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Novel lipids in *Myxococcus xanthus* and their role in chemotaxis

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Summary

Organisms that colonize solid surfaces, like *Myxococcus xanthus*, use novel signalling systems to organize multicellular behaviour. Phosphatidylethanolamine (PE) containing the fatty acid 16:1ω5 (Δ^{11}) elicits a chemotactic response. The phenomenon was examined by observing the effects of PE species with varying fatty acid pairings. Wild-type *M. xanthus* contains 17 different PE species under vegetative conditions and 19 at the midpoint of development; 13 of the 17 have an unsaturated fatty acid at the *sn*-1 position, a novelty among Proteobacteria. *Myxococcus xanthus* has two glycerol-3-phosphate acyltransferase (PlsB) homologues which add the *sn*-1 fatty acid. Each produces PE with 16:1 at the *sn*-1 position and supports growth and fruiting body development. Deletion of *plsB1* (MXAN3288) results in more dramatic changes in PE species distribution than deletion of *plsB2* (MXAN1675). PlsB2 has a putative N-terminal eukaryotic fatty acid reductase domain and may support both ether lipid synthesis and PE synthesis. Disruption of a single *sn*-2 acyltransferase homologue (PlsC, of which *M. xanthus* contains five) results in minor changes in membrane PE. Derivatization of purified PE extracts with dimethyldisulfide was used to determine the position of the double bonds in unsaturated fatty acids. The results suggest that Δ^5 and Δ^{11} desaturases may create the double bonds after synthesis of the fatty acid. Phosphatidylethanolamine enriched for 16:1 at the *sn*-1 position stimulates chemotaxis more strongly than PE with 16:1 enriched at the *sn*-2 position. It appears that the deployment of a rare fatty acid (16:1ω5) at an unusual position (*sn*-1) has facilitated the evolution of a novel cell signal.

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Introduction

In nature many bacteria are attached to surfaces where they are subject to different physical stresses from planktonic cells. As a consequence, surface dwelling bacteria often employ distinctive mechanisms, including different motility motors, chemical signals and sensory transduction mechanisms. A model surficial organism is *Myxococcus xanthus*, a soil-dwelling δ-proteobacterium with a complex life cycle. Vegetative cells travel in swarms, feeding on other bacteria. When starved, cells aggregate into large fruiting bodies and then differentiate into spores. During development, swarms of *M. xanthus* maintain significant species purity (Fiegna and Velicer, 2005), even in a complex soil microbial community, through mechanisms that are as yet unknown. Lipid signals may be important. *Myxococcus xanthus* cells respond chemotactically to purified *M. xanthus* membrane phosphatidylethanolamine (PE) (Kearns and Shimkets, 1998), containing the fatty acid 16:1ω5 (Kearns *et al*., 2001a). This response is dependent on the presence of the extracellular matrix and has only been observed under starvation conditions, suggesting developmental relevance. Chemotaxis is also promoted by PE containing 18:1ω9 (Kearns and Shimkets, 1998), a fatty acid not found in *M. xanthus* (Kearns *et al*., 2001a) but common in prey bacteria such as *Escherichia coli* (Cronan and Rock, 1996). The response to PE containing 18:1ω9 uses a different sensory pathway from the response 16:1ω5 and may be involved in sensing prey (Kearns *et al*., 2000; Bonner *et al*., 2005).

Mutations that alter the fatty acid composition of the membrane can have dramatic effects on development. For example, mutation of the genes encoding the E1α and E1β subunits of the branched-chain keto-acid dehydrogenase complex (BCKAD) (involved in the synthesis of branched-chain fatty acids) decreases the abundance of branched-chain fatty acids, with a subsequent rise in unsaturated fatty acids (Toal *et al*., 1995). These mutants are deficient in fruiting body formation and sporulation. Partial restoration of branched-chain fatty acid synthesis by feeding cells isovalerate improves

fruiting body formation and sporulation (Toal *et al*., 1995). These results argue that a balance of fatty acids is necessary to maintain the complex life cycle of the organism.

Phosphatidylethanolamine is usually the most abundant phospholipid in Proteobacteria membranes. Its biosynthesis starts with the addition of an acyl chain (fatty acid) at the *sn*-1 position of glycerol-3-phosphate by glycerol-3-phosphate acyltransferase PlsB (Cronan and Rock, 1996). The second acyl chain is added at the *sn*-2 position by PlsC (1-acyl-*sn*-glycerol-3-phosphate acyltransferase), forming phosphatidic acid. A serine molecule is added to the phosphate group, forming phosphatidylserine, which is then decarboxylated to create the PE. The fatty acid diversity of PE molecules is due in part to the activities of the PlsB and PlsC acyltransferases and in part to the pool of available fatty acids.

In this work the molecular diversity of membrane PE was examined in wild-type and mutant strains, showing that *M. xanthus* contains significant amounts of unsaturated fatty acids at the *sn*-1 position. Mixtures of PE molecules enriched in *sn*-1 16:1 are stronger attractants than molecules with *sn*-2 16:1. This effect is associated with 16:1ω5 as it is the major monounsaturated fatty acid in *M. xanthus*.

Results

Analysis of double bond position in unsaturated fatty acids

Fatty acid methyl esters (FAMEs) were prepared from wild-type cells (Härtig *et al*., 2005), separated by gas chromatography (GC), and analysed by mass spectrometry (MS) (Fig. 1A). The most abundant fatty acid was iso15:0 while the second most abundant was 16:1ω5; this result agrees with previous analyses (Kearns *et al*., 2001a; Ware and Dworkin, 1973). In order to identify the positions of the double bond, unsaturated fatty acids were derivatized with dimethyldisulfide (DMDS), which derivatizes the carbons flanking the double bond and facilitates fragmentation between the derivatized carbons (Fig. 1B, inset). The resulting mass spectrum verifies the position of unsaturation. In the case of the major 16:1 GC peak, the double bond was as the ω5 position (Fig. 1B). The minor 16:1 species was the ω11 isomer. 15:1 exists as both ω10 and ω4 isomers (data not shown), although the ω4 isomer is more abundant (Fig. 1A). Finally, an iso17:1 was found. Because it eluted from the GC column earlier than the iso17:0, it appeared to be a branched monounsaturated fatty acid. The fragmentation of the DMDS-derivitized product indicated unsaturation at the ω5 position (Δ^{11}, data not shown).

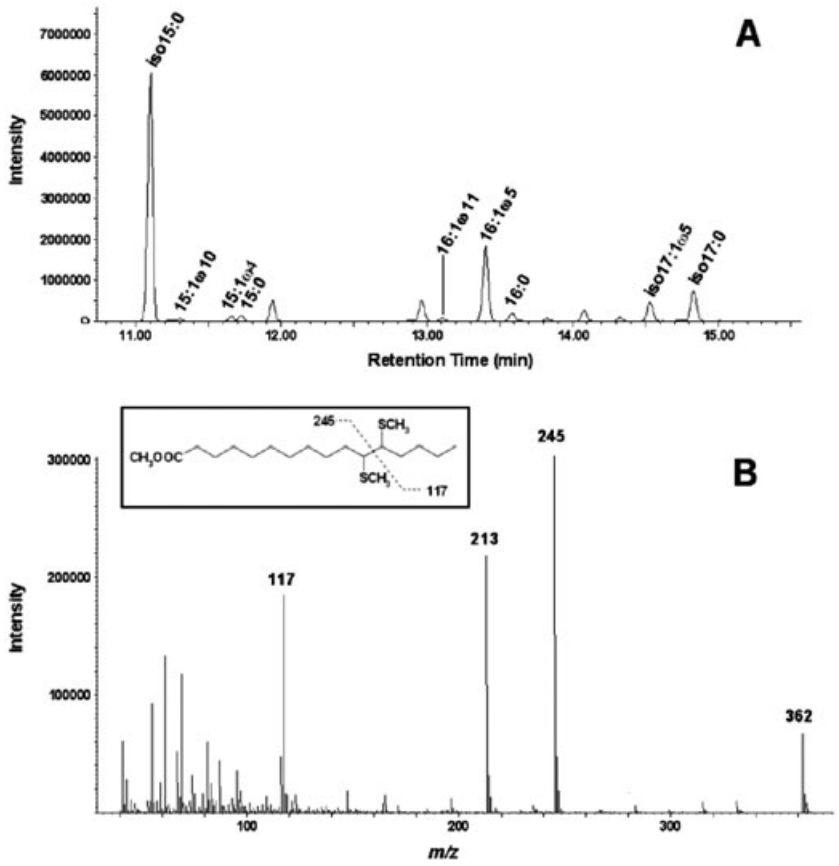

Fig. 1. Analysis of fatty acids from *Myxococcus xanthus*.
A. Fatty acid methyl esters were prepared from wild-type *M. xanthus* DK1622 cells, separated by gas chromatography, and analysed by mass spectrometry.
B. Unsaturated fatty acid methyl esters were derivatized with dimethyldisulfide (DMDS) to determine the point of unsaturation by detecting the size of resultant fragments after derivatization and fragmentation. The mass spectrum of the 16:1ω5 DMDS derivative is depicted. Fragmentation between the ω5 and ω6 carbons would result in *m/z* 245 and 117 fragments respectively (inset). The parent ion (*m/z* 362) and fragment ions are detected in addition to the ion *m/z* 213 resulting from the loss of methanol from the fragment *m/z* 245.

