Identification of a developmental chemoattractant in *Myxococcus xanthus* through metabolic engineering

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Fruiting body formation of *Myxococcus xanthus* requires the ordered migration of tens of thousands of cells by using a form of surface motility known as gliding and chemical signal(s) that have yet to be elucidated. Directed movement is regulated by phosphatidylethanolamine (PE) purified from *M. xanthus* cell membranes. Because the purified PE preparation contains a remarkably diverse mixture of fatty acids, metabolic engineering was used to elucidate the biologically active fatty acid component. The mutational block in an *esg* mutant, which renders it defective in producing primers for branched-chain fatty acid biosynthesis, was bypassed with one of a series of primers that enriches for a particular family of branched-chain fatty acids. Each PE enrichment was observed for chemotactic activity by using an excitation assay and for fatty acid content. The excitation activity of a PE preparation was generally proportional with the concentration of the fatty acid 16:1ω5c. 1,2-*O*-Bis[11-(*Z*)-hexadecenoyl]-*sn*-glycero-3-phosphoethanolamine (PE-16:1ω5c/16:1ω5c) was synthesized and elicited an excitation peak at 2 ng. This peak activity occurred at a 1,000-fold lower concentration than dilauroyl PE (PE-12:0/12:0) and the peak magnitude was 2-fold higher. PE containing 16:1ω5c is likely to play a role in development because it is active at physiological concentrations and only under developmental conditions.

The myxobacteria exhibit many social behaviors during their remarkable life cycle. The most striking of these is fruiting body morphogenesis, which involves the directed movement of nearly 10^5 cells to an aggregation center. The fruiting body then develops a species-specific shape, which can involve further vertical and lateral movement of portions of the population. Genetic approaches have suggested that many intercellular signals are involved in development of *Myxococcus xanthus* (see ref. 1 for review). Many of the genes involved in signal production have been cloned and sequenced, but this genetic approach has provided little insight into the chemical nature of each signal. For example, the signal(s) directing fruiting body morphogenesis remain to be elucidated.

Myxobacteria move by gliding on solid surfaces at a rate of several micrometers per minute. In *M. xanthus* there are two motility systems known as adventurous (A) and social (S). S motility, which is similar if not identical to twitching motility of many pathogens, requires the production of type IV pili, which apparently provide propulsion by attaching to a substrate and then retracting to pull a cell along (2–4). The mechanism of A motility has not been elucidated. Although a chemoattractant might be involved in directing cells to an aggregation center during fruiting body morphogenesis, attempts to demonstrate chemotaxis to soluble chemical signals have been unsuccessful (5). Dworkin and Eide noted that the use of soluble chemical signals for slow-moving, surface-motile organisms was unlikely in that the diffusion rate of the attractant could exceed the motility rate (5). This observation prompted us to examine the cell for insoluble molecules that might direct movement, and resulted in the identification of the hydrophobic chemoattractant phosphatidylethanolamine (PE) (6). PE directs cell movement up chemical gradients and regulates the cell reversal frequency.

The PE fraction exhibiting chemotactic activity is extracted directly from the cell membrane and purified by adsorption and TLC based on the polar head group. The PE extract contains dozens of different molecules because of the diversity of *M. xanthus* fatty acids, which includes saturated, unsaturated, branched-chain, and hydroxylated species (7). Although synthetic dilauroyl PE (PE-12:0/12:0) and dioleoyl PE (PE-18:1ω9c/18:1ω9c) have modest chemotactic activity, neither is present in sufficient concentrations to account for the activity of the PE preparation. Techniques for fractionating PE based on the constituent fatty acids are difficult and are further compromised by the fact that the *sn*-1 and *sn*-2 positions of the glycerol backbone are usually occupied by different fatty acids.

