Two Δ9-Stearic Acid Desaturases Are Required for Aspergillus nidulans Growth and Development

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Two Δ9-stearic acid desaturases are required for *Aspergillus nidulans* growth and development

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Abstract

Unsaturated fatty acids are important constituents of all cell membranes and are required for normal growth. In the filamentous fungus *Aspergillus nidulans*, unsaturated fatty acids and their derivatives also influence asexual (conidial) and sexual (ascospore) sporulation processes. To investigate the relationship between fatty acid metabolism and fungal development, we disrupted the *A. nidulans sdeA* and *sdeB* genes, both encoding Δ9-stearic acid desaturases responsible for the conversion of palmitic acid (16:0) and stearic acid (18:0) to palmitoleic acid (16:1) and oleic acid (18:1). The effects of *sdeA* deletion on development were profound, such that growth, conidial and ascospore production were all reduced at 22 and 37°C. Total fatty acid content was increased over 3-fold in the Δ*sdeA* strain, reflected in up-regulation of the expression of the *fasA* gene encoding the α chain of the fatty acid synthase, compared to wild type. Stearic acid accumulated approximately 3-fold compared to wild type in the Δ*sdeA* strain, while unsaturated fatty acid production was decreased. In contrast, disruption of *sdeB* reduced fungal growth and conidiation at 22°C, but did not affect these processes at 37°C compared to wild type. Interestingly, ascospore production was increased at 37°C for Δ*sdeB* compared to wild type. Total fatty acid content was not increased in this strain, although stearic acid accumulated 2-fold compared to wild type, and unsaturated fatty acid production was decreased. Combining the Δ*sdeA* and Δ*sdeB* alleles created a synthetic lethal strain requiring the addition of oleic acid to the medium for a modicum of growth. Taken together, our results suggest a role for Δ*sdeA* in growth and development at all temperatures, while Δ*sdeB* is involved in growth and development at lower temperatures.

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Keywords: *Aspergillus nidulans*; Fatty acid biosynthesis; Δ9-Stearic acid desaturase; Fungal development; Stearic acid

1. Introduction

Oleic and linoleic acid are the most common unsaturated fatty acid components of *Aspergillus nidulans* (Calvo et al., 2001; Evans et al., 1986). The previously described Δ12-oleic acid desaturase (OdeA) of *A. nidulans* converts monounsaturated oleic acid (18:1) to polyunsaturated linoleic acid (18:2) (Calvo et al., 2001), while the activities of Δ9-stearic acid desaturase nominally produce oleic acid (18:1) from unsaturated stearic acid (18:0). Δ9-Stearic acid desaturases additionally function to convert palmitic acid (16:0) to palmitoleic acid (16:1). Studies of many fungal genera clearly show that fatty acid composition is important for normal growth and survival (Chattopadhyay et al., 1985; Scott, 1977; Stukey et al., 1989), with unsaturated fatty acids being critical for maintaining cell membrane fluidity, and hence cell viability, at low temperatures. In the genus *Aspergillus*, biochemical data suggests an additional requirement of unsaturated fatty acids and their derivatives for the production of multicellular developmental structures such as conidiophores, cleistothecia, and sclerotia (Calvo et al., 1999).

To elucidate the role of unsaturated fatty acids and their derivatives on fungal development, knowledge of...
the genetics and regulation of fatty acid metabolism is required. The importance of polyunsaturated fatty acids for normal Aspergillus fungal development has already been demonstrated by the A. nidulans odeA mutant, where loss of OdeA function leads to accumulation of large amounts of oleic acid and its derivatives, abolition of polyunsaturated fatty acid production, and a concomitant aberration in both sexual and asexual development (Calvo et al., 2001).