Analysis of M. xanthus *vegetative and developmental PE*

Phosphatidylethanolamine from vegetative and developmental cells was purified by thin-layer chromatography (TLC) and liquid chromatography (LC). Finally, high-performance liquid chromatography (HPLC)-coupled electrospray ionization tandem mass spectrometry (HPLC-ESI-MS-MS) was used to identify the location and abundance of specific acyl chains in PE. The molecular ions of PE molecules were isolated by their mass (*m/z*, amu) in the negative mode $(M–H)^-$, fragmented with N_2 causing collision-induced dissociation (CID), and the fragments were analysed. The fatty-ester bonds at *sn*-1 and *sn*-2 exhibit different stabilities during CID, enabling the positions of fatty acids in PE molecules to be determined by the relative abundance of the fragment ions (Ekroos *et al*., 2002; Taguchi *et al*., 2005).

Phosphatidylethanolamine from vegetative *M. xanthus* cells contains at least 17 different molecular species; this number rises to at least 19 during development (Table 1). The most abundant PE species during vegetative growth is PE-15:0/15:0 (PE with the fatty acid 15:0 at the *sn*-1 and *sn*-2 positions, respectively, 20.3%) followed by PE-16:1/15:0 (12.0%). The lipids PE-16:1/16:1 and PE-17:2/15:0 (combined 12.0%) have the same *m/z* ratio and could not be separated. Because CID is a partial hydrolysis, it is difficult to separately quantify the abundances of different PE species with the same molecular weight. In this example, however, relative abundance of the two species is easy to infer from the CID. The abundance of 15:0 after CID is 30-fold lower than 16:1, indicating that there is much more PE-16:1/16:1 than PE-17:2/15:0. This conclusion is supported by GC-MS which shows that 17:2 is a low abundance fatty acid (data not shown) (Kearns *et al*., 2001a).

Table 1. Mass spectrometry – collision-induced dissociation (CID) analysis of phosphatidylethanolamine (PE) in *Myxococcus xanthus* strains.

Progenitor ion (*m/z*, amu)	PE species Fragmention		Per cent of total PE (wild type and mutants)							
	sn-1	*sn*-2	WT Veg. (DK1622)	WT Dev. (DK1622)	*plsB1* (LS2300)	*plsB2* (LS2301)	*esg*[a] (JD300)	*plsC2* (LS2305)	*plsC4* (LS2306)	*plsC5* (LS2307)
646.6	14:1	15:0		2.5		2.8	3.0	3.3		3.9
	16:1	13:1								8.1
648.6	14:0	15:0	5.6	5.3	8.0	4.4	6.3	6.8	6.1	
658.6	16:1	14:1					3.2			
	15:1	15:1								7.2
660.6	15:1	15:0	10.2	11.0	2.9	5.8	9.6[b]	14.2	10.7	
	16:1	14:0								19.7
662.4	15:0	15:0	20.3	12.2	29.9	21.2	10.3	21.5	27.9	
672.3	16:1	15:1	7.1			4.3	6.1	4.6	5.0	6.2
	16:2	15:0								12.3
674.4	16:1	15:0	12.0	6.1	13.1	7.9	11.3	10.9	10.3	
676.6	15:0	16:0			4.7		5.0			
684.6	16:1	16:2	7.1	6.5	3.0	4.7	9.7	4.4	4.6	4.0
686.3	16:1	16:1	12.0	15.5	7.3	8.2	27.8	9.5	12.2	11.6
	17:2	15:0								6.3
688.5	17:1	15:0	4.1	7.0	9.3	3.6	7.8[c]	6.0	6.5	
	16:0	16:1								4.3
690.5	17:0	15:0	4.5	3.3	14.4	6.1				
698.4	17:2	16:1	3.4	4.5				5.7	5.3	3.9
700.6	17:1	16:1	6.0	9.4		3.5		8.7	6.1	8.0
702.3	17:0	16:1	7.5	8.6		4.0		4.6	5.3	4.7
	18:1	15:0								
704.7	17:0	16:0				2.6				
712.2	n/d	n/d		3.3						
714.4	n/d	n/d		4.9						
716.7	n/d	n/d				2.5				
718.7	n/d	n/d				3.5				

a. Strain JD300 (*esg*) was created previously (Toal *et al*., 1995).
b. PE-16:1/14:0 is more prevalent than PE-15:1/15:0 in this strain.
c. PE-17:1/15:0 is undetectable and both PE-16:1/16:0 and PE-16:0/16:1 are detected in this strain.
Phospholipids were extracted from whole cells and purified by thin-layer chromatography. The PE fraction was extracted from the silica and separated by liquid chromatography followed by electrospray ionization tandem mass spectrometry, wherein collision with nitrogen gas causes a preferential fragmentation of the *sn*-2 fatty-ester linkage. The most abundant fatty acid after CID is the *sn*-2 fatty acid. The abundance of the PE species was determined by signal intensity (cps). In the case that two PE species have the same mass (*m/z*, amu), the more abundant species is listed above the less abundant as determined by the ratio of *sn*-2 fatty acid abundances after CID. Phosphatidylethanolamine species below 2% of total were not found consistently and have been omitted.

The *sn*-1 position in vegetative PE is occupied by five saturated fatty acids and 12 unsaturated fatty acids, while the *sn*-2 position is occupied by nine saturated fatty acids and eight unsaturated fatty acids. The 16:1 fatty acid that is associated with chemotaxis is found in the *sn*-1 position of five PE species. In sum, they account for approximately 38.2% of all PE molecules. 16:1 is also found at the *sn*-2 position, where it accounts for about 28.9% of total.

The fatty acid pairings in vegetative PE species did not change during development (Table 1), although some species changed in abundance. In vegetative cells, PE-15:0/15:0 was most abundant (20.3%), whereas under developmental conditions PE-16:1/16:1 was most abundant (15.5% including a small amount of PE-17:2/15:0). Four new PE species appear at the midpoint of development and two disappear. Development induces a notable increase in larger PE species, many of which contain 16:1 in the *sn*-2 position. Concordantly, the summed percentage of PE containing 16:1 at the *sn*-2 position rises from 28.9% to 38.0%.

Phosphatidylethanolamine from M. xanthus plsB1 *and* plsB2 *mutants*

PlsB initiates PE biosynthesis by transferring a fatty acid from the acyl carrier protein to the *sn*-1 position of glycerol-3-phosphate (Cronan and Rock, 1996). The *plsB* gene is considered to be one of the minimal set of 256 that are essential in bacteria (Gil *et al*., 2004). Synthesis of PEs with unsaturated fatty acids at the *sn*-1 position could involve a PlsB that uses unsaturated fatty acyl substrates. While there is typically one *plsB* gene per organism, a TBLASTN search with the *E. coli* PlsB protein sequence identified two significant *M. xanthus* matches. PlsB1 (MXAN3288) showed 29% identity and 49% similarity to *E. coli* PlsB over the full length of the protein (5e-56 expect). As seen in Fig. 2, PlsB1 contains the conserved amino acids shown to be essential for cell viability in *E. coli* (Heath and Rock, 1998; Lewin *et al*., 1999). The TIGR annotation of MXAN3288 places 22 additional amino acids at the N-terminus of the product; these may be incorrectly assigned. Our proposed *plsB1* start site has a near-consensus ribosome binding site (RBS), AGGACG, 12 bp upstream of the start site, whereas MXAN3288 has no recognizable RBS. Using our start site, *plsB1* was deleted from the chromosome, leaving only the start codon separated from the stop codon by a 6 bp XbaI site. The resulting mutant, LS2300, exhibited no growth or motility defects, and formed fruiting bodies containing 99% viable spores, compared to the wild type.