Fatty acid biosynthesis typically begins with a two-carbon acetyl-CoA primer, and chain elongation occurs with condensation of two-carbon units from malonyl-CoA. Repeated condensation with malonyl-CoA leads to the synthesis of saturated fatty acids containing an even number of carbons. To synthesize branched-chain fatty acids, leucine, isoleucine, and valine are deaminated and then decarboxylated by branched-chain keto acid dehydrogenase (BCKAD) to produce branched-chain CoA primers that substitute for acetyl-CoA (8). The branched-chain primers are also elongated by recursive condensation with malonyl-CoA to produce the iso-odd, anteiso-odd, and iso-even families, respectively (Fig. 1). The *esg* mutant, which lacks BCKAD, has reduced levels of branched-chain fatty acids unless cultured in the presence of isovalerate, 2-methylbutyrate, or isobutyrate (8). These substrates bypass the mutational block and feed into the fatty acid biosynthesis pathway through an unknown enzyme (9).

In this report we used the *esg* mutation and bypass technology to elucidate the chemical structure of an endogenous *M. xanthus* lipid signal. The *esg* mutation was bypassed with isovalerate, 2-methylbutyrate, or isobutyrate to enrich for specific families of fatty acids. The PE preparations were evaluated for chemotactic excitation and fatty acid composition to identify the most likely fatty acid component of the attractant. We synthesized this molecule and showed that it behaves as a chemoattractant under developmental conditions.

Methods

Strains and Conditions. *M. xanthus* DK1622 (wild type; ref. 10) and JD300 (*esg*; ref. 8) were grown in casetone/Tris (CTT) broth (11) with vigorous shaking at 32°C. When appropriate, JD300 was grown in CTT supplemented with 1 mM isovalerate, 1 mM 2-methylbutyrate, or 1 mM isobutyrate. Lipid was extracted and purified as described (6, 12). Fatty acid analysis of purified PE was performed by Microbial ID (Newark, DE).

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Abbreviations: PE, phosphatidylethanolamine; BCKAD, branched-chain keto acid dehydrogenase; CTT, casetone/Tris; TPM, Tris phosphate magnesium.

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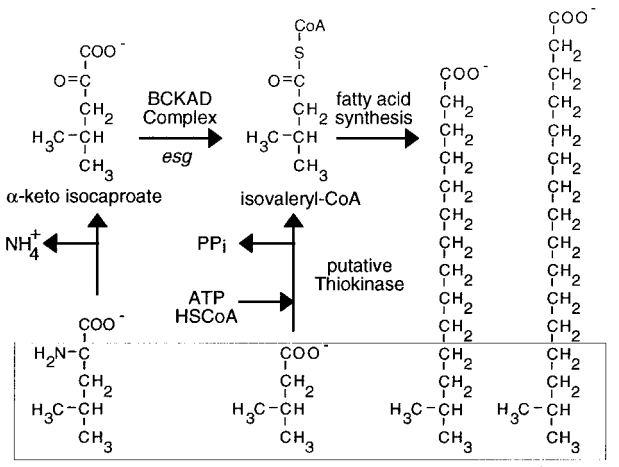

Amino acid	Bypass primer	Fatty acid family
L-leucine	isovalerate	iso-odd
L-isoleucine	2-methyl-butyrate	anteiso-odd
L-valine	isobutyrate	iso-even

Fig. 1. The branched-chain fatty acid biosynthesis pathway. (*Upper*) Leucine is deaminated and then converted to a branched-chain fatty acyl-CoA primer by BCKAD, the product of the *esg* locus. The primer is extended in multiples of two carbons during fatty-acid biosynthesis to generate the iso-odd fatty acid family. A bypass pathway exists by which isovalerate is converted to the acyl-CoA derivative by another enzyme. *Lower* shows that the nature of the amino acid or bypass primer determines the fatty acid family that is produced. The *esg* mutation inhibits the synthesis of all three fatty acid families. A bypass primer exists for each of the three families.