We undertook to create additional fatty acid mutants of A. nidulans. Two Δ9-stearic acid desaturase encoding genes, sdeA and sdeB, were disrupted. Both mutations decreased unsaturated fatty acid production and increased saturated fatty acid accumulation. AsdeA, the most severe mutation, led to up-regulation of a fatty acid synthase gene, fasA, and produced 3.5-fold more fatty acids than wild type. sdeB gene expression was also up-regulated in this AsdeA strain. From the results presented below, we see that the sdeA and sdeB genes play differing roles in fungal growth and development, with sdeA important for fungal viability at all temperatures studied, while sdeB is mainly required for optimal growth and development at lower temperatures.

2. Materials and methods

2.1. Fungal strains and growth conditions

Aspergillus nidulans strains used in this study were listed in Table 1. Cultures were maintained on glucose minimal medium (GMM) (Calvo et al., 2001) at 37˚C. For asexual conidial production, cultures were grown on GMM at 22 or 37˚C in continuous white light using an incubator equipped with a General Electric 15-W broad spectrum fluorescent light bulb (F15T12CW) positioned 50 cm from the agar surface with a light intensity of 66 mE/m2/s. For sexual ascospore production, cultures were grown in the dark at 22 or 37˚C on YGT medium (Calvo et al., 2001). These temperatures were chosen because 37˚C is the optimum growth condition for A. nidulans, while 22˚C represents a significantly lower than optimal temperature suitable for studying the effects of the desaturase mutations on cold tolerance. For RNA studies 10^6 spores of each strain were inoculated into 50 ml flasks of liquid GMM and incubated at 37˚C with vigorous shaking at 220 rpm. Mycelium was harvested at the given time points by vacuum filtration through sterile filter paper.

2.2. Molecular biology techniques

For Southern analysis, 10 µg of restriction digested genomic DNA was separated by gel electrophoresis in a 1% agarose gel and transferred by capillary action to Hybond membrane (Amersham). Total RNA was extracted from mycelia using Trizol reagent (Life Technologies). Ten micrograms of RNA was separated on a 1.2% agarose–1.5% formaldehyde gel. RNA was transferred to Hybond membrane (Amersham) by capillary action. Northern and Southern blot analysis of the sdeA gene was performed using a radiolabelled 1.8 kb EcoRI–XhoI fragment from the sdeA cDNA clone pj502a1. Northern and Southern blot analysis of the sdeB gene was performed using a radiolabelled 1 kb DNA fragment amplified from genomic DNA by the primers 5’sdeBsaIF (see below) and sdeBR1 (GGAGAGGAG CTGGCAACAG). fasA expression was analyzed using a 0.5 kb PCR amplified fragment of fasA (GenBank Accession No. U75347) generated from genomic DNA with the primers fasAF (GGATTCCACACCGG) and fasAR (GGGAGCACG GAGAG). fasA encodes the β-subunit of fatty acid synthase and is exclusively involved with the primary metabolism of fatty acids (Brown et al., 1996).

DNA fragments to use as probes were radiolabeled with 32P using the random primer method (Sambrook et al., 1989). Following prehybridization and addition of the probe, the membranes were hybridized overnight at 60˚C, and washed with increasing stringency up to 0.1× SSC, 0.1% SDS at 60˚C.

The sdeA and sdeB genes were sequenced using previously described techniques and software (Calvo et al., 2001).

PCR conditions were 96˚C for 3 min, followed by 40 cycles of 96˚C denaturation (30 s), 55˚C annealing (1 min) and 74˚C extension (2 min), unless otherwise stated.

2.3. Identification of the Δ9 stearic acid desaturase encoding genes, sdeA and sdeB, from A. nidulans

The sdeA EST clone pj502a1 (Calvo et al., 2001) was used to probe the pWE15 and pLORIST A. nidulans genomic cosmid libraries (Fungal Genetics Stock Cen-