PlsB2 (MXAN1675) showed 33% identity and 48% similarity over the 452 C-terminal amino acids of the *E. coli* PlsB sequence (807 amino acids total). PlsB2 also contains the conserved amino acids shown to be necessary for *E. coli* viability (Fig. 2). The N-terminal half of PlsB2 showed some homology to eukaryotic fatty acid reductases (FARs). An alignment of PlsB2 amino acids 1–309 with eukaryotic FARs is shown in Fig. 3. The NAD(P)H-binding motif [I, V, F]-X-[I, L, V]-T-G-X-T-G-F-L-[G, A] conserved among eukaryotic FARs is indicated by the black box (Aarts *et al*., 1997; Metz *et al*., 2000; Moto *et al*., 2003; Cheng and Russell, 2004). The *M. xanthus* sequence has a near-consensus NAD(P)H binding site as well as other conserved blocks of amino acids, suggesting that the N-terminal portion of PlsB2 may function in fatty acid reduction. MXAN1675 has a consensus RBS (AGGAGG) 8 bp upstream of the predicted start. The portion of the gene containing the PlsB2 active site, corresponding to amino acids 416–868, was deleted to eliminate putative acyltransferase activity. The *plsB2* mutant had no obvious growth, motility or developmental defects (102% viable spores compared with the wild type).

Phosphatidylethanolamine was purified from vegetative *plsB1* and *plsB2* cells and analysed by LC-ESI-MS-MS (Table 1). The most significant finding is that each mutant contains unsaturated fatty acids at the *sn*-1 position. While

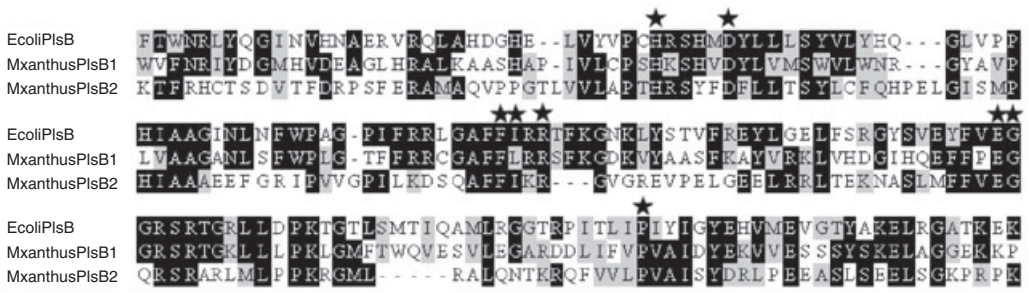

Fig. 2. Alignment of *Escherichia coli* PlsB and *Myxococcus xanthus* homologues. The *E. coli* PlsB protein (Accession No. P0A7A7, amino acids 273–446) was aligned with the *M. xanthus* PlsB1 (MXAN3288; 313–487) and PlsB2 (MXAN1675; 469–635) proteins. Stars indicate conserved amino acids known to be essential for viability in *E. coli* (Heath and Rock, 1998; Lewin *et al*., 1999).

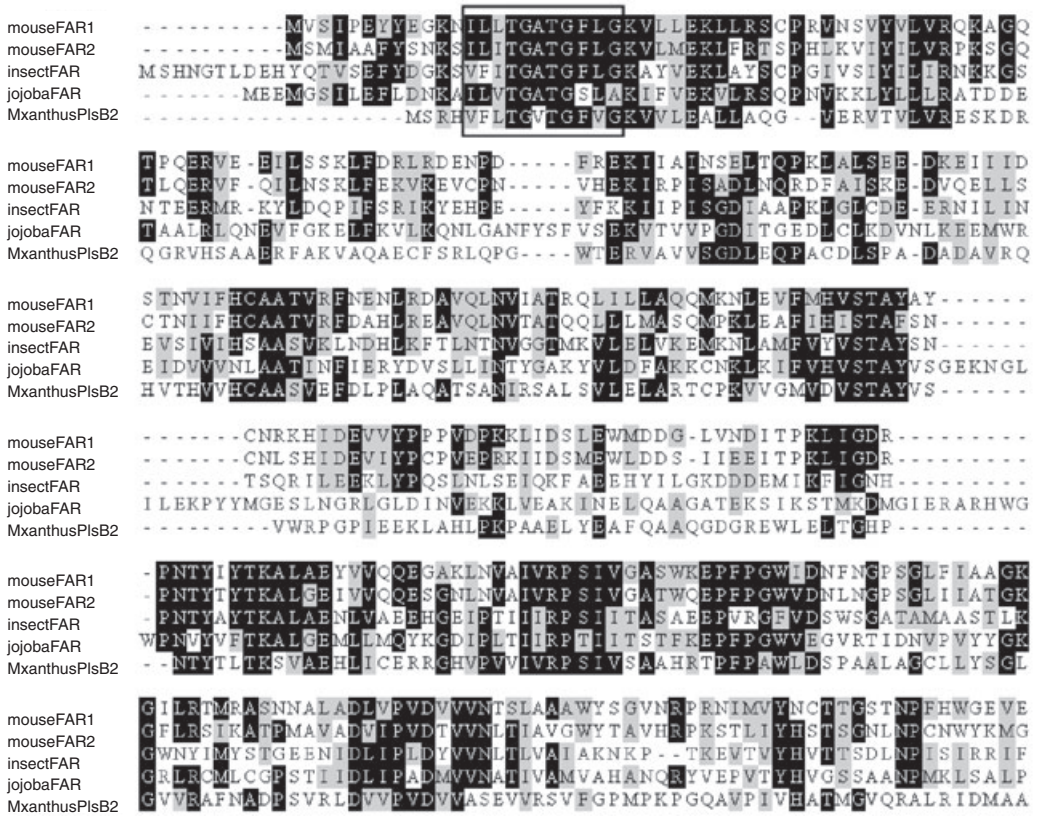

Fig. 3. Alignment of several eukaryotic fatty acid reductases (FARs) and *Myxococcus xanthus.* PlsB2 (amino acids 1–309). Mouse *Mus musculus* FAR 1, BC007178 amino acids 1–319, mouse FAR 2, BC055759 1–319, silkworm *Bombyx mori* FAR, BAC79426, 1–328, jojoba plant *Simmondsia chinensis* FAR, AD38040, 1–353. The boxed portion indicates the NAD(P)H-binding motif [I, V, F]-X-[I, L, V]-T-G-X-T-G-F-L-[G, A] (Aarts *et al.*, 1997; Metz *et al.*, 2000; Moto *et al.*, 2003; Cheng and Russell, 2004).

the majority of the fatty acid pairings for individual PE species remain the same, there are some changes in PE composition for these mutants. The *plsB1* mutant lacks many of the high-molecular-weight PE species present in wild-type, notably the species with *m/z* 698.4, 700.6 and 702.3. Each of these species has 16:1 at the *sn*-2 position. Furthermore, *m/z* 686.3, the principal species with 16:1 at the *sn*-2 position, declines by nearly 40%. For this reason, total unsaturated fatty acids at the *sn*-2 position decline from about 44% to roughly 10% in the *plsB1* mutant. The *plsB2* mutant contains the high-molecular-weight PE species missing in the *plsB1* mutant in addition to other larger PE species (*m/z* 704.7, 716.7 and 718.7). Despite these changes, the three most abundant PE species are present in quantities similar to those of the wild type. These results argue that PlsB1 and PlsB2 have overlapping substrate specificities.

The fatty acid composition at the *sn*-2 position appears to be dependent on the *sn*-1 fatty acid, given that a mutation that alters *sn*-1 acyltransferase activity also alters the *sn*-2 fatty acid content. If the *sn*-2 acyltransferase added fatty acids irrespective of which fatty acids occupy the *sn*-1 position, then the *sn*-2 fatty acid content would remain unchanged in this mutant.

Analysis of PE from an esg *mutant*

The *esg* mutant (JD300) is deficient in the BCKAD necessary for branched-chain fatty acid synthesis, resulting in PE that is enriched for 16:1 (Toal *et al.*, 1995). As previously reported, this mutant has reduced amounts of branched-chain fatty acids 15:0 and 17:0 (Table 1) (Toal *et al.*, 1995). PE-15:0/15:0 in the *esg* mutant decreased nearly twofold compared with the wild type. Branched-chain fatty acid synthesis is not abolished in this mutant as a bypass mechanism has been recently elucidated (Mahmud *et al.*, 2002). The mass-spectral signal corresponding to PE-16:1/16:1 increased 2.3-fold, constituting 27.8% of the total PE. In the *sn*-1 position, the amount of 16:1 rises from roughly 38.2% in wild type to approximately 71.6% in the *esg* mutant. While this mutant lacks many of the high-molecular-weight PE species found in the wild type due to the fact that the 17-carbon branched-chain fatty acids are missing, the *sn*-2 16:1 content is

similar to that in the wild type, largely due to the increase in PE-16:1/16:1. Fatty acid pairings are similar in the *esg* mutant and in the wild type, but the total fatty acid content is changed. This again suggests that the fatty acids added to the *sn*-2 position are dependent on the *sn*-1 acyl chain, and not the overall fatty acyl-ACP pool. Again, the 16:1 acyl chains can be attributed mainly to the ω5 species, which is by far the most abundant 16:1 isomer in *M. xanthus*.