Excitation Assay. The excitation assay measures changes in the period between reversals (reversal period) in response to a uniform concentration of attractant (6). A Tris phosphate magnesium (TPM) agar plate was dried for 10 min at 37°C. PE was dissolved in chloroform, and 4 μl of PE solution was applied to TPM agar in an area of about 0.4 cm^2 and dried for 20 min at 37°C. Five microliters of *M. xanthus* cells diluted to 5×10^6 cells per ml in Mops buffer [10 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.6/8 mM $MgSO_4$] was dried on top of the test compound for 15 min at 32°C to allow the liquid-grown cells to adjust to the surface. Plate cultures were observed at room temperature 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 color video camera and a Macintosh 9500 with Adobe PREMIERE software (Adobe Systems, Mountain View, CA; frame capture rate, 12 frames per min). The cellular reversal periods of 20 isolated cells were manually enumerated.

Synthesis of 1,2-*O*-Bis(11-(*Z*)-hexadecenoyl)-*sn*-glycero-3-phosphoethanolamine. Synthesis of the phospholipid proceeded in three stages: (*i*) synthesis of the unsaturated fatty acid chain, (*ii*) synthesis of the glycerol core and attachment of the fatty acids, and finally (*iii*) attachment of the phosphoethanolamine moiety.

Synthesis of hexadecenoyl ethyl ester was performed according to a procedure by Danheiser and Cha (13). The Z/E ratio 98:2 was determined by ^{1}H-NMR (14). The ester group was saponified with lithium hydroxide to provide the acid. The 2,3-*O*-isopropylidene-*sn*-glycerol was synthesized (15, 16) and its enantiomeric purity confirmed by examination of the ^{1}H-NMR spectrum of the corresponding Mosher ester [(*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenyl acetate], which indicated the presence of a single enantiomer (17). The glycerol derivative was then transformed into the corresponding 1,2-*O*-diacyl-*sn*-glycerol after a sequence of reactions described by Alcaraz *et al.* (18) and Martin *et al.* (19). The attachment of the phosphoethanolamine moiety to the 1,2-*O*-diacyl-*sn*-glycerol was performed stepwise (19), with *N*-(*tert*-butoxycarbonyl)ethanolamine as starting material, and the appropriate deprotection sequence provided the final product. The purity of all the intermediates and of the final product was checked by TLC and by ^{1}H-NMR at 500 MHz. Appropriate NMR experiments (^{1}H, ^{13}C, ^{31}P-NMR) were used to confirm the structure of the intermediates and final product.

Results

Although the *esg* mutant is defective in branched-chain fatty acid synthesis, the PE is not devoid of branched-chain fatty acids, which declined from about 50% in wild-type cells to 25% when *esg* cells were cultured in the absence of bypass primers (Fig. 2). We considered several reasons why the *esg* cells were not devoid of branched-chain fatty acids. First, there could be a second BCKAD complex in the organism. A search of the genome sequence did not reveal a second BCKAD complex, although it did indicate the presence of a pyruvate dehydrogenase complex. The pyruvate dehydrogenase complex is structurally and functionally related to BCKAD and could conceivably metabolize other β-keto acids. To the best of our knowledge this possibility has not been reported for pyruvate dehydrogenase complex in other organisms and was not explored further with *M. xanthus*. Second, the bypass primers could be supplied in the CTT growth medium or produced by amino acid catabolism. To avoid these possibilities we attempted to culture *esg* cells in A1 minimal media with and without the bypass primers (20). We were unable to obtain growth of *esg* cells in A1 medium and resorted to enrichments in CTT broth.

The PE fatty acid composition was selectively enriched for each branched-chain fatty acid family by growing *esg* cells in CTT broth supplemented with isovalerate, 2-methylbutyrate, or isobutyrate. PE was extracted from the cell membrane of each preparation and purified by adsorption and TLC (6). Despite the inherent leakiness of the system, the enrichment works fairly well. *esg* cells grown in the presence of isovalerate, 2-methylbutyrate, and isobutyrate were enriched for iso-odd, anteiso-odd, and iso-even fatty acids, respectively (Fig. 2). One of the most dramatic enrichments was observed in isovalerate-grown *esg* cells where the levels of 15:0 iso increased to about 75% of the total fatty acids.