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Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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</thead>
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<tr>
<td>FGSC773</td>
<td>wA3; pyrG89; pyroA44; veA1</td>
<td>FGSC*</td>
</tr>
<tr>
<td>TRAW50.410</td>
<td>wA3; pyrG89; pyroA44; AsdeA::pyrG; veA1</td>
<td>This study</td>
</tr>
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<td>RRAW6.1</td>
<td>biA1; pyrG89; AsdeA::pyrG</td>
<td>This study</td>
</tr>
<tr>
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<td>pyrG89; AsdeA::pyrG</td>
<td>This study</td>
</tr>
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<td>Prototroph</td>
<td>This study</td>
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<td>argB2; pyrG89; metG81</td>
<td>D. Tsitsiagisianis</td>
</tr>
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<tr>
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</tr>
<tr>
<td>RRAW5.2</td>
<td>AsdeA::argB; argB2</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Fungal Genetics Stock Center (Kansas City, KS).
ter, Kansas City, KS). Two cosmids, pWBH6 from pWE15 and pLAO12 from pLORIST, were isolated and sequenced using primers designed from the cDNA. The sequence of the sdeA gene has been deposited with GenBank under the Accession No. AF510861. This genomic sequence was then blasted against A. nidulans sequences deposited at the Whitehead Institute website (http://www-genome.wi.mit.edu/annotation/fungi/aspergillus/index.html) to identify the coding region of sdeB, Accession No. AY504633.

2.4. Construction of the sdeA and sdeB gene disruption vectors

The sdeA disruption vector was designed as follows. First, a 2.2 kb EcoRI-BamHI fragment from the pPYRG plasmid, containing the A. nidulans pyrG gene (Oakley et al., 1987), was ligated into pK19 previously digested with EcoRI and BamHI to give pRAW12. Next, the primer pairs 5'EIF (CCAGCGACGAGAATTCAGGAG TAATAAC) and 5'EIR (TGCTATTTCCGCCGAAAT TCTGGAACGTGA) were used to PCR amplify a 1.1 kb region 5' to the sdeA gene using Thermolase Taq polymerase (Invitrogen). 5'EIF and 5'EIR were designed to introduce EcoRI sites at either end of the PCR product, enabling the fragment to be subcloned into pRAW12 at the EcoRI site to give pRAW13. Finally, the primer pairs 3'BIF (GTCTATGGGATCCTGCTACTCCGCC AC) and 3'BIR (CTACACCAGCACAGGATCCACAA ACTCCGCCG) were used to PCR amplify a 1.1 kb region located 700 bp downstream from the translation start site. 3'BIF and 3'BIR were designed to introduce BamHI sites at the ends of the PCR fragment, enabling the fragment to be subcloned into pRAW13 at the BamHI site to give pRAW14. pRAW14 represents a disruption vector containing 1.1 kb each of 5' upstream and 3' coding regions of the sdeA gene, with 700 bp of coding region, including the translation start site, replaced by the pyrG cassette. pRAW14 was used to transform the recipient strain FGSC773 using standard techniques (Calvo et al., 1999), and the sdeA gene was amplified using the primers 5'sdeBsacIF (GGCAGAGCTCGGCGGTTCAGAGA TCAATTTAAGATAAGC) and 3'sdeAR (CCCGCGGCATGCGAC UATTAAAGCTATGCGG) to give a 1 kb fragment with SacI restriction sites introduced at the ends. The plasmid pUG11-41, containing the metG gene including its promoter and terminator regions (Sienko and Paszewski, 1999), and the sdeB PCR fragment were digested with SacI and the sdeB 5' flank was ligated into the pUG11-41 to give pRAW17. Next, the 3' end of the sdeB gene was amplified by 3'sdeBsphl (GGACTGCA TGCGGGGCGATTTGCGGGGGG) and 3'sdeB HindIII (CGAACCAACCTTTGCAGGAAGATGCT CTTGCCGGGCC) to give a 1 kb fragment. pRAW17 and this fragment were digested with SphI and HindIII and the 3' fragment ligated into pRAW17 to give pRAW18. Therefore, pRAW18 represents the coding region of sdeB split in the center by the metG coding region. This vector was used to transform the metG requiring strain RDIT1.1, and putative disruptants were selected initially by metG prototrophy.