Analysis of PE from three M. xanthus plsC *mutants*

PlsC is an essential enzyme that adds a fatty acid to the *sn*-2 position of 1-acyl-*sn*-glycerol-3-phosphate (Cronan and Rock, 1996). Typically, there are one to two *plsC* genes per organism (Cronan and Rock, 1996; Shih *et al*., 1999). Searching the *M. xanthus* genome with the *E. coli* PlsC sequence (TBLASTN) revealed five homologues: *plsC1* (MXAN3330), *plsC2* (MXAN3969), *plsC3* (MXAN5578), *plsC4* (MXAN0955) and *plsC5* (MXAN0147). The related protein products had 28–36% identity and 49–56% similarity to the *E. coli* protein over the entire length of the protein. PlsC enzymes have conserved amino acid blocks similar to PlsB enzymes (Li *et al*., 2003; Weier *et al*., 2005), and the *M. xanthus* PlsC homologues contain many of the conserved residues (Fig. 4). All the homologues had the characteristic small size of PlsC enzymes. The *plsC2*, *plsC4* and *plsC5* genes were each mutated by homologous integration of a plasmid bearing an internal fragment of each gene. This method results in a merodiploid in which both copies of the gene are truncated. Phosphatidylethanolamine from each mutant was purified and analysed by LC-ESI-MS-MS (Table 1). Although some changes were observed, such as an increase in PE-15:0/15:0 in the *plsC4* mutant, most values stay within 1.5-fold of each other for comparable PE species. These results suggest that the homologues are either functionally redundant or biologically inactive.

Chemotactic excitation by PE from different strains

Previous work demonstrated that *M. xanthus* alters its motility in response to PE with specific fatty acids (Kearns and Shimkets, 1998). Solitary *M. xanthus* cells travel over solid surfaces in one direction for approximately 7 min before reversing direction (reversal period) (Blackhart and Zusman, 1985). In the presence of certain PEs, *M. xanthus* cells are stimulated to travel longer distances before reversing directions. Not all PEs produce this response indicating a molecular specificity related to a particular fatty acid. PE-12:0/12:0 and PE-18:1ω9/18:1ω9 elicit a response, whereas PE-14:0/14:0, PE-16:0/16:0, PE-17:0/17:0 and PE-18:0/18:0 do not (Kearns and Shimkets, 1998). Cells adapt over time and the stimulated reversal period falls back to the unstimulated level. Stimulation and adaptation are hallmarks of chemotaxis (Kearns and Shimkets, 1998).

Neither of the chemoattractants, PE containing 12:0 or PE containing 18:1ω9, is found in *M. xanthus*. Phosphati-

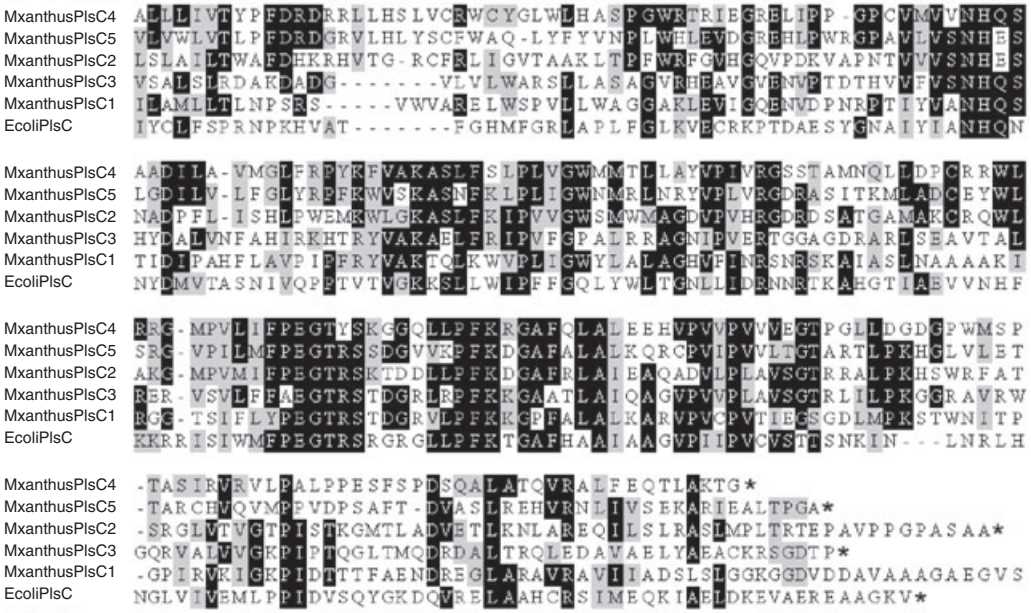

Fig. 4. Alignment of *Escherichia coli* PlsC and *Myxococcus xanthus* PlsC homologues. The *E. coli* PlsC protein (P26647, amino acids 22–245) was aligned with the five *M. xanthus* PlsC homologues: PlsC1 (MXAN3330; 22–254), PlsC2 (MXAN3969; 30–263), PlsC3 (MXAN5578; 26–245), PlsC4 (MXAN0955; 45–264) and PlsC5 (MXAN0147; 61–282).

dylethanolamine containing 18:1 is found in many bacteria *M. xanthus* preys on and may be involved in prey recognition. The nature of a native attractant found in the *M. xanthus* membrane was revealed through metabolic engineering and linked to the fatty acid 16:1ω5 (Kearns *et al.*, 2001a). Phosphatidylethanolamine 16:1ω5/16:1ω5 was synthesized and found to be about 1000-fold more active than the other two attractants and active at physiological concentrations. The chemical synthesis was performed with identical fatty acids at both positions simply because it was easier and was completed without prior knowledge of the PE composition of the *M. xanthus* membrane. Table 1 illustrates the unexpected finding that PE 16:1/16:1 is both present and abundant in *M. xanthus* and there are a wide variety of other species containing 16:1 at the *sn*-1 position. This raises the question of whether the chemotactic response is more sensitive to 16:1ω5 at the *sn*-1 position, or the *sn*-2 position.

The per cent PE with 16:1 at a given *sn* position was determined from the data in Table 1 by summing the percentages for each PE containing 16:1 at that position. In cases where the PE contained multiple fatty acid pairings, only the most abundant species was considered. The PE with *m/z* 688.5 peak in the *esg* mutant did not contain PE-17:1/15:0 (like the wild type) but instead had a mixture of PE-16:0/16:1 and PE-16:1/16:0, as represented by equal amounts of each fatty acid after CID. For the purpose of this analysis, 3.9% was assigned to *sn*-1 and 3.9% to *sn*-2 16:1. The 16:1 composition for vegetative wild type, 24 h developing wild type, *plsB1* and *esg* cells is reported in Table 2 and represent the most varied examples of 16:1 distribution among the strains reported in Table 1. *esg* PE had by far the largest amount of *sn*-1 16:1 at 71.6%. The next highest is vegetative wild-type PE at 38.2%, then developmental wild-type PE at 28.1% and finally *plsB1* PE at 23.4%. However, this order is not preserved for the *sn*-2 position. The highest *sn*-2 16:1 composition is developmental wild-type PE at 38.0%, followed by *esg* PE at 31.6%, vegetative wild-type PE at 28.9% and *plsB1* PE at 7.3%.

Table 2. Phosphatidylethanolamine (PE) enriched in *sn*-1 16:1 stimulates chemotaxis.

PE type	Reversal period	*sn*-1 16:1	*sn*-2 16:1	PE-16:1/16:1
None	7.0 ± 1.0	–	–	–
plsB1	16.0 ± 1.9	23.4	7.3	7.3
Dev WT	17.4 ± 5.2	28.1	38.0	15.5
Veg WT	18.4 ± 1.1	38.2	28.9	12.0
esg	47.3 ± 17.0	71.6	31.7	27.8
R^2		0.95	0.09	0.88

Phosphatidylethanolamine was purified from vegetative DK1622 (Veg WT), 24 h developing DK1622 (Dev WT), LS2300 (*plsB1*) and JD300 (*esg*) cells. Relative amounts of 16:1 at the *sn*-1 and *sn*-2 positions were calculated from the data in Table 1 by summing the percentages of each molecular PE species containing 16:1. Where two molecular species pairings have the same *m/z* only the most abundant pairing was considered. Phosphatidylethanolamine from these same strains was used to assess chemotaxis of wild-type DK1622 cells using the stimulation assay. In this assay, attractants increase the reversal period from an unstimulated level of 7 min depending on the abundance of attractant molecules in the preparation. Phosphatidylethanolamine preparations are listed in order of increasing stimulated reversal period. Linear regression was performed between the reversal period stimulated by each sample and the amount of 16:1 at the *sn*-1 position, *sn*-2 position or the species PE-16:1/16:1. Regression analysis was performed using Microsoft Excel.

