated as a product of number, size and density of fruiting bodies. The WT at 72 h was set to 100.
required for the biosynthesis of iso-15:0 (Bode et al., 2006, 2009) and therefore the amount of signal produced can be regulated during the biosynthesis, leading to a physiological amount of signals and the expected saturation (Fig. S23). Other complementing lipids were 2, 3 and 11 (Figs S24 and S25), all bearing an iso-15:0 chain. In contrast to 1 and 10, these lipids did not complement at the same level (3) or they did not lead to an overdose (2, 11). That might be due to decreased uptake efficiency by the cells, as these lipids carry acetyl (2) or an acetonid moiety (3, 11) instead of a free hydroxyl group. Moreover, triglycerides used in this work showed only small or no effect in complementation. Presumably, the hydrophobic properties of these lipids impede their uptake by the cells, as they bear the same acyl residues as the monoalkylglycerols but have little or no effect on complementation. In particular, TG1 (4), which has been identified as a signalling factor before (Bhat et al., 2014), was expected to have a much higher effect on development. Compound 14 was also expected to have a higher complementing effect as it theoretically provides a threefold amount of iso-15:0 to the cells compared with 10, which showed a strong effect on complementation in HB015 (Fig. 2).

**Spores from complemented HB015 fruiting bodies are defective in germination**

In order to test whether the complementation of fruiting bodies correlates with the formation of viable myxospores, fruiting bodies from HB015 developed with 40 nmol lipids per 10⁹ cells were harvested and disrupted to give individual spores. Subsequently, isolated spores were counted (=total spores), plated on CTTYE plates and allowed to develop for 7 days (=viable spores). Total spore counts showed results similar to the fruiting body complementation (Fig. 3a). As expected, cells treated with DMSO or palmitic acid did not develop any spores, as they were also not capable of forming fruiting bodies (Figs 2, S1 and S3). While the addition of triglycerides (4, 5, 6 and 14) only led to very low levels of complementation (Figs S22–S26), with only a few fruiting bodies, spore formation was restored to about 5% of the WT level. The addition of isovalerate, iso-15:0 FA and the monoglycerides with iso-15:0 caused obvious improvements in spore formation, especially the monoglycerides, as 1 and 10 exhibited strong complementation potentials of 42 and 67%, respectively (Figs 2, S23 and S25). The subsequent germination experiments showed that, even if some lipids were able to restore the formation of myxospores, only a very small number of these spores were actually viable and could germinate (Fig. 3b). A possible explanation for the discrepancy between total and germinating spores could be the defect in strain HB015. Recent studies showed that during germination all iso-15:0 FAs are synthesized de novo (Ahrendt et al., 2015). Hence, the loss of isovaleryl-CoA biosynthesis in HB015 might prevent or delay spore germination as iso-FAs already present in the spore membranes cannot be used (Ahrendt et al., 2015). However, addition of

![Fig. 3. Sporulation and germination data for complementation of M. xanthus mutants HB015 and elbD. Lipids were added to a concentration of 40 and 20 nmol per 10⁹ cells for HB015 and elbD, respectively. Myxospores from complementation assays with HB015 (a) and elbD (c) were isolated and counted. Afterwards spore suspensions of HB015 (b) and elbD (d) were plated on CTTYE germination medium and incubated for 7 days at 30 °C. Values were calculated with regard to DK1622 WT set to 100% and are the result of triplicates.](http://mic.microbiologyresearch.org)
1 mM isovalerate to the germination medium did not increase the number of germinating cells, indicating a fine-tuned regulation of iso-FA biosynthesis or function during germination that might be dependent on a very specific iso-FA concentration.