An interesting relationship was observed between unsaturated fatty acid biosynthesis and branched-chain saturated fatty acid biosynthesis. *esg* cells responded to the branched-chain fatty acid deficiency by increasing production of unsaturated fatty acids, particularly 16:1ω5c. Conversely, when isovalerate enriched *esg* cells for 15:0 iso, 16:1ω5c declined 3-fold. These results may reflect the fact that the two types of fatty acids have similar physical properties and can substitute for each other in maintaining membrane fluidity. Unsaturated fatty acids and branched-chain fatty acids have lower melting temperatures than straight chain fatty acids enabling a fluid membrane at low temperatures (21, 22).

Each PE preparation was evaluated for chemotactic activity by using the excitation assay (6). Excitation is well defined in the swimming bacterium *Escherichia coli*, which swims through liquid with the aid of flagella. *E. coli* alternates periods of smooth swimming in a nearly straight trajectory with circular tumbling after which a new trajectory is chosen. Excitation occurs when a chemoattractant stimulates a sensory system that increases the duration of the period of smooth swimming, enabling the cell to swim for a longer period up a chemoattractant gradient. *E. coli* cells exposed to a uniform concentration of attractant also suppress direction reversals (23). *M. xanthus* cells glide over solid

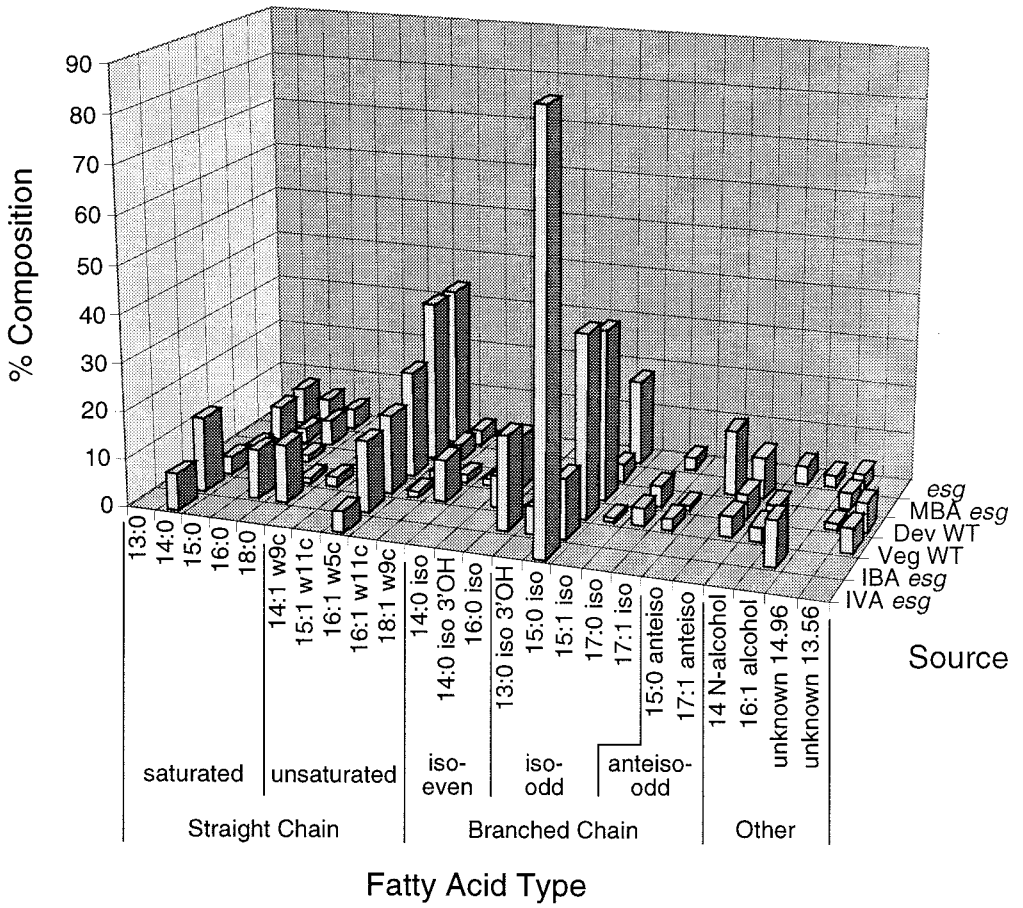

Fig. 2. Fatty acid composition of *M. xanthus* PE purified from various enrichments. PE purified from vegetative wild-type cells grown in CTT broth (Veg WT), PE purified from wild-type cells in the process of directed movement into fruiting bodies (Dev WT), PE purified from *esg* cells in CTT broth (*esg*), PE purified from *esg* cells in CTT broth supplemented with isovalerate (IVA *esg*), PE purified from *esg* cells in CTT broth supplemented with isobutyrate (IBA *esg*), and PE purified from *esg* cells in CTT broth supplemented with 2-methylbutyrate (MBA *esg*).

surfaces along linear paths and reverse their direction of movement once every 6.8 min (24). Suppression of direction reversals in response to a chemoattractant is analogous to *E. coli* excitation (6, 25). In our excitation assay the PE was applied to an agar surface, the delivery solvent was evaporated, and the period between cell reversals was examined by time-lapse video microscopy.