The ability of the collective pool of PE from each strain to stimulate chemotaxis was assayed by tracking the movement of wild-type cells by time-lapse video microscopy. The reversal period of cells in the absence of PE was 7.0 ± 1.0 min and provides the baseline for unstimulated cells (Table 2). In trial experiments the optimal response was found with 2 μg of PE distributed over an area of about 0.16 mm^2 and overlayed with about 5×10^4 cells. Phosphatidylethanolamine from the *esg* mutant was most active and produced a reversal period of 47.3 ± 17.0 min (Table 2). *esg* PE has the largest abundance of 16:1 at the *sn*-1 position and more average levels of *sn*-2 16:1. Vegetative and developmental wild-type PE and *plsB1* PE produced comparable reversal periods. Phosphatidylethanolamine from the *plsB1* mutant has a similar amount of *sn*-1 16:1 to wild-type cells, but much lower *sn*-2 16:1. Together these results argue that the *sn*-1 position is more important than the *sn*-2 position for stimulating chemotaxis. A linear regression analysis was performed and an excellent relationship was observed with *sn*-1 16:1 ($R^2 = 0.95$), but not *sn*-2 16:1 ($R^2 = 0.09$).

Although the *sn*-1 position appears to be more important for chemotactic stimulation than *sn*-2, this relationship may be driven by a particular PE species. The most dramatic correlation between reversal period and specific species containing 16:1 was PE-16:1/16:1 ($R^2 = 0.88$, Table 2). PE-16:1ω5/16:1ω5 has been synthesized and is the strongest attractant described for *M. xanthus* (Kearns *et al.*, 2001a; Bonner *et al.*, 2005). Whether other PE species with 16:1 at the *sn*-1 position are chemoattractants and contribute to the observed stimulation is unknown, but it is quite evident that the abundance of 16:1 at the *sn*-1 position is strongly correlated with chemotaxis.

Abundance of 16:1ω5 in analysed strains and communities

The fatty acid 16:1ω5 is rarely found in cultured bacteria and generally represents a small proportion of the total fatty acids (Table 3). There are only a few exceptions. For example, 16:1ω5 has been suggested as a biomarker for arbuscular mycorrhizal fungi in soil (Olsson *et al.*, 1995). Elevated proportions of 16:1ω5 and a high ratio of 16:1ω5 to 16:1ω7 (including 16:1ω6) were found in the phospholipid fatty acids (PLFA) of a strain of *Flexibacter flexilis*

Table 3. 16:1ω5 and 16:1ω7/16:1ω6 content in cultured bacteria and environment samples.

Organism	% 16:1ω5	% 16:1ω7/ω6	Ratio[a]	Reference
Bacillus firmus	3.8	0.9	4.2	Jackoway[b]
Bradyrhizobium japonicum GC subgroup B	6.4	2.1	3.0	Jackoway
Bradyrhizobium japonicum GC subgroup A	1.8	0.7	2.6	Jackoway
Mycobacterium marinum	8.0	6.3	1.3	Jackoway
Roseomonas cervicalis	3.5	2.9	1.2	Jackoway
Hyphomonas neptunium	1.3	1.8	0.7	Jackoway
Roseomonas genomospecies 5	1.2	1.8	0.6	Jackoway
Empedobacter brevis	8.5	21.4	0.4	Jackoway
Streptococcus mitis	2.2	6.0	0.4	Jackoway
Exiguobacterium acetylicum	2.6	7.3	0.4	Jackoway
Geobacter metallireducens	1.5	40.7	0.04	Lovley *et al*. (1993)
Caulobacter-like aerobic dye degrader	1.4	25.7	0.05	Govindaswami *et al*. (1993)
Desulfomonile tiedjei	2.1	23.7	0.1	Ringelberg *et al*. (1994)
Subsurface Sphingomonas	0.3	2.1	0.1	Balkwill *et al*. (1997)
Pseudoalteromonis tunicata	0.4	47.5	0.008	Holmstrom *et al*. (1998)
Syntrophomonas wolfei (valerate)	5.7	0.2	28.5	Henson *et al*. (1988)
Soil methanotrophs	5.3	10.3	0.5	Nichols *et al*. (1987)
Surface soil	1.7	3.5	0.5	Ringelberg *et al*. (1989)
Propanotrophs	3.2	22.0	0.1	Ringelberg *et al*. (1989)
Oak rhizosphere	9.3	11.2	0.8	Ringelberg *et al*. (1997)
Myxococcus xanthus	24.0	0.3	80.0	Ware and Dworkin (1973)
Myxococcus xanthus	16.3	0.13	125.4	This study

a. Ratio is determined by dividing the per cent 16:1ω5 by the per cent 16:1ω7 (including 16:1ω6)
b. Gary Jackoway, MIDI, Newark, DE (pers. comm.).

which contained 51% of its PLFA as 16:1ω5 with a ratio = [16:1ω5]/([16:1ω7] + [16:1ω6]) of 96 (Nichols *et al*., 1986). This is unusual for the cultured Flavobacterium/Cytophaga. Another unusual strain from this group, *Cytophaga hutchinsonii*, also has a high level of 16:1ω5. Cultured type I methanotrophs like *Methylomonas* sp. 761 contain high levels of 16:1ω5 (16% of its PLFA as 16:1ω5 and a ratio of 0.94) (Nichols *et al*., 1985), whereas other cultured methanotrophs do not. In the environmental database compiled by MIDI (Newark, DE) 16:1ω5 was found in only 46 of 906 bacterial species. Of these 46, only three contained 16:1ω5 above 4% of the total fatty acids released by alkaline saponification: *Bradyrhizobium japonicum* 6.4%, *Mycobacterium marinum* 8.0% and *Empedobacter brevis* 8.6% (data kindly supplied by Gary Jackoway, MIDI, Newark, DE). In these cases, however, the 16:1 fatty acids are mixtures of isomers with different points of unsaturation. The ratio of 16:1ω5 to 16:1ω7 + 16:1ω6 is 3.0 for *B. japonicum*, 1.3 for *M. marinum* and 0.4 for *E. brevis*. In a previous study, 16:1ω5c in *M. xanthus* constitutes an even larger percentage of the total fatty acids (24%) than in the previous examples and is in much higher proportion to other 16:1 isomers (a ratio of 80.0, Table 3) (Ware and Dworkin, 1973). While, in this study, the relative abundance of 16:1ω5 was smaller (16.3%, Table 3), the ratio of isomers still strongly favours the ω5 isomer at 125.4. A high ratio of 16:1ω5 to 16:1ω7 + 16:1ω6 in PLFA can also be found in *Syntrophomonas wolfei/Desulfovibrio* sp. co-cultures growing with valerate instead of butyrate (28.5; Henson *et al*., 1988); however, the total 16:1ω5 was comparatively low at 5.7%. 16:1 found in *Stigmatella aurantiaca*, another myxobacterium, is predominantly in the ω5 form and is relatively high in abundance (Dickschat *et al*., 2005).

The conclusion that *M. xanthus* and *S. aurantiaca* are unusual in having both a high level of 16:1ω5 and a high ratio of 16:1ω5 relative to other 16:1 isomers is strengthened by examining microbial communities. In surface soil and in an oak rhizosphere 16:1ω5 can be detected but is not the most abundant 16:1 isomer (Table 3). A similar result is obtained with soils enriched with different carbon sources such as methane and propane.

Discussion

Myxococcus xanthus vegetative PE contains at least 17 different PE species (Table 1), rising to 19 during development. Among all the strains examined, 27 different PE species were detected. The number is surprising as most Proteobacteria have on average five to seven different PE species (Cronan and Rock, 1996; Rahman *et al*., 2000). Even more unusual is the fact that 12 of the 17 fatty acids found at the *sn*-1 position in *M. xanthus* PE are unsaturated (Table 1), which violates the well-established paradigm for Proteobacteria (Cronan and Rock, 1996).

In addition to the diversity observed in PE species, there is a large amount of diversity in the fatty acids themselves, as exemplified by the multiple points of unsaturation found in unsaturated fatty acids. 15:1 fatty acids were represented by ω4 (Δ^{11}) and ω10 (Δ^{5}) isomers, 16:1 by ω5 (Δ^{11})

and ω11 (Δ^5) isomers and iso17:1 by ω5 (Δ^{11}). However, the mechanism for generating this diversity is unclear. In the well-characterized *E. coli* fatty acid biosynthesis pathway, unsaturated fatty acids are created by the action of FabA (Cronan and Rock, 1996). FabA isomerizes the *trans*-Δ^2 double bond intermediate (which is usually reduced to create a fully saturated acyl chain) to the *cis*-Δ^3 double bond, which is preserved in further acyl-chain elongation. Given that only one point of unsaturation is observed in *E. coli*, FabA must have molecular specificity for the intermediate to be isomerized. If *M. xanthus* produces unsaturated fatty acids using a similar pathway, it would require multiple FabA homologues to introduce unsaturations at different positions on different fatty acids.