An iso-15:0 ether lipid is capable of complementing the elbD mutant

The elbD mutant is delayed in fruiting body formation and barely forms any spores (Lorenzen et al., 2014). Fig. 4 illustrates fruiting body formation in *M. xanthus* WT DK1622 and the elbD mutant over 48 h. Mutant cells with DMSO exhibited almost no fruiting body formation within the first 20 h and only reached about 57% of WT development after 24 h. This indicates no complementing effect of DMSO in early fruiting body formation in elbD. Nevertheless, no matter which lipid was added to the mutant, fruiting body formation was comparable to WT after 48 h. The focus was therefore set on complementation during early fruiting body formation between 12 and 20 h. As an initial assay showed the best complementation for 1 at 20 nmol per 10⁹ cells (Figs S27 and S28), this concentration was used for all lipids in this assay. The best level of complementation in early fruiting body formation was observed for 1, being an iso-15:0 ether lipid (Fig. 4; 18 h). The most striking observation in this assay was the strong complementing effect of the C14:0 ether lipid 7 (Fig. 4), showing the second-highest level of complementation. Lipid 7 had no effect on complementation for HB015 (Fig. 2). As C14:0 ether lipids have never been found in *M. xanthus* and the absence of any effect of the glycerol backbone is demonstrated with 12, the ether bond must be responsible for the effect. Lipid 10 had only a small effect on complementation in early fruiting body development, and 12 did not have any effect (Figs 4 and S9). Comparison of the complementing effect for iso-15:0 lipids 1 and 10, as well as the C14:0 lipids 7 and 12, emphasizes the importance of the ether linkage for elbD complementation. Ether lipids 1 and 7 had a higher effect on complementation in early development than 10 and 12, which do not bear an ether linkage (Fig. 4, 18 h). However, iso-15:0 supported complementation, as 1 and 10 both showed a higher effect than their C14:0 equivalents 7 and 12. In contrast to previous results (Lorenzen et al., 2014), elbD was able to form about 10% of the WT

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**Fig. 4.** Fruiting body formation in *M. xanthus* WT DK1622 and elbD complemented with various lipids. Five microlitres of cell suspensions with a density of 5 × 10⁶ ml⁻¹ were allowed to develop on TPM agar plates for 48 h at 30 °C. Lipids were added to a concentration of 20 nmol per 10⁹ cells. Numbers indicate the level of complementation. For details see Fig. 2.
spore level (Fig. 3c). The addition of 1, which had already exhibited the highest effect on fruiting body formation, improved sporulation to over 40% of WT level. However, surprisingly, most added lipids led to a similar spore yield of 20 to 30%. The germination assay produced a clearer result, as the amount of viable spores was indeed higher for 1, 7 and 10, similar to their complementation potential in early fruiting body formation (Fig. 3d). The results indicate that ether lipids play a more important role in early fruiting body formation than in sporulation. The addition of different lipids could have provided a new energy source required for spore formation, resulting in a higher amount of total and viable spores.

**DL-1-O-(13-methyltetradecyl)glycerol (1) includes the pharmacophore of the E-signal**

The E-signal has been described as a combination of iso-15:0 FA and TG1 (4) (Bhat et al., 2014). The fact that complementation of HB015 development was only dependent on iso-15:0 and complementation of elbD was promoted by ether bonds identifies these features as the actual pharmacophore of the E-signal, both present in 1. However, in contrast to TG1, no such monoglyceride as 1 has ever been detected in any lipid analysis in M. xanthus. Nevertheless, both molecules 1 and 4 exhibit the same molecular features, aside from the acyl chains in 4, which obviously hindered efficient uptake by the cells, resulting in only low complementing effects by 4. Previous research has shown that developing cells form TG1-filled lipid bodies and secrete them into the fruiting body, where they disappear gradually during development (Hoiczyk et al., 2009). This would argue for a mechanism that transmits TG1, or a TG1-like lipid, as the actual E-signal. Fig. 5 shows a proposed mechanism for E-signal transmission. During development ether lipid biosynthesis produces 4, which leaves the cell through lipid body formation by an unknown mechanism. Probably lipid bodies are also released by cell lysis, as the majority of cells are lysed during fruiting body formation in M. xanthus. Once the lipid bodies or the lipids they contain are taken up by a receiving cell, 4 is hydrolysed to 1, which might be further modified to yield the actual E-signal. Although uptake of lipids or lipid bodies is unknown so far, the proposed intermediate 1 can indeed be taken up directly, as we have shown in this work. Nevertheless, the fate of the ether bond leading to the E-signal after degradation of the lipid bodies remains unexplored, and there is no known mechanism for the degradation of ether lipids in myxobacteria. Although previous studies have identified TG1 as the only physiological neutral lipid mechanism or by cell lysis. Subsequent uptake of these lipid bodies (or lipid vesicles) containing 4 by the receiving cell (bottom), hydrolysis to 1 and further modifications then possibly form the E-signal. Bkd, branched-chain-2-keto-acid dehydrogenase.
containing the E-signal pharmacophore identified in this work, more research is needed to identify the true structure of the E-signal as well its underlying mechanisms for signal detection and integration.

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REFERENCES


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