Isolation of a Δ⁵-Fatty Acid Desaturase Gene from Mortierella alpina*

(Received for publication, April 20, 1998, and in revised form, May 21, 1998)

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Arachidonic acid (C20:4 Δ⁵,8,11,14) is a polyunsaturated fatty acid synthesized by the Δ⁵-fatty acid desaturation of di-homo-γ-linolenic acid (C20:3 Δ⁸,11,14). In mammals, it is known to be a precursor of the prostaglandins and the leukotrienes but it is also accumulated by the filamentous fungus Mortierella alpina. We have isolated a cDNA encoding the Δ⁵-fatty acid desaturase from M. alpina via a polymerase chain reaction-based strategy using primers designed to the conserved histidine box regions of microsomal desaturases, and confirmed its function by expression in the yeast Saccharomyces cerevisiae. Analysis of the lipids from the transformed yeast demonstrated the accumulation of arachidonic acid. The M. alpina Δ⁵-desaturase is the first example of a cloned Δ⁵-desaturase, and differs from other fungal desaturases previously characterized by the presence of an N-terminal domain related to cytochrome b₅.

The filamentous fungus M. alpina is unusual in that it can produce a wide range of polyunsaturated fatty acids. It differs from higher plants in its fatty acid unsaturation as it is able to produce arachidonic acid (C20:4 Δ⁵,8,11,14) and eicosapentaenoic acid (C20:5 Δ⁸,11,14,17) (1). In filamentous fungi, polyunsaturated fatty acids are present both in the phospholipids and the triacylglycerols, indicating that they have a role in membrane structure and as storage oils (2). In animals, arachidonic acid is a precursor of the short-lived regulatory molecules, the eicosanoids, which comprise the prostaglandins, the leukotrienes, and the thromboxanes (3). All mammalian cells except erythrocytes synthesize eicosanoids, and among their many functions they are involved in inflammatory response, reproductive function, and regulation of blood pressure (4).

In fungi, unsaturated fatty acids are formed in the endoplasmic reticulum by the action of integral membrane-bound fatty-acid desaturase enzymes, which sequentially insert double bonds into the acyl chain (5, 6). The Δ⁵-desaturase is responsible for the conversion of di-homo-γ-linolenic acid (C20:3 Δ⁸,11,14) to arachidonic acid. It is thought to function like the other microsomal desaturases from higher plants and yeast, catalyzing aerobic reactions requiring cytochrome b₅ as a cofactor. Electrons are transferred from NADH-dependent cytochrome b₅ reductase, via the heme-containing cytochrome b₅ molecule to the fatty acid desaturase (7, 8).

Biochemical characterization of membrane-bound desaturases has been limited because they are difficult to purify due to their hydrophobic nature. Hence, molecular genetic approaches, particularly the use of Arabidopsis mutants, have provided much information on desaturation reactions (9, 10). Analysis of the predicted protein sequences for the higher plant desaturases together with those from cyanobacteria, yeast, and mammals revealed the presence of eight highly conserved histidine residues (11). Mutagenesis studies on the microsomal Δ⁵-desaturase from rat and the Δ¹₂-desaturase from Synechocystis (11, 12) showed that all of the conserved histidines were catalytically essential. Variation is present within the amino acid sequences between the conserved histidine residues, but sequence alignments have permitted the design of degenerate oligonucleotides capable of amplifying novel desaturase gene fragments from various sources (13–15). Although the approach has yielded gene probes for a variety of microsomal desaturase activities including Δ⁵, Δ⁷, and Δ¹₂, no information is available yet for a Δ⁵-desaturase. Here, we describe the successful application of this approach to the isolation of a Δ⁵-fatty acid desaturase from the fungus Mortierella alpina.

EXPERIMENTAL PROCEDURES

Cultures—M. alpina CBS 210.32 was grown in liquid culture in potato dextrose medium (Life Technologies, Inc.) at 28 °C with shaking at 200 rpm. After 48 h, mycelium was harvested and used in fatty acid analysis and various DNA and RNA extractions.

Mucor circinelloides was grown in liquid culture in potato dextrose medium (Life Technologies, Inc.) at 25 °C with shaking at 200 rpm. Mycelium was harvested after 48 h, and DNA was extracted.