2.5. Confirmation of sdeA and sdeB gene disruption

Prototrophic transformants were screened for homologous gene replacement of sdeA with pyrG using PCR. PCR conditions were 96 °C for 3 min, followed by 40 cycles of 96 °C denaturation (30 s), 52 °C annealing (1 min), and 74 °C extension (5 min) using PCR primers 5'EIF and 3'BIR. Wild type sdeA yielded a 3.1 kb PCR fragment, while AsdeA yielded a 4.4 kb fragment. Single copy integration was confirmed by Southern hybridization.

For sdeB, disruption of this gene by metG was confirmed using a PCR approach followed by Southern analysis. PCR conditions were 96 °C for 3 min, followed by 40 cycles of 96 °C denaturation (30 s), 62 °C annealing (1 min), and 74 °C extension (4 min) using the primers 5'sdeBsacIF and 3'sdeBR1. Wild type sdeB gene yielded a 1.5 kb PCR fragment, the distance between 5'sdeBsacIF and 3'sdeBR1. AsdeB yielded a 4 kb fragment representing the distance between 5'sdeBsacIF and 3'sdeBR1 in addition to the 2.5 kb metG gene cassette located between these two primers. Single copy integration was confirmed by Southern hybridization.

To ensure the severe phenotype of the AsdeA strain was due solely to the single replacement of this gene by pyrG, the full-length sdeA gene was re-introduced into a AsdeA strain using the vector pRAW19. pRAW19 was constructed as follows: a 2.7 kb fragment, containing the sdeA coding region and upstream elements, was amplified from genomic DNA using pfu proof reading Taq and the primers 5'UapCsphIF (CCCAGCGCATGCCA TCAATTTAAGATAAGC) and 3'sdeAR (CCAGCAT GCCGAAGTGGATGCATAT) to introduce SphI sites at either end. The resulting PCR fragment and pUG11-41 were digested with SphI, and the sdeA containing fragment was ligated into this vector to give pRAW19. pRAW19 was then transformed into AsdeA strain RRAW6.1 and restored sdeA function was selected for by remediated growth and sporulation at lower temperatures (22 °C) compared to wild type, followed by Southern confirmation. Five transformants were single spore purified and analyzed.
The double mutant AsdeA; AsdeB was constructed by sexual cross of RRAW6.2 and TRAW128 (Table 1) using standard techniques (Pontecorvo et al., 1953). As mutations in both Δ9-stearic acid desaturases were lethal, AsdeA; AsdeB double mutant strains were selected on GMM supplemented with 1% (v/v) Tween 80, and confirmed by PCR.

2.6. Physiological studies

Conidiation studies were performed on plates containing 25 ml GMM. For each plate, a 5 ml top layer of cool but molten agar that contained 10⁶ spores of the appropriate strain was added. For each strain, there was a minimum of four replicate plates. A core of 15 mm was removed from the plates at the appropriate time interval, and homogenized in 2 ml of 0.01% Tween 80 water to release the spores. Colony radial growth was recorded as colony diameter and measured at the appropriate strain. For each strain, there was a minimum of four replicate plates. Strains were grown in constant light conditions, mycelia was lyophilized, and the remediation of the Δ9-stearic acid desaturase was assessed by gas chromatography (GC) analysis of the Δ9-stearic acid desaturase from Ajellomyces capsulata (83%) (GenBank sequence Accession No. S52745). SdeA contains the conserved histidine clusters, composing the Fe-binding active centers of the enzyme, present in all Δ9-desaturases (Los and Murata, 1998).