The PE preparations varied widely in their ability to suppress direction reversals suggesting that the fatty acid composition determines activity (Fig. 3). PE purified from the isovalerate enrichment elicited a modest decline in activity relative to wild-type vegetative and developmental PE suggesting that fatty acid 15:0 iso, which accounts for about 75% of total PE fatty acid, is not likely to be an attractant. In contrast, the 2-methylbutyrate enrichment increased the *M. xanthus* reversal period dramatically in comparison with wild-type PE. PE purified from *esg* cells grown in the absence of branched-chain fatty acid primers contained the highest activity. These results suggest the presence of a strong attractant in the most active samples (Fig. 3).

Of the fatty acids present in all the samples, only the concentration of 16:1ω5c increased with the excitation activity (Figs. 2 and 3). To examine the possibility that PE containing 16:1ω5c is an attractant, the lipid was synthesized. Although naturally occurring PE rarely contains the same fatty acid at both positions, for the sake of simplicity, 1,2-*O*-bis[11-(*Z*)-hexadecenoyl]-*sn*-glycero-3-phosphoethanolamine (PE-16:1ω5c/16:1ω5c) was synthesized. The activity of the lipid was examined with the excitation assay and compared with that of dilauroyl PE (PE-12:0/12:0), which we have used for several years as a model attractant (Fig. 4). Under starvation conditions, peak activity of PE-16:1ω5c/16:1ω5c occurred at 2 ng, whereas that of PE-12:0/12:0 was 2 μg. The excitation curve for PE-16:1ω5c/16:1ω5c exhibited a sharp optimum with little response at concentrations exceeding 5 ng. The sharpness of the peak led us to repeat the assay with a second preparation of synthetic lipid. Again the peak was found at 2 ng (Fig. 4).

At least two sensory pathways are involved in PE perception. The response to PE-12:0/12:0 occurs only when cells are starved, a requirement for fruiting body morphogenesis (25). On the other hand, the response to PE-18:1ω9c/18:1ω9c occurs under both vegetative and developmental conditions. An excitation assay dose-response curve was performed on nutrient-rich CTT agar to determine whether response to PE-16:1ω5c/16:1ω5c is vegetative or developmental. The presence of nutrients inhibited the cellular response to PE-16:1ω5c/16:1ω5c (Fig. 4). These results argue that cells respond to the lipid only under environmental conditions that favor development.

