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The dihydroceramide desaturase is not essential for cell viability in *Schizosaccharomyces pombe*

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Abstract **Recent studies have identified a new family of desaturase-like polypeptide sequences in many higher eukaryotes. Functional characterisation of one member of this family, from *Schizosaccharomyces pombe*, revealed the enzyme to be a sphingolipid desaturase. This *S. pombe* gene designated SDCB3b8.07c was identified as the dihydroceramide Δ^4-desaturase, responsible for the synthesis of sphingosine. Homologous recombination was used to disrupt the endogenous *S. pombe* dihydroceramide Δ^4-desaturase. Surprisingly, this had no effect on cell viability, indicating that sphingosine may not be crucial for normal *S. pombe* functions. This observation has implications for our understanding of the role of sphingosine and its phosphorylated metabolite sphingosine-1-phosphate in lower eukaryotes.**

Key words: Ceramide; Desaturase; Long-chain base; Sphingolipid; Sphingosine

1. Introduction

Sphingolipids are ubiquitous and essential components of eukaryotic cells, and have recently attracted considerable interest in view of their roles in cellular signalling [1]. Sphingolipids comprise a sphingoid long-chain base (LCB) that is amide-linked through the 2-amino group to a very-long-chain fatty acid, with various additions to the C-1 OH group of the resultant ceramide [1]. The biological activity of sphingolipids, in terms of either biophysical or signalling properties, is defined by heterogeneity within all three of these components [2,3]. In terms of the LCB, this heterogeneity takes the form of desaturation and/or hydroxylation of the carbon atoms (usually 18 or 20 in number) which make up the backbone of the sphingoid base [3]. In animals and some fungi, dihydrosphingosine (annotated as d18:0, where d = dihydroxy, 18 is the length of the carbon chain) may be Δ^{4trans}-desaturated to yield sphingosine (d18:1^{4t}, where the position and orientation of the double bond is indicated by the superscript) [1,4]. This Δ^{4trans}-desaturation has been shown to occur on dihydroceramide (i.e. dihydrosphingosine *N*-acylated to the fatty acid moiety) rather than free dihydrosphingosine; thus the Δ^4-LCB desaturase is more precisely referred to as the dihydroceramide Δ^4-desaturase, since this defines the true substrate of the enzyme [4]. In the yeast *Saccharomyces cerevisiae*, the C-4 position of dihydrosphingosine is hydroxylated to yield phytosphingosine (t18:0, where t = trihydroxy), a reaction carried out by the SUR2 C-4 hydroxylase; however, this enzyme uses both free LCBs and ceramides as substrates [5]. Higher plants display further levels of LCB modification, with (stereo-unselective) desaturation at the C-8 position as well as either hydroxylation or desaturation occurring at the C-4 position [6,7].

In animals, sphingosine serves as a substrate for a LCB kinase, yielding sphingosine-1-phosphate (S-1-P) which has been shown to be a potent signalling molecule [8]. S-1-P has been implicated in a number of responses including cellular proliferation, motility, cytoskeletal organisation and apoptosis, and is clearly an important lipid-derived second messenger [8]. In *S. cerevisiae*, the related LCBs dihydrosphingosine and phytosphingosine are phosphorylated and play a key role in the response to heat shock and trehalose synthesis [1,9]. S-1-P has also been implicated in stress responses and apoptosis in higher plants [10].

Whilst genes encoding the C-4 hydroxylase SUR2 and the sphingolipid Δ^8-desaturase have been identified from a number of organisms [5,8,11,12], the identity of the dihydroceramide Δ^4-desaturase has only very recently been reported [13]. Here we describe the functional characterisation of an open reading frame (ORF) from *Schizosaccharomyces pombe* (fission yeast), which when expressed in the *sur2*Δ mutant of *S. cerevisiae* resulted in the accumulation of Δ^4-desaturated LCBs. We also demonstrate the non-essential nature of the dihydroceramide Δ^4-desaturase in *S. pombe*.

2. Materials and methods

2.1. Growth of yeasts

S. cerevisiae strains W303-1A and *sur2*Δ were obtained from EUROSCARF (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/index.html).

S. pombe wildtype strain (accession number NCYC1354; 972 h−) and ura4− strain (NCYC2036; h− ura4− D18) were obtained from the National Collection of Yeast Cultures (http://www.ifr.bbsrc.ac.uk/ncyc/). Yeast strains were maintained on solid rich medium (YPD) at 30°C; cultures were grown in liquid YPD at 30°C with shaking, as previously described [11].

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Abbreviations: LCB, long-chain base; ORF, open reading frame; S-1-P, sphingosine-1-phosphate

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2.2. Polymerase chain reaction (PCR) amplification of S. pombe desaturase ORF and functional expression

PCR amplification was carried out on genomic DNA from *S. pombe* using primers pombeKpn.For (5′-GCGGTACCATGGCCGAATCAACTGCTACA-3′) and pombeSac.Rev (5′-CGCGAGCTCTTATGAAACATGAAGGTGCAT-3′). The amplified sequence was restricted with *Kpn*I and *Sac*I (underlined in the forward and reverse primers respectively) and cloned into the pYES2 expression vector (Invitrogen). *S. cerevisiae* strains W303-1A and *sur2*Δ were transformed using the lithium acetate-based method [14]. Transformed cells were maintained on synthetic dextrose minimal medium minus uracil. Cultures were grown at 22°C with shaking in the presence of 2% (v/v) raffinose, induction of the transgene was via the addition of 2% (w/v) galactose. All cultures were grown for a further 72 h.

2.3. Sphingoid base analysis

Sphingolipid analysis of yeast cells was carried out according to the method of Sperling et al. [7]. LCBs were liberated from yeast cells by alkaline hydrolysis and extracted with chloroform/dioxane/water (6/5/1, v/v). The LCB fraction was converted to dinitrophenol (DNP) derivatives, extracted with chloroform/methanol/water (8/4/3, v/v), purified by thin-layer chromatography (TLC) on silica plates and analysed by reversed-phase high-performance liquid chromatography (HPLC) using an Agilent 1100 LC system, with mass spectrometry (MS) analysis carried out on a Thermoquest LCQ system with an APCI source. Free LCB extraction and subsequent TLC analysis was carried out as described [5].

2.4. Deletion of the S. pombe dihydroceramide Δ^4-desaturase gene

The PCR product generated by primers pombeKpn.For and pombeSac