Examination of the Whitehead A. nidulans database with the sdeA genomic sequence revealed a second putative Δ9-desaturase, sdeB, located on scaffold IV (GenBank sequence Accession No. AY504633) encoding an approximately 460 amino acid protein. It had 55% identity to sdeA at the nucleotide level and 66% identity at the amino acid level including the conserved His cluster motifs. In GenBank BLAST analysis, SdeB also had high identity (75% at the amino acid level) to the Δ9-stearic acid desaturase from Neurospora crassa (GenBank sequence Accession No. XM_324615). Examination of the Whitehead database with either sdeA, sdeB or other known Δ9-stearic acid desaturases did not produce significant matches to any other gene, indicating that sdeA and sdeB are likely the only Δ9-stearic acid desaturase encoding genes present in A. nidulans.

The two desaturase genes were disrupted by homologous recombination as described in materials and methods.

For sake of clarity, A. nidulans strains carrying the gene mutations AsdeA and AsdeB are referred by these genotypes and not by strain name (i.e., TRAW50.410) for the rest of this presentation.

2.7. Fatty acid analysis

Fatty acid methyl esters were generated from Aspergillus mycelia for gas chromatography (GC) analysis as follows. After growth in GMM for 72 h at 37°C, constant light conditions, mycelia was lyophilized, ground into fine powder, and the lipids extracted three times in chloroform:methanol (2:1) following Bligh and Dyer (1959). About 0.64 ng heptadecanoic acid (C17:0) was added as an internal standard. The samples were dried down, resuspended in 1 ml of 5% HCl in 90% methanol, and placed at 95°C for 30 min to generate fatty acid methyl esters (FAME). The methylation reaction was quenched with water, and the FAMEs extracted three times in hexane. The samples were concentrated, and 1 μl of the hexane layer was examined by gas chromatography. Identification of peaks was achieved by comparison of sample retention times to those of palmitic-, palmitoleic-, stearic-, oleic-, linoleic-, and linolenic acid standards (FAME-GC mix, Sigma).

2.8. Statistical analysis

Spore data and colony diameters were evaluated by analysis of variance (ANOVA) using SAS.

3. Results

3.1. The sdeA and sdeB genes of A. nidulans encode Δ9-stearic acid desaturases

Sequencing of the A. nidulans genomic cosmids pWBH6 and pLAO12 by sdeA cDNA specific primers revealed a coding sequence of 1419 bp, interrupted by one intron, encoding a 455 amino acid Δ9-stearic acid desaturase (GenBank sequence Accession No. AF510861). The deduced amino acid sequence has highest identity to the fungal Δ9-stearic acid desaturase from Ajellomyces capsulata (83%) (GenBank sequence Accession No. S52745). SdeA contains the conserved histidine clusters, composing the Fe-binding active centers of the enzyme, present in all Δ9-desaturases (Los and Murata, 1998).
was not significantly different from wild type at 37°C, however ΔsdeB conidiated significantly less ($p \leq 0.05$) than wild type when grown at 22°C (Fig. 1). ΔsdeA did not conidiate at this temperature. This reduction in asexual sporulation at 22°C of the two mutant strains compared to wild type suggests the importance of unsaturated fatty acids, or at least normal fatty acid profiles, for growth of the fungus at lower temperatures ($p \leq 0.05$).

Conidiation of ΔsdeA was remediated by transformation with the full-length sdeA gene. This strain neither grew nor developed significantly different ($p \leq 0.05$) to wild type even at 22°C (data not shown).

3.3. Effect of sdeA and sdeB mutations on A. nidulans ascospore production at 37 and 22°C

Ascospore production was assessed on YGT medium after 240 h. At 37°C, ascospore production was significantly ($p \leq 0.05$) reduced in ΔsdeA compared to ΔsdeB and wild type. Interestingly, ΔsdeB ascospore production was significantly increased ($p \leq 0.05$) compared to wild type at this temperature (Fig. 2).

Ascospore production was significantly reduced ($p \leq 0.05$) in ΔsdeA compared to ΔsdeB and wild type at 22°C (Fig. 2). ΔsdeB ascospore production was not significantly different to wild type ($p \leq 0.05$) at this temperature.