Starvation induces the production of an extracellular matrix that is secreted in long thin fibrils that emanate from many points on the cell surface (26). The fibrils are essential for fruiting body development and chemotactic excitation to PE-12:0/12:0 but not PE-18:1ω9c/18:1ω9c (27). To determine whether fibrils are essential for excitation to PE-16:1ω5c/16:1ω5c we examined excitation of a *dsp* mutant, which lacks surface fibrils (26). The

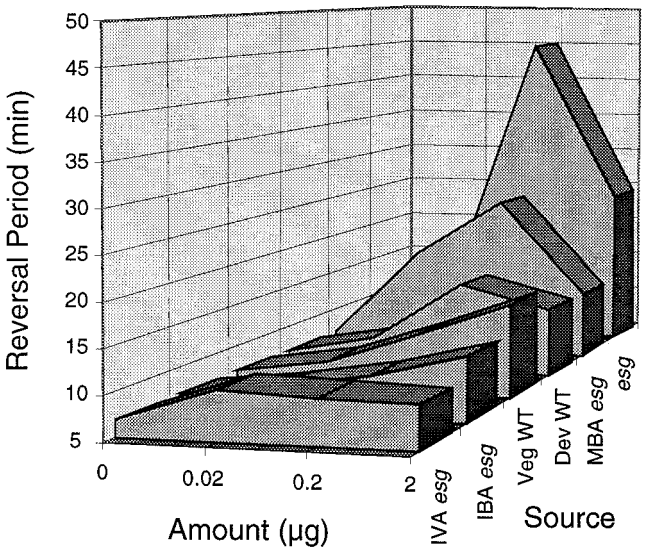

Fig. 3. Excitation magnitude varies with the PE source. The interval between reversals for isolated wild-type *M. xanthus* cells was measured in response to increasing amounts of PE purified from various *M. xanthus* PE enrichments: PE purified from vegetative wild-type cells grown in CTT broth (Veg WT), PE purified from wild-type cells in the process of directed movement into fruiting bodies (Dev WT), PE purified from *esg* cells in CTT broth (*esg*), PE purified from *esg* cells in CTT broth supplemented with isovalerate (IVA *esg*), PE purified from *esg* cells in CTT broth supplemented with isobutyrate (IBA *esg*), and PE purified from *esg* cells in CTT broth supplemented with 2-methylbutyrate (MBA *esg*). For convenience, the PE enrichments are given in the same order front-to-back as those in Fig. 2.

dsp cells showed no response to PE-16:1ω5c/16:1ω5c (data not shown). The requirement for both starvation and fibrils indicates that PE-16:1ω5c/16:1ω5c resembles the PE-12:0/12:0 class of attractants. Because fibrils are necessary for development, PE-16:1ω5c/16:1ω5c apparently has a developmental function.

Discussion

In previous work we identified two attractants for *M. xanthus*, PE-12:0/12:0 and PE-18:1ω9c/18:1ω9c (6). However, neither is present in sufficient concentrations to account for the activity of the PE preparations from wild-type cells. To help identify the native attractant species we used metabolic engineering to enrich for particular fatty acid families. This approach led to the identification and chemical synthesis of one species, PE-16:1ω5c/16:1ω5c, that has 1,000-fold greater activity than PE-12:0/12:0. Three lines of evidence suggest that PE-16:1ω5c/16:1ω5c facilitates directed movement during fruiting body morphogenesis. First, the biologically active concentration range of this lipid encompasses the amount produced by a monolayer of cells (about 1 ng), the minimum cell density necessary for rapid development. These calculations are based on an estimate of 9×10^{-11} μmol lipid phosphorous per cell in vegetative cells (28) and the fact that 16:1ω5c accounts for about 10% of the total fatty acid in vegetative cells. Therefore the lipid is produced at concentrations sufficient to serve as a cell–cell signal. Second, the proportion of PE containing 16:1ω5c increases about 30% during development. Third, cells respond to PE-16:1ω5c/16:1ω5c only under conditions that promote development, namely nutrient limitation. Starvation is necessary to produce fibrils which, in turn, are necessary for PE-16:1ω5c/16:1ω5c detection. Together these arguments suggest a role for PE containing 16:1ω5c in directed movement during fruiting body development.

Many issues remain to be resolved. Foremost is the precise structure of the lipid. Because few organisms use the same fatty acid at the *sn*-1 and *sn*-2 positions of PE, it remains to be determined whether 16:1ω5c must be at one particular position or both positions for activity. Second is the question of what role this lipid plays in development. This question is best addressed by devising a method to limit the concentration of PE containing 16:1ω5c during development to examine the morphological and behavioral consequences.