RNA Isolation and Manipulation—Total RNA was isolated from 400 mg wet weight of mycelium using the RNeasy plant mini kit (Qiagen) and 5 μg was reverse transcribed with the Ready-To-Go® T-primed first strand kit (Amersham Pharmacia Biotech) according to the manufacturers’ instructions. The cDNA was used as a template for PCR amplification with degenerate primers.

Genomic DNA Isolation—Approximately 4 g wet weight of mycelium was ground to a fine powder under liquid nitrogen using a precooled mortar and pestle. The ground tissue was added to 10 ml of extraction buffer (10 mM Tris-HCl, pH 8, 10 mM EDTA, 0.5% SDS), thawed, and mixed gently by inversion. Ten ml of phenol:chloroform:isoamyl alcohol (16:24:1) were added and mixed gently for 15–30 min. The organic and aqueous phases were separated by centrifugation and the aqueous layer removed to a fresh centrifuge tube. This extraction was repeated until the interface between the two phases was clear, after which an extraction with chloroform:isoamyl alcohol (24:1) was carried out. DNA was

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF054824.

‡ Supported by a Biotechnology and Biological Sciences Research Council studentship and a Cooperative Awards in Science and Engineering award from Horticulture Research International, Wellesbourne. To whom correspondence should be addressed. Tel.: 44-117-928-7574; Fax: 44-117-925-7374; E-mail: louise.michaelson@bristol.ac.uk

1 The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s); GC, gas chromatography; FAME, fatty acid methyl ester; MS, mass spectroscopy.

This paper is available online at http://www.jbc.org

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concentrated by ethanol precipitation and purified by two rounds of equilibrium centrifugation in a CsCl ethidium bromide density gradient. The band containing genomic DNA was removed from the gradient and diluted with TE buffer.

PCR-based Cloning—Highly degenerate primers were synthesized using inosine for 4-base redundancy and extended at the 5' ends to include EcoRI restriction sites to facilitate cloning PCR products. The forward primer was, 5'−GGGAATTCA/G/TGGGACA/TG/CA/TG/TO/T/ CGGGACA−3', and the reverse primer was 5'−GGGAATTCA/TATTGC/ TGGGACA/G/AAAGA/G/A/G/TA/G/AG/G/TA/−3', where 1 stands for inosine and the EcoRI sites are underlined. The primers were used for PCR amplification of cDNA reverse transcribed from total RNA. After initial denaturation at 94 °C for 2 min, amplification was performed in 32 cycles of 45 s at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, followed by a final extension at 72 °C for another 10 min. Amplification products were fractionated on 1% agarose gels from which selected DNA bands were purified. They were ligated directly into pGEM-T (Promega) and the plasmids used to transform into *Escherichia coli* DH5α cells. Plasmid DNA was purified for sequencing using the Qiagen QIAprep mini prep kit. Nucleotide sequences were determined using a Perkin Elmer ABI-377 DNA sequencer, and analyzed using the University of Wisconsin GCG software package (17).

Library Screening—End-adapted cDNA was synthesized from *M. alpina* poly(A) + mRNA using a cDNA synthesis kit (Amersham Pharmacia Biotech) and ligated into the *EcoRI* site of AMOSSlox (Amersham Pharmacia Biotech). The resultant DNA was packaged into phage particles to produce a library of 4×10⁸ plaque-forming units with an average insert size of 1.4 kilobases. The library was screened by standard techniques (16) using cloned PCR products as probes. DNA fragments were labeled with [α-³²P]dCTP using the Ready To Go™ DNA labeling reaction mix (Amersham Pharmacia Biotech). The full-length cDNA clone L11.1 was purified by successive rounds of plating and hybridization before it was subjected to plasmid excision in *vivo* and the insert was sequenced on both strands.