3.4. Effect of sdeA and sdeB mutations on A. nidulans radial colony growth at 37 and 22°C

The effect of the Δ9-stearic acid desaturase gene mutations on colony growth was determined by measuring radial colony diameters after five days growth. Fig. 3 shows that at 37°C, ΔsdeA was significantly ($p \leq 0.05$) impaired in growth compared to ΔsdeB and wild type, which are not significantly different at this temperature. Colony growth of both ΔsdeA and ΔsdeB was significantly reduced ($p \leq 0.05$) compared to wild type at 22°C, with ΔsdeA proving again to be more severely impaired in growth compared to ΔsdeB (Fig. 3). The significant ($p \leq 0.05$) reduction in growth of ΔsdeB at 22°C, coupled with reduced conidiation compared to wild type at low temperatures, suggests a role for the SdeB protein in the physiological adaptation to cold.

The ΔsdeA; ΔsdeB double mutant was constructed by sexual crossing of the parental disruptant strains. This combination of mutated genes is synthetically lethal and the strain, carrying no intact Δ9-stearic acid desaturase loci, is incapable of growth or development on unsupplemented GMM or YGT at any temperature (data not shown). Fig. 4 illustrates the qualitative differences and similarities in radial growth of these mutants and wild type at 37°C.

3.5. Fatty acid analysis

Mycelial fatty acid content of the desaturase mutants was assessed using GC analysis. Table 2 shows the total amount of fatty acids produced by each mutant and wild type as well as the proportion of each fatty acid detected in the FAME mixture. In ΔsdeA, total fatty acid content was increased 3.5-fold over the amount produced by wild type. For ΔsdeB, total fatty acid content was reduced to 72% of the amount produced by wild type.
One action of Δ9-stearic acid desaturase is to convert palmitic acid to palmitoleic acid. Consequently, accumulation of palmitic acid increased by 125 and 150% in ΔsdeA and ΔsdeB, respectively, compared to wild type, while palmitoleic acid content was reduced in ΔsdeA and ΔsdeB to 1 and 35%, respectively, of the levels produced by wild type.

The second function of Δ9-stearic acid desaturases is to convert stearic acid to oleic acid. Disruption of sdeA led to more than a 3-fold increase in stearic acid compared to wild type, while disruption of the sdeB gene increased stearic acid production 1.3-fold. Compared to wild type, oleic acid levels were reduced approximately 4-fold in both disruption strains compared to wild type. Linoleic acid was almost undetectable in ΔsdeA, and reduced 3-fold in ΔsdeB.

For ΔsdeA, fatty acid desaturation, measured as a ratio of unsaturated to saturated fatty acids, was reduced to only 4.5% the amount of desaturation seen for wild type. Total desaturation was reduced to 18% of the wild type level in ΔsdeB.

### 3.6. Feedback regulation of fatty acid metabolism

RNA transcript accumulation was studied in the ΔsdeA, ΔsdeB and wild type strains (Figs. 5A and B). In wild type, at 37 °C, sdeA was transcribed at high levels from 36 to 48 h before diminishing at 72 h. sdeB was expressed at much lower levels than sdeA in wild type, and expression was strongest at 36 h before diminishing to undetectable levels by 72 h. This suggests different roles for the two Δ9-stearic acid desaturases under these physiological conditions. At 22 °C sdeA and sdeB expression in wild type was similar to that seen at 37 °C with the exception that sdeB, while still expressed at very low levels, was expressed for longer time points in the wild type (Fig. 5B). Levels of sdeA transcript were similar in ΔsdeB and wild type at both temperatures. However, in ΔsdeA, up-regulation of sdeB expression occurred at both temperatures compared to wild type, with sdeB expression being particularly strong in the ΔsdeA background at 22 °C. This indicates a degree of rescuing of the ΔsdeA genetic lesion by sdeB. The expression of fasA (Fig. 5), encoding the fatty acid synthase α-subunit gene (Brown et al., 1996) was shown to be elevated in ΔsdeA compared to ΔsdeB and wild type at both temperatures studied. fasA expression was not altered in ΔsdeB compared to wild type. Increased expression of fasA in ΔsdeA could reflect the increase in total fatty acid content seen for ΔsdeA at 37 °C in Table 2.