At least four FabA homologues are found in the *M. xanthus* genome. Additionally, in this model, fatty acids with even numbers of carbons must have unsaturations with odd-numbered ω positions (and the opposite case for fatty acids with odd numbers of carbons). In fact, this pattern is observed. There is, however, a far simpler explanation for the pattern of unsaturations. In the case of 15:1ω4, 16:1ω5 and iso17:1ω5, the point of unsaturation in each fatty acid is conspicuously 11 carbons from the Δ terminus. Similarly, for 15:1ω10 and 16:1ω11, the point of unsaturation is five carbons from the Δ terminus. Therefore, the pattern of unsaturations observed could easily be explained by the action of a Δ^{11} desaturase and a Δ^5 desaturase, and in fact desaturases are found in the genome. This model for desaturation is similar to the method used by *Bacillus* (Aguilar *et al*., 1998) and cyanobacteria (Hongsthong *et al*., 2004). In *Bacillus* and in cyanobacteria, the acyl-lipid desaturases introduce double bonds at specific positions (usually relative to the Δ terminus) in fatty acids that have already been esterified to glycerolipids. While the *E. coli* model for biosynthesis of unsaturated fatty acids is viable for *M. xanthus*, the desaturase model is the simplest explanation for the pattern of unsaturations observed. Further testing will be needed to determine exactly what role each model has in contributing to unsaturated fatty acid diversity in this organism.

The chemotactic activity of *M. xanthus* PE is associated with the fatty acid 16:1ω5. This compound is rare in bacteria and in natural samples. It may be a chemoattractant for the myxobacteria, as *M. xanthus* and *S. aurantiaca* both contain high levels of the ω5 isomer. High levels of 16:1 are also present in other myxobacteria *Nannocystis exedens* and *Sorangium cellulosum*, although the isomer(s) is unknown (Iizuka *et al*., 2003). Synthetic PE-16:1ω5/16:1ω5 is a potent chemoattractant for *M. xanthus* (Kearns *et al*., 2001a), and here we show that PE-16:1/16:1 is one of the major PE constituents. However, there are several other PE species containing 16:1 at either *sn* position. *Myxococcus xanthus* may respond to PE with 16:1 at the *sn*-1 position, the *sn*-2 position or a particular 16:1-containing species. In addition, PE may be hydrolysed by a phospholipase and the free fatty acid detected. Regression analysis with chemotactic stimulation by PE of varying composition shows strong linear relationships with *sn*-1 16:1 and with the species PE-16:1/16:1. However, the alternate hypothesis of position-specific cleavage by phospholipase A1 cannot be ruled out. Taken as a whole, the results argue that the wide diversity in PE species by *M. xanthus* is due to the fact that some species play a role in cell–cell signalling. It appears that *M. xanthus* has deployed a rare unsaturated fatty acid (16:1ω5) at an unusual position in PE (*sn*-1) to obtain a species or group-specific signalling molecule.

A key to understanding the evolution of this signalling system is the enzymology leading to the deployment of unsaturated fatty acids at the *sn*-1 position. One case of unsaturated fatty acid localization at the *sn*-1 position has been examined previously; phospholipids of *Clostridium butyricum* have both saturated and unsaturated fatty acids at the *sn*-1 position while the *sn*-2 position has only saturated fatty acids (Heath *et al*., 1997). The *sn*-1 fatty acid bias in *C. butyricum* involves a unique PlsC-like *sn*-1 acyltransferase, PlsD. However, there is no evidence that *M. xanthus* uses a similar strategy as PlsB1 and all five PlsC homologues found in the *M. xanthus* genome closely resemble their *E. coli* counterparts (Figs 2 and 4). In addition, *M. xanthus* PE does not have a reversed fatty acid bias like *C. butyricum* (variable saturation at the *sn*-1 position and predominantly saturated at the *sn*-2) but rather an aberrant one (predominantly unsaturated at the *sn*-1 and variable at the *sn*-2). Therefore, it appears that *M. xanthus* PE has properties that differ from not only the established lipid paradigm, but also other unusual cases.

Myxococcus xanthus is unusual in the large number of putative acyltransferase genes present in the genome. We suspected that one of the two *plsB* genes might be the source of the unsaturated fatty acids at the *sn*-1 position. However, deletion of *plsB1* or *plsB2* did not eliminate unsaturated fatty acids at the *sn*-1 position, suggesting that both acyltransferases contribute unsaturated fatty acids. The N-terminal half of PlsB2 is similar to several eukaryotic FARs. These enzymes reduce fatty acyl-CoA substrates to free fatty alcohols (Kolattukudy, 1970) either for synthesis of waxes, a reaction where a fatty alcohol is esterified to another fatty acid, or for ether-linked phospholipids synthesis (for review, see Nagan and Zoeller, 2001). Prokaryotic fatty acid reduction utilizes two enzymes, one to reduce the fatty acyl-CoA to the fatty aldehyde, and a second to reduce the fatty aldehyde to the fatty alcohol (Reiser and Somerville, 1997). Eukaryotic FARs use a single enzyme with a NAD(P)H-binding motif to reduce fatty acyl-CoA to the fatty alcohol in a single enzyme (Kolattukudy, 1970). In both eukaryotic and

prokaryotic systems, the reductases are separate enzymes from the acyltransferases. Therefore, PlsB2 is unique in that the FAR is more similar to eukaryotic reductases, and is uniquely coupled to an acyltransferase domain. It appears unlikely that PlsB2 functions solely in ether lipid production, although ether lipids are detected in myxobacteria (Kleinig, 1972; Caillon *et al*., 1983), because the *plsB1* mutation would be lethal. The *plsB1* gene was deleted without complication and while there is a loss of some of the higher-molecular-weight species, the majority of the species and their abundances remain similar to the wild type (Table 1). Therefore, it seems likely that the PlsB2 acyltransferase domain functions in PE biosynthesis.

The *plsB2* gene is located next to a putative alkyl-dihydroxyacetone phosphate synthase gene; the stop codon of the synthase overlaps the start codon of *plsB2* by 1 bp suggesting translational coupling. This enzyme catalyses the exchange of a fatty alcohol for a fatty acid at the *sn*-1 position of 1-acyl-dihydroxyacetone phosphate, creating an intermediate in eukaryotic ether lipid biosynthesis. The proximity of these genes combined with the nature of the FAR domain would suggest that PlsB2 functions in ether lipid biosynthesis. Therefore, it is possible that PlsB2 has a dual role in both PE biosynthesis and ether lipid biosynthesis.

Lipid signalling may be involved in maintaining culture specificity during fruiting body formation in the midst of a complex microbial community. The output from lipid signalling assayed in these experiments is an alteration of motility. The chemotaxis signal passes through the *dif* chemosensory pathway (Kearns *et al*., 2000; Bonner *et al*., 2005), which also regulates extracellular matrix production, another component of surface-dwelling organisms that is essential for fruiting body formation (Yang *et al*., 2000; Bellenger *et al*., 2002; Black and Yang, 2004). The coupling of matrix production and lipid sensing is unusual and may reflect the fact that extracellular matrix is essential for a response to some lipid attractants.

Myxococcus xanthus responds chemotactically to another lipid, PE-18:1ω9/18:1ω9. The fatty acid 18:1ω9 was not observed in the cells of *M. xanthus*, but is found in many other proteobacteria, such as *E. coli* (Cronan and Rock, 1996), which *M. xanthus* preys on in the soil (Yamanaka *et al*., 1987; Shimkets *et al*., 2006). It is speculated that 18:1 chemotaxis is used for finding prey. Correlatively, neither the conditions nor part of the machinery for the 18:1 response has much in common with the 16:1 response. Whereas 16:1 stimulation requires starvation conditions and production of the extracellular matrix (factors for the developmental cycle), 18:1 can stimulate chemotaxis under nutrient-rich conditions and independently of the presence of the extracellular matrix (Kearns *et al*., 2000). It was also recently shown that the 18:1 signalling pathway is partially independent of the Dif chemosensory pathway used for 16:1 signalling; 18:1 requires the histidine kinase (DifE) and response regulator (DifD) but not the methyl-accepting chemotaxis protein (DifA) or the coupling protein (DifC) to stimulate chemotaxis (Bonner *et al*., 2005). Therefore, it appears that *M. xanthus* has two largely independent lipid sensory systems.