Southern Blot Analysis—Aliquots (10 μg) of genomic DNA from *M. alpina* and *M. circinelloides* were digested with restriction enzymes and fractionated by agarose gel electrophoresis. DNA was transferred to a Zeta-Probe™ nylon membrane (Bio-Rad) by alkaline capillary electrophoresis for 5–8 h using 0.4 m NaOH as transfer buffer. To improve transfer of the larger DNA fragments (greater than 4 kilobases), the gel was soaked in 0.25 m HCl for 15 min to partially depurinate the DNA and then briefly rinsed in water prior to blotting. The filters were probed with the 660-bp fragment amplified between histidine boxes 1 and 3 using the gene-specific primers 5'-CATGATGCTCTCAGCTTTTCA-3' (forward) and 5'-GGGAATTCAACACTGCTGGTATT-3' (reverse). Over-night hybridization was performed at 55 °C in 0.25 m sodium phosphate pH 7.2, 7% SDS, after which the filters were washed in 2× SSC, 0.1% SDS at the hybridization temperature and x-ray film exposed to it at −80 °C using an intensifying screen (18).

**Functional Analysis: Yeast Transformation**—PCR with the primers MYfor, 5'-GGCGGCTACCATGGTACGGACAAAGA-3' (annealing to the initiating methionine indicated by the boldface type), and MYrev, 5'-GGCGGACCTTCACCTCCTTGAGGGACG-3' (annealing to the complement of the stop codon, indicated in boldface type) was used to amplify the pL11 coding region and to flank it with *KpnI* and *SacI* restriction sites. The amplified PCR product was ligated into the vector pYES2 (Invitrogen) to generate the plasmid pYES2/L11 and cloned in *E. coli*. The fidelity of the cloned PCR product was checked by in vitro transcription and translation using the TNT™ system (Promega). Translation products labeled with [³⁵S]methionine were generated, separated by SDS-polyacrylamide gel electrophoresis, and visualized by autoradiography. The plasmid was transformed into *Saccharomyces cerevisiae* DBY746 by the lithium acetate method (19), and expression of the transgene was induced by the addition of galactose to 1% (w/v). The yeast culture medium was supplemented with 0.5 m di-homo-γ-linolenic acid or 0.5 m linoleic acid in the presence of 1% tergitol as a coculture medium, and 0.2 m di-homo-γ-linolenic acid or 0.5 m linoleic acid in the presence of 1% tergal as described by Napier et al. (20).

**Fatty Acid Analysis**—Total fatty acids extracted from yeast cultures were analyzed by gas chromatography (GC) of methyl esters. Lipids were transmethylated with 1 m HCl in methanol at 80 °C for 1 h, then fatty acid methyl esters (FAMEs) were extracted in hexane. GC anal-

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**Fig. 1. Comparison of deduced amino acid sequences.** The translation of the coding sequence of L11.1 (labeled *M. A5*) is aligned with the cyanobacterial Δ⁵-desaturase (*SYN6D*) and the borage Δ⁵-desaturase (*BORD6*). Identical or conserved residues are shaded, and the conserved histidines are underlined. The sequence of L11.1 has been deposited in the GenBank data base (accession no. AF054824).
Analysis of FAMEs was conducted using a Hewlett Packard 5880A Series gas chromatograph equipped with a 25 m × 0.32-mm RSL-500 BP bonded capillary column and a flame ionization detector. Fatty acids were identified by comparison with retention times of FAME standards (Sigma). Relative percentages of the fatty acids were estimated from peak areas. Arachidonic acid was identified by GC-MS using a Krats MS80RFA operating at an ionization voltage of 70 eV, with a scan range of 500–40 daltons.

RESULTS

Cloning of a M. alpina Membrane-bound Desaturase—The strategy employed to obtain probes for M. alpina desaturase genes involved reverse transcriptase PCR with the degenerate primer sequences used to amplify a plant Δ⁵-desaturase gene (15). Inosine was used at all positions of 4-base degeneracy to maximize the relative concentrations of individual primers within the degenerate mixtures. The forward primer mixture was designed to encode histidine box 1, and the reverse primer mixture encoded the complement of histidine box 3. Total RNA extracted from M. alpina was reverse-transcribed, and the cDNA product was used as a template in a PCR that yielded products of various sizes. Several PCR products were generated, and those of the expected length (600–700 bp) were isolated from a gel for cloning and sequencing. The DNA sequence of one of the clones (L11) predicted an open reading frame of 224 amino acids. It displayed some similarity to other membrane-bound desaturases, including the presence of the second characteristic histidine box (11). Alignment of the deduced amino acid sequence of this PCR product with the corresponding region of known desaturases indicated the greatest similarity to the Δ⁶-desaturase from a cyanobacterium (21), although the actual level of identity was low and less than 25%.