In this article we use the *esg* mutant as a tool to make fatty-acid enrichments for the identification of a highly active PE che-

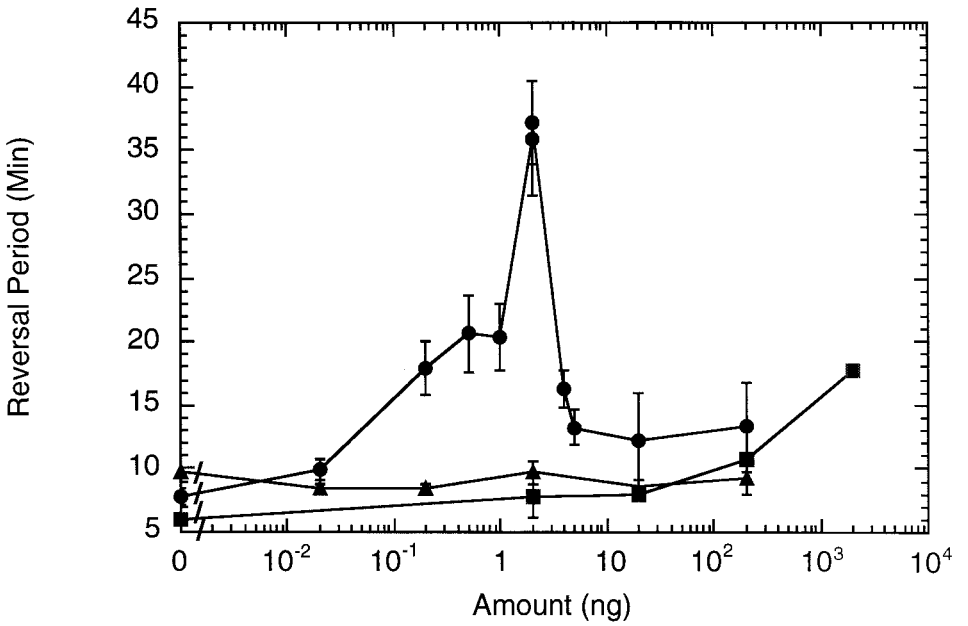

Fig. 4. Dose–response curve for chemotactic excitation. Wild-type (DK1622) cells were placed on an agar surface coated with lipid, and the interval between reversals was quantified for individual cells. Bars indicate the standard deviation of three replicates. PE-16:1ω5c/16:1ω5c under starvation conditions (TPM agar; ●); PE-16:1ω5c/16:1ω5c under growth conditions (CTT agar; ▲); and PE-12:0/12:0 under starvation conditions (TPM agar; ■). The excitation peak of PE-16:1ω5c/16:1ω5c was determined with two independent synthetic preparations as denoted by the two data points at the 2-ng concentration.

moattractant. The *esg* mutant is also interesting from a biological perspective in that it is defective in the production of a developmental signal, the E-signal, whose structure is unknown (8). The *esg* mutant arrests development before fruiting body morphogenesis, suggesting that the E signal plays a role in aggregation. Although these results might suggest that *esg* fails to produce a signal containing a branched-chain fatty acid, it came as a surprise that the unsupplemented *esg* mutant contained the greatest amount of chemoattractant activity. We wonder whether the *esg* defect may be caused by overproduction of a signaling molecule, rather than a branched-chain fatty acid deficiency, because PE-16:1ω5c/16:1ω5c is not active at high concentrations.

Traditionally, the role of unsaturated and branched-chain fatty acids has been thought to be the maintenance and regulation of membrane fluidity at low temperatures, and biosynthesis of either type of fatty acid is sufficient for most organisms. It is rare and seemingly redundant that *M. xanthus*, an organism with a narrow temperature tolerance (29), should produce both types of fatty acids. Our data suggest that lipid diversity in the cell membrane also carries information pertinent to directed movement during multicellular development.

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