Pseudomonas aeruginosa also travels up gradients of PE-18:1ω9/18:1ω9 and PE-12:0/12:0 (Kearns *et al*., 2001b; Barker *et al*., 2004). Interestingly, the response to PE-18:1ω9/18:1ω9 is dependent on the activity of a phospholipase C (PlcB) while the response to PE-12:0/12:0 is not (Barker *et al*., 2004), indicating that *P. aeruginosa*, like *M. xanthus*, has two lipid-signalling pathways. Indeed, *P. aeruginosa* shares several characteristics in common with *M. xanthus*. They are both soil-dwelling microbes, utilize surface motility, produce an extracellular matrix and display forms of multicellular behaviour (biofilm formation in the case of *P. aeruginosa*). Lipids may be ideal signals for surface-translocating organisms; surface motility is prohibitively slow compared with flagellar motility of planktonic cells. Whereas soluble chemical signals could freely diffuse and therefore collapse gradients before surface-motile organisms have a chance to respond, lipids will establish more stable gradients due to the insoluble nature of the molecules. Other microorganisms may provide a broad variety of unique fatty acids and lipid species (signature lipid biomarkers) and may also utilize lipid-based signalling.

Experimental procedures

Strains and growth conditions

Myxococcus xanthus wild-type strain DK1622 (Kaiser, 1979), and mutant strains LS2300 (*plsB1*; MXAN3288), LS2301 (*plsB2*; MXAN1675), JD300 (Downard and Toal, 1995), LS2305 (*plsC2*; MXAN3969), LS2306 (*plsC4*; MXAN0955) and LS2307 (*plsC5*; MXAN0147) were grown at 32°C in CYE [1.0% Bacto Casitone, 0.5% Difco yeast extract, 10 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS), pH 7.6 and 0.1% $MgSO_4$] broth with vigorous shaking (Campos *et al*., 1978). Cultures were grown on plates containing CYE with 1.5% Difco agar. To induce development, 7.5 ml of 5×10^9 cells ml^{-1} were plated on TPM agar [10 mM Tris (hydroxymethyl) aminomethane HCl, 8 mM $MgSO_4$, 1 mM $KHPO_4$-KH_2PO_4, 1.5% Difco agar, pH 7.6] in a 33×22 cm tray and incubated at 32°C for 24 h.

Construction of plsB *mutants*

Preliminary sequence data were obtained from The Institute for Genomic Research through the website at http://www.tigr.org. Two *M. xanthus plsB* homologues were identified using the *E. coli* PlsB protein sequence (P0A7A7) to search the *M. xanthus* genome (available at http://

cmr.tigr.org/tigr-scripts/EMR/CmrHomePage.cgi) and analysed using the CLUSTALW (http://align.genome.jp/) and BOXSHADE (http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html) programs. In-frame deletion mutants of *plsB1* and *plsB2* were constructed as described previously (Julien *et al*., 2000). Wild-type *M. xanthus* chromosomal DNA was purified (Easy-DNA kit, Invitrogen) and approximately 750 bp of sequence upstream of *plsB1* was amplified using primers INFRB1P1F and INFRB1P1R, which add BamHI and XbaI restriction sites respectively (Table 4). Approximately 750 bp of downstream sequence was amplified using primers INFRB1P2F and INFRB1P2R, which add XbaI and HindIII sites respectively. Polymerase chain reaction (PCR) products were separated on 1.0% agarose and the desired fragments were extracted using the UltraClean 15 DNA Purification Kit (Mo Bio Laboratories). Extracted fragments were used in a third PCR reaction with primers INFRB1P1F and INFRB1P2R and the products were separated on 1.0% agarose. A band of approximately 1.5 kb was extracted and cloned into pCR2.1-TOPO (TOPO TA Cloning Kit, Invitrogen) to create plasmid pINFR6. The integrity of the construct was verified by DNA sequencing. Plasmids pBJ113 (Julien *et al*., 2000) and pPDC4 were digested with BamHI and HindIII and gel purified. The ~1.5 kb insert from pPDC4 was ligated with pBJ113. The resultant plasmid (pPDC5) was electroporated in *M. xanthus* DK1622 and transformants were selected on CYE agar plates containing 50 μg ml^{-1} kanamycin. Transformants were then grown in CYE broth in the absence of selection and plated on CYE agar plates supplemented with 1.0% galactose. Galactose-resistant colonies were screened for kanamycin sensitivity and analysed by PCR reactions with primers INFRB1P1F and INFRB1P2R. Polymerase chain reaction fragments of ~1.5 kb were verified as deletions by digestion of the PCR fragments with XbaI into approximately equal size fragments. A colony with appropriate amplification size and digestion pattern was kept as strain LS2300.

Similarly, the *plsB2* mutant (LS2301) was generated using primers INFRB2P1F and INFRB2P1R in the first PCR reaction, INFRB2P2F and INFRB2P2R in the second reaction, INFRB2P1F and INFRB2P2R in the third reaction and INB2DIAGF and INB2DIAGR in the verification reaction. Polymerase chain reaction fragments were cloned, digested and ligated exactly as described above.

Table 4. Primers used in construction and examination of *plsB* and *plsC* mutants.

Primer name	Sequence (5′→3′)
INFRB1P1F	GGA TCC GCA CCG TCC ACG TCG CGT TCG
INFRB1P1R	CAT CTA GAC ATG GGG CCG AAT TCG TCC TTC AGC
INFRB1P2F	ATG TCT AGA TGA GCA GCC CTC CCT GGC CCC
INFRB1P2R	AAG CTT GGC GCT GAA CAC CAC GGC GGA G
INFRB2P1F	GGA TCC GGT GGT GGG CAT GGT CGA CGT G
INFRB2P1R	TCA TCT AGA CAT GCC CTC GTT CAC CAC GCG C
INFRB2P2F	GTC TAG ATG AAG ACA CTC GTG ACG GGA GCC
INFRB2P2R	AAG CTT CGA GCA CCA CGG GGT CCG GCA GC
INB2DIAGF	CAT GTT CGT CGG CCG CAA GGA C
INB2DIAGR	CGA GCT GTC GAT GTT GAA GCG C
MYXplsC2F	AAG CAA CCA CGA GTC CAA
MYXplsC2R	TTG GTG GAG ATG GGC GTG
MYXplsC4F	TCA TTG GTC TGT CGT TGG
MYXplsC4R	GTC TGG ATG CAG CAG CCC
MYXplsC5F	CCG TGC TCG TGT CCA ACC
MYXplsC5R	GAG GAC CAC GGG GAT GAC

Construction of plsC *mutants*

Five *M. xanthus plsC* homologues were identified by genome search using the *E. coli* PlsC protein sequence (P26647). They were named *plsC1*, *plsC2*, *plsC3*, *plsC4* and *plsC5*. Campbell insertion mutants were created by amplifying internal fragments of approximately 450 bp. These were amplified using primers MYXplsC2F and MYXplsC2R for the *plsC2* gene, MYXplsC4F and MYXplsC4R for *plsC4* and MYXplsC5F and MYXplsC5R for *plsC5*. The PCR products were separated on a 1.0% agarose gel, purified, and cloned into pCR2.1-TOPO to create plasmids pPDC1, pPDC2 and pPDC3 respectively. Each plasmid was electroporated in DK1622 and grown on CYE + 50 μg ml^{-1} kanamycin. Transformants were examined by Southern hybridization and probed with labelled PCR fragments generated using the same primers.

Characterization of mutants

Mutants were examined for motility, fruiting body formation and sporulation. For motility, the edges of colonies on CYE agar were viewed under 400× magnification on a Leitz Laborlux D phase-contrast microscope. To analyse fruiting body formation and sporulation, 1.5×10^9 cells were plated on TPM agar plates and incubated at 32°C with observation on a Wild Heerbrugg dissecting microscope over 5 days. Fruiting bodies were removed with a sterile razor blade and resuspended in 1.0 ml of TPM buffer. The fruiting bodies were then incubated at 55°C for 2 h and sonicated at a 60% duty cycle for 2 × 15 s on an Ultrasonic Processor Sonicator (Heat Systems–Ultrasonics). Refractile myxospores were quantified using a Petroff–Hauser counting chamber. Spores were diluted and plated on CYE plates to enumerate viable spores.

Fatty acid methyl ester analysis

Lyophilized cells of *M. xanthus* were extracted and derivatized according to the whole-cell hydrolysate procedure of the Microbial Identification System (MIDI, Newark, DE) (Härtig *et al*., 2005). Samples were resolved in hexane containing the FAME 21:0 as an internal standard (59 pmol μl^{-1}) for analysis by GC-MS (Geyer *et al*., 2005).