Lipid analysis has shown that in cultures of M. alpina harvested after 48 h arachidonic acid comprises up to 25% of the total lipid. It was therefore considered possible that L11 could encode part of either a Δ⁵- or a Δ⁶-desaturase from M. alpina.

To isolate a complete coding region corresponding to the L11 PCR product, the insert was used to probe a M. alpina cDNA library that had been constructed in λMOSStox. A sample of the primary library containing 1.48 × 10⁸ plaque-forming units

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**Fig. 2.** Hybridization of L11.1 to genomic DNA from M. alpina and M. circinelloides. Samples of genomic DNA from M. circinelloides (lanes 1–3) and M. alpina (lanes 4–6) were digested with EcoRI (lanes 1 and 4), BamHI (lanes 2 and 5), and HindIII (lanes 3 and 6). A is the 1× TAE, 1% agarose gel stained with ethidium bromide (M = 100-bp marker, Boehringer Mannheim). B is the autoradiograph of the Southern blot of this gel probed with the fragment amplified between histidine boxes 1 and 3.

**Fig. 3.** Identification of arachidonic acid synthesized in transgenic yeast by GC. Fatty acid methyl esters of total lipids of S. cerevisiae grown under inducing conditions in the presence of di-homo-γ-linolenic acid were analyzed by GC, using flame ionization detection. A, FAMEs extracted from yeast transformed with control (empty) vector pYES2; B, FAMEs extracted from yeast transformed with pYES2/L11. The common peaks were identified as C16:0 (peak 1), C16:1 (peak 2), C18:0 (peak 3), C18:1 (peak 4), and C20:3 (peak 5 supplied exogenously). The additional peak (in B), which corresponds to the retention time of arachidonic acid, is indicated by the arrow.

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was plated and screened for hybridization to L11. Fifteen individual phage plaques gave positive signals; they were cored out of the agar plates and eluted into SM buffer. The resultant phage suspensions were checked for the presence of desaturase-encoding cDNAs by PCR with the degenerate histidine box primers. Clone L11.1 was determined as having the longest insert in a PCR screen involving the histidine box 3 primer and vector arm (T7 and SP6) primers, and was purified by additional rounds of plating and hybridization screening. The plasmid pL11.1 was released from a clone L11.1 by in vivo excision, and the cDNA insert was sequenced.

The 1410-bp cDNA insert of clone pL11.1 includes a 1341-bp reading frame encoding 446 amino acids and a TAG stop codon. The coding region is flanked by 3 bp from the 5′-untranslated region of the mRNA and the full 3′-untranslated region, followed by part of the poly(A) tail. Fig. 1 shows the deduced amino acid sequence of the predicted coding region. It shows 22% identity with the Δ⁵-desaturase from the cyanobacterium *Synechocystis* (21) and 20% identity with the *Borago officinalis* (borage) Δ⁶-desaturase (15). The three conserved histidine boxes that are characteristic of other membrane desaturases are present. The third histidine box contains a H → Q substitution, which is also evident in the *B. officinalis* and the *Caenorhabditis elegans* Δ⁶-desaturase sequences (15, 20).

**L11.1 Encodes a Protein with a Cytochrome b₅-like Heme-binding Domain at the N Terminus**—A heme-containing electron donor is required for fatty acid desaturation and cytochrome *b₅* fulfils this function for the membrane-bound desaturases (5, 8, 22). Fig. 1 shows the presence of a heme binding region characterized by the H-P-G-G motif toward the N terminus and hence the cytochrome *b₅* and desaturase may exist as a fusion protein. Previously identified fungal desaturases also contain cytochrome *b₅* fusions, but with the heme-binding region located toward the C terminus (5, 14).