Previous studies have shown that like ΔsdeA, the ΔodeA mutation also leads to an increase in total fatty acid production (Calvo et al., 2001). Therefore, the effect of the ΔodeA mutation on fasA gene expression was also investigated. fasA was elevated in ΔodeA to levels intermediate between sdeA and wild type as shown in Fig. 6. Because mutations which increase endogenous stearic acid and oleic acid content appear to stimulate fasA gene expression and fatty acid biosynthesis (Table 2, Fig. 6 and Calvo et al., 2001), while linoleic acid is known to down regulate fatty acid biosynthesis (Clarke and Jump, 1993), the individual effects of these lipids on wild type fasA expression was investigated.

### Table 2

**Fatty acid composition of mycelia of A. nidulans wild type (WT), ΔsdeA, and ΔsdeB**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total FAME</th>
<th>Weight % of individual FAME</th>
<th>Desaturation</th>
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<tr>
<td></td>
<td></td>
<td>16:0</td>
<td>16:1</td>
</tr>
<tr>
<td>WT</td>
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<td>48.25 ± 0.85</td>
<td>0.65 ± 0.15</td>
</tr>
<tr>
<td>ΔsdeA</td>
<td>3.02 ± 0.5</td>
<td>60.55 ± 1.45</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>ΔsdeB</td>
<td>0.62 ± 0.04</td>
<td>72.43 ± 4.51</td>
<td>0.23 ± 0.15</td>
</tr>
</tbody>
</table>

Analysis was carried out on 72 h old mycelia grown in stationary liquid glucose minimal medium under continuous dark conditions at 37 °C.

*a Abbreviations: FAME, fatty acid methyl esters, given as ng FAME per µg lyophilized weight of mycelia; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; and 18:3, linolenic acid. Values are means of four replicates. Standard deviation is shown.

*b Desaturation represents the ratio of unsaturated to saturated lipids (i.e., 16:1 + 18:1 + 18:2/16:0 + 18:0).
and oleic acid induced fasA gene expression, while linoleic acid had no discernable affect (data not shown). The fluxes in transcript level in both Figs. 5 and 6 support a case for fatty acid feedback regulation of fatty acid metabolism genes, similar to previous reports (Bassilian et al., 2001; Calvo et al., 2001; Clarke and Jump, 1993; Lee et al., 1998).

3.7. Failure to remediate Δ9-stearic acid desaturase mutants to wild type growth by addition of exogenous fatty acid

To determine if wild type phenotype could be restored to the desaturase mutants by exogenous fatty acids, ΔsdeA, ΔsdeB, wild type, and the ΔsdeA; ΔsdeB double mutant were grown on GMM supplemented with 1% (v/v) Tween 80 at 37 and 22 °C (Fig. 7). Despite supplementation, ΔsdeA grew significantly less than wild type and ΔsdeB (p ≤ 0.05) at 22 and 37 °C, while ΔsdeB still grew significantly less than wild type (p ≤ 0.05) at 22 °C. Although none of the mutants were remediated to wild type growth by exogenous unsaturated fatty acids, this is the only condition that allows some growth of the ΔsdeA; ΔsdeB double mutant. Fatty acid analysis of ΔsdeA and wild type grown in liquid GMM supplemented with 1% (v/v) Tween 80 showed that while the unsaturated fatty acid profile of ΔsdeA approached that of wild type, stearic acid content is still increased in ΔsdeA (Table 3).