Dimethyldisulfide derivatization

Fatty acid methyl esters were derivatized using a modified procedure of Leonhardt and deVilbiss (1985). Samples for derivatization with dimethyldisulfide were dissolved in 1.0 ml of diethylether/hexane (50:50). Approximately 4.0 mg of iodine and 300 μl of DMDS were added and the mix was incubated at 37°C for 30 min. The reaction was completed by adding sodium thiosulfate (10% solution) dropwise until the

mixtures remained colourless. The upper organic phase was transferred, dried over sodium sulfate, evaporated to dryness and resuspended in 500 μl of hexane. An aliquot of 1.0 μl was analysed with GC-MS using the same temperature programme as for FAME analysis. Results showed a nearly complete derivatization. Details to interpretation of mass spectra from DMDS derivatives are outlined elsewhere (Christie, 2006).

Phosphatidylethanolamine purification

Phosphatidylethanolamine was purified using a modified method of Bligh and Dyer (1959). Cells were grown in CYE to a density of 7.5×10^8 cells ml^{-1} (approximately mid-log phase) and pelleted by centrifugation at 12 100 *g* for 10 min. To 0.8 g (wet cell weight) of *M. xanthus* cells was added 7.5 ml of methanol/chloroform (2:1). The mixture was vigorously shaken for 1 h. Insoluble material was pelleted by centrifugation at 12 100 *g* for 5 min and the supernatant was collected. The pellet was re-extracted with 9.5 ml of methanol/chloroform/water (2:1:0.8), centrifuged again, the supernatant collected and combined with the supernatant from the first extraction. Chloroform (5.0 ml) and 5.0 ml of water were added to the combined supernatants and mixed by vortexing. The phases were separated by centrifugation at 3020 *g* for 30 s. The chloroform layer was air-dried, resuspended in 0.75 ml of methanol/chloroform (2:1) and spotted onto silica gel 60 TLC plates (EM Science). The plates were developed for 3 h in 100 ml of chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1) and dried. Two-centimetre horizontal swaths of silica were scraped from the plate, leaving only a small vertical section of silica for staining. Lipid was extracted from the scraped silica with methanol/chloroform (2:1) followed by centrifugation at 15 400 *g* to pellet the silica, and the extract was dried. The extract was re-extracted 3×0.5 ml methanol/chloroform (2:1), centrifuged twice at 15 400 *g* for 5 min to remove residual silica, and the final extract was dried for analysis. The remainder of the plate was then stained with 0.5% ninhydrin (3% acetic acid in 1-butanol) and incubated at 100°C for 5 min. Phosphatidylethanolamine resolved with an R_f value of approximately 0.55.

Phosphatidylethanolamine analysis

The polar lipid extracts were analysed by LC-ESI-MS-MS performed on an Applied Biosystems/MDS SCIEX 365 tandem MS system with an electrospray interface (LC-ESI-MS-MS). The LC system (Agilent 1100 LC) used for introduction of the sample into the mass spectrometer consisted of an in-line vacuum degasser, a quaternary solvent pump, an autosampler, a column oven and a diode-array detector (Agilent 1100, Agilent, Palo Alto). The autosampler was equipped with a 100 μl sample loop. Details of phospholipid analyses at this system were described elsewhere (Lytle *et al.*, 2000; White *et al.*, 2002). A Thermo Dash-8 20×2.1 mm HPLC column was used for the reversed phase chromatographic separation of phospholipids. A gradient solvent system composed of solvent A (water) and solvent B (methanol/acetonitrile 90:10 + 0.002% piperidine) was used with a flow rate of 100 μl min^{-1}. At the beginning of the gradient, the mobile phase was 80% of B for 0.5 min. Solvent B was increased to 100% at 15 min. The mobile phase was then held isocratically for 10 min. Solvent B was decreased within 0.5 min to the starting value and the column equilibrated for 5 min. The column oven was held at 40°C.

The mass spectrometer was operated in the positive ionization mode to uniquely detect the PE molecules and determine their mass (*m/z*) by a neutral-loss scan for the PE head group (*m/z* 141). The acyl-chain composition within the detected PEs was determined by a product ion scan (MS2) in the negative ionization mode. Overviews of methods for ESI-MS-MS of phospholipids were recently given (Sturt *et al.*, 2004; Taguchi *et al.*, 2005).

With a palmitoyl-oleoyl PE standard (PE-16:0/18:1, Avanti Polar Lipids) infused into the ESI source, the instrument source parameters (e.g. curtain gas, nebulizer gas, source heater) were optimized as follows. The source temperature was 450°C. For positive ionization, the ion transfer voltage (IS) between electrospray needle and vacuum interface was set to 5000 V or in the negative mode to −4400 V, with the skimmer held at ground potential. The declustering potential (DP, voltage between orifice plate and ground), the focusing potential (FP, voltage between skimmer and ring potential) and the collision energy (CE) for CID were optimized at 30 V, 290 V and 30 V in the positive mode and at −40 V, −280 V and −45 V in the negative mode respectively. Nitrogen was used as collision gas. The nitrogen curtain gas, and the ion-source gases 1 (nebulizer gas) and 2 (turbo gas at the heater) were set to pressures of approximately 20, 30 and 60 psi (1.4, 2.0, 4.1 bar) respectively. The mass spectrometer was tuned from *m/z* 5 to 2000 amu according to the protocol provided by the manufacturer.

The fragmentation of the $[M+H]^+$ ions of PEs is dominated by the *m/z* $[M-141]^+$ ion, which unequivocally characterizes molecules of this lipid class. This decomposition, releasing the PE head group as a neutral molecule $[H_2PO_4CH_2CH_2NH_2]$, can be assessed by tandem mass spectrometer as loss of *m/z* 141. The obtained masses uniquely characterized the *m/z* $[M+H]^+$ of PEs.

The relative concentration of PE within a sample was assessed based on the ion currents of the individual phospholipids set in relation to the sum of all detectable PE species in the neutral-loss scan. To exclude molecules with low abundances and with background signals, a cut-off of 15% signal intensity was applied. Absolute concentrations of PE species were calculated based on a calibration curve obtained with a PE 16:0/18:0 standard (Avanti Polar Lipids).

In the negative mode, subsequent collision-induced fragmentation of the proposed PE ion *m/z* $[M-H]^-$ revealed the acyl-chain composition within the individual PE. The position on the glycerol backbone was assigned based on the signal intensities of the abundant carboxylate anion as the *sn*-2-derived anion is usually preferred at a ratio greater than 2 to 1 (Pulfer and Murphy, 2003). The assignment as PEs could be additionally verified based on the diagnostic ion of the PE headgroup at *m/z* 140 $[HPO_4CH_2CH_2NH_2]^-$, which was visible at a low abundance.

Fragmentation scans in the negative mode indicated that the samples still contained phosphatidylglycerol (PG) characterized by the diagnostic ions *m/z* 153

$[CH_2C(OH)CH_2HPO_4]^-$ and *m/z* 171 $[HPO_4CH_2CH(OH)CH_2OH]^-$. However, the PG did not compromise either the quantification of PE or analysis of fatty acid composition.

Phospholipids were designated as follows: PL-C1:d1/C2:d2 (e.g. PE-16:0/18:1), where C1 and C2 are the numbers of carbon atoms in the fatty acyl chains on the *sn*-1 and *sn*-2 positions, respectively; d1 and d2 are the numbers of double bonds of the *sn*-1 and *sn*-2 fatty acyl chains, respectively; and PL is the abbreviation for phospholipids class.

Stimulation assay

Chemotaxis was quantified using the stimulation assay (Kearns and Shimkets, 1998). This assay measures the period of time between cell reversals (reversal period, which is approximately 7 min for unstimulated cells) (Blackhart and Zusman, 1985). Attractants increase the reversal period (Kearns and Shimkets, 1998). The stimulation assay was performed as previously described with minor modifications (Kearns and Shimkets, 1998). Phosphatidylethanolamine purified from DK1622, LS2300 or JD300 was solubilized in chloroform to 0.5 mg ml^{-1}. A TPM agar plate was dried for 10 min at 37°C. Then 4 μl of PE solution was applied to the surface of the agar plate and the solvent was evaporated by incubation at 37°C for 15 min. Five microlitres of DK1622 cells diluted to 5×10^7 cells ml^{-1} in MOPS buffer (10 mM MOPS, 8 mM $MgSO_4$, pH 7.6) were applied to the PE spot and incubated at 32°C for 15 min. Cells were then observed at 25°C with a Leitz Laborlux D microscope for 45 min at 640× magnification. Stop-motion digital movies were produced with a microscope-mounted Sony Power HAD 3CCD colour video camera and a Macintosh 9500 with Adobe PREMIERE software (Adobe Systems, Mountain View, CA; frame capture rate, 12 frames per minute). The reversal period was manually enumerated for 15–20 isolated cells per movie. The mean and standard deviation were calculated from three movies.

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