**Southern Blot Analysis—** *M. circinelloides* is a filamentous zygomycete, which accumulates a triacylglycerol oil rich in γ-linolenic acid but lacking arachidonic acid. In all other respects, it is very similar to *M. alpina* (6). It thus has genes encoding Δ⁵-, Δ¹₂-, and Δ⁶-desaturase activities and is likely to lack a Δ⁷-desaturase gene. Genomic DNA was isolated from *M. alpina* and *M. circinelloides*, digested with three restriction enzymes and fractionated by agarose gel electrophoresis. A Southern blot of the gel was probed with the 660-bp fragment amplified by PCR between histidine boxes 1 and 3 of clone L11.1. Fig. 2 shows single hybridizing bands in each of the digests of *M. alpina* DNA, but no hybridization was observed to *M. circinelloides* DNA. In a similar experiment, a putative Δ⁵-desaturase cDNA was found to hybridize strongly to both the *M. circinelloides* genome as well as the *M. alpina* genome. These results indicate that the desaturase isolated from *M. alpina* is encoded by a single copy gene that is not present in *M. circinelloides*. This suggests that the gene is likely to encode a Δ⁵-desaturase rather than a Δ⁶-desaturase.

**Functional Analysis of L11.1 in Yeast—** The complete coding region (446 amino acids) of L11.1 was amplified by PCR and inserted into the yeast expression vector pYES2 downstream of the GAL1 promoter. This construct was transformed into *E. coli*. The fidelity of the PCR-generated insert in plasmid pYES2/L11 was confirmed *in vitro* by coupled transcription/translation under the control of the T7 RNA polymerase promoter in the vector. The translation products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. A product of *M. circinelloides* was produced from the plasmid pYES2/L11, whereas the empty vector control (pYES2) failed to yield any protein products (data not shown).