4. Discussion

In a number of fungi, including N. crassa (Scott, 1977), Aspergillus niger (Chattopadhyay et al., 1985), and Saccharomyces cerevisiae (Stukey et al., 1989), loss of Δ9-stearic acid desaturase function is lethal and these mutants require the addition of unsaturated fatty acids for growth. Searches of the relevant genomic databases reveal only one Δ9-stearic acid desaturase gene for each of these species (data not shown). A. nidulans more closely resembles Mortierella alpina (MacKenzie et al., 2002) and higher organisms such as mice (Miyazaki et al., 2001 and references therein) which carry multiple Δ9-stearic acid desaturase genes. Consequently, deletion of
The fatty acid profile of \textit{A}sdeA shows that although stearic- and palmitic acid levels were increased and oleic acid levels were reduced, it still produced measurable amounts of oleic acid, indicating SdeB function. This was supported by elevation of \textit{sdeB} gene expression in the \textit{A}sdeA strain at both temperatures studied. The reduction in \textit{A}sdeA development and growth might be attributed to the delay in \textit{sdeB} up-regulation seen in \textit{A}sdeA (Fig. 5A). \textit{A}sdeA accumulated large amounts of stearic acid, while \textit{AsdeB} saw a greater increase in palmitic acid production (Table 2). Therefore, one could speculate that under normal growth conditions, \textit{sdeA} encodes a protein whose affinity for stearic acid is higher than palmitic acid, while the SdeB protein is mainly involved with converting palmitic acid to palmitoleic acid.

An additional affect of stearic acid accumulation appears to be stimulation of fatty acid biosynthesis, as seen by the increase in total fatty acids (Table 2) and \textit{fasA} expression (Fig. 5) for \textit{A}sdeA compared to wild type. The increase in total fatty acid content, as seen previously with \textit{A}sdeA, could be attributed to two antagonistic mechanisms. One mechanism would be lack of a negative fatty acid feedback mechanism involving linoleic acid. In \textit{A}odeA, \textit{fasA} expression is increased compared to wild type, though not to the same high level seen for \textit{A}sdeA (Fig. 6). As \textit{A}odeA and \textit{A}sdeA generated only trace amounts of linoleic acid (Calvo et al., 2001; Table 2), this suggests \textit{fasA} expression is regulated negatively by linoleic acid, as for other organisms (Clarke and Jump, 1993). The second mechanism is through positive feedback involving stearic acid and oleic acid. \textit{A}sdeA accumulated stearic acid to levels 3-fold greater than seen for wild type (Table 2), while \textit{A}odeA accumulates oleic acid 4-fold compared to wild type (Calvo et al., 2001). In wild type, exogenous stearic acid and oleic acid stimulated \textit{fasA} expression, while linoleic acid did not increase \textit{fasA} expression (data not shown). Therefore, fatty acid biosynthesis is likely controlled through negative regulation of \textit{fasA} expression by linoleic acid, as described for other organisms (Clarke and Jump, 1993; Kim et al., 2002; Xu et al., 1999) and positive regulation by stearic acid and oleic acid.
acid. This latter observation contrasts with the situation in mammals where exogenous fatty acids, including stearic acid, inhibit de novo lipogenesis and the activities of fatty acid syntheses (Bassilian et al., 2001; Lee et al., 1998).

The reduced growth capacity of the sde mutants could not be restored to wild type levels by the addition of exogenous oleic acid, as 1% (v/v) Tween 80, even though this addition markedly improved growth of the double mutant (Fig. 7). For AsdeA, although levels of oleic acid, linoleic acid, and linolenic acid were approaching wild type when grown on 1% (v/v) Tween 80 supplemented media (Table 3), indicating cellular uptake and incorporation of exogenous fatty acids, stearic acid content was still elevated and percent desaturation still reduced compared to wild type. Therefore, exogenous fatty acids are unable to prevent stearic acid accumulation, which in turn affects Aspergillus growth.

Taken together, these results suggest a strong genetic link between fatty acid biosynthesis and morphological development in A. nidulans. Continuing investigations of this connection will also reveal interesting insights into the regulation and control of fatty acid metabolism in filamentous fungi.

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References