For functional analysis of the L11.1 coding region, the recombinant plasmid was transferred to yeast. Cells were cultured overnight in a medium containing raffinose as a carbon source, and supplemented by the addition of either linoleic acid (18:2 Δ⁹,12) or di-homo-γ-linolenic acid (C20:3 Δ⁹,12,15). These fatty acids are not present in *S. cerevisiae* but serve as the specific substrates for either the Δ⁶- or Δ⁵-desaturase, respectively. Expression of the L11.1 coding region from the GAL1 promoter of the vector was induced by the addition of galactose to 1%. Growth of the cultures was continued for 16 h, when aliquots were removed for the analysis of fatty acids by GC. Fig. 3 shows the result of GC analysis of the fatty acid methyl esters of transformed yeast strains. An additional peak is apparent in the trace obtained from induced pYES2/L11 grown in the presence of di-homo-γ-linolenic acid compared with an empty vector control. This peak was also absent from uninduced cultures grown on di-homo-γ-linolenic acid and it is also important to note that pYES2/L11 grown in the presence of linoleic acid failed to accumulate any novel peaks indicating that this fatty
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acids is not a substrate for the enzyme encoded by the *M. alpina* cDNA. The retention time of the additional peak is identical to that of the authentic methyl-arachidonic acid standard. The fatty acid produced from di-homo-γ-linolenic acid was further characterized by GC-MS and identified as arachidonic acid (Fig. 4). The results show, therefore, that yeast cells transformed with the plasmid pYES2/L11 had acquired functional Δ5-desaturase activity and were now capable of synthesizing arachidonic acid from the substrate di-homo-γ-linolenic acid. The Δ5-desaturase in the transformed yeast appeared to be a very efficient catalyst, with 21.3% of the substrate converted to arachidonic acid under the conditions of the experiment. **Identification of a Similar Sequence in C. elegans**—Data base searching with the *M. alpina* cDNA identified a high scoring match to one of the sequences on *C. elegans* cosmid T13F2 (GenBank accession number Z81122). The *C. elegans* ORF of T13F2.1 predicts a protein of 454 amino acids. It contains the characteristics of other membrane-bound desaturases including the three histidine box domains. It also contains an N-terminal cytochrome *b*5 region, and the third histidine box contains the H → Q variant. As the nematode performs Δ5 desaturation (23), it is possible that this homologue encodes a Δ5-desaturase gene. **DISCUSSION** A cDNA isolated from *M. alpina* has been characterized and found to encode a protein with three histidine box motifs indicative of a microsomal fatty acid desaturase (11). The deduced protein sequence also contained the diagnostic features of a heme-binding cytochrome *b*5 domain located toward the N terminus. Evidence that the L11.1 clone encodes a Δ5-desaturase was obtained by Southern blot analysis, which indicated the absence of homologous sequences in the genome of *M. circinelloides*, a related fungus that produces γ-linolenic acid but not arachidonic acid. Functional analysis of the L11.1 clone in transformed yeast confirmed that it encoded a Δ5-desaturase gene, since cells growing in a medium supplemented with di-homo-γ-linolenic acid produced arachidonic acid in significant quantities. The fungal Δ5-desaturase appears to differ from the other fungal and plant desaturases that have been characterized (5, 14). The most closely related sequence identified was that of the cytochrome *b*5 desaturase involved in GLA formation (21), with which the fungal Δ5-desaturase showed only some 22% amino acid identity. Data base searches have also enabled us to identify a putative animal Δ5-desaturase in the nematode *C. elegans*. It is interesting that the Δ5-desaturase may exist as a cytochrome *b*5 fusion protein. Such fusions have been identified in a hypothetical desaturase-like sunflower protein (24) and the Δ5-desaturase in *B. officinalis*, both of which contain N-terminal cytochrome *b*5 domains (15). Δ5-Desaturases characterized from other fungi have also been found to contain cytochrome *b*5 domains, but in these examples the diagnostic heme binding site was located toward the C terminus (5, 14). The Δ5-desaturase from *M. alpina*, therefore, appears to be the first fungal fatty acid desaturase described with an N-terminal cytochrome *b*5 domain and in this respect is similar to the “front end” Δ5-desaturase of *B. officinalis* (15, 25) and the Δ5-desaturase of *C. elegans* (20). In contrast to other microsomal desaturases, the microsomal desaturation with N-terminal cytochrome *b*5 domains use a polyunsaturated fatty acid as a substrate and insert a double bond between the methyl end of the fatty acid and existing double bonds, but it is unclear how significant a role the fused cytochrome *b*5 region plays in this reaction. The OLE1 gene isolated from *S. cerevisiae*, which encodes a Δ5-desaturase, contains a C-terminal cytochrome *b*5 domain. OLE1 rescued double mutants deficient in both OLE1 and the separate microsomal cytochrome *b*5. However, when the cytochrome *b*5 region was deleted from this gene, the yeast cells remained fatty acid auxotrophs, even in the presence of the endogenous yeast cytochrome *b*5 (5). This suggests that the cytochrome *b*5 domain plays an essential role in the desaturation reaction. Whether the cytochrome *b*5 desaturation fusion proteins are more efficient awaits further assessment. Recently, another N-terminal cytochrome *b*5 fusion protein, Fah1p, has been identified in yeast; it is required for the α-hydroxylation of sphingolipid-associated very-long-chain fatty acids (26). Interestingly, it contains two of the histidine box motifs characteristic of membrane-bound desaturases. The *Arabidopsis* homologue of Fah1p has no cytochrome *b*5 domain, but it is able to functionally complement a *fah1* deletion mutant. This indicates that, in the case of Fah1p, the N-terminal cytochrome *b*5 may not be essential for function (26). Desaturases isolated from fungal sources have much potential for further exploitation, particularly in the genetic manipulation of oil crops to produce novel and diverse polyunsaturated fatty acid products for the pharmaceutical and nutraceutical industries (27). The Δ5-desaturase is an essential enzyme in the production of eicosanoids, and the identification of a *M. alpina* cDNA encoding this activity is the first example of its type.

**Acknowledgments**—We thank Prof. S. Shimizu (Kyoto University, Kyoto, Japan) and Prof. R. Herbert (Dundee University, Dundee, UK) for providing *M. alpina* and *M. circinelloides*, respectively. We thank Mervyn Lewis for the GC-MS analysis. Institute of Arable Crops Research-Long Ashton receives grant-aided support from the Biotechnology and Biological Sciences Research Council. A. K. S. thanks the Royal Society for equipment grants and the Wolfson foundation for laboratory refurbishment. L. M. thanks Dr. Steve Screen for helpful discussions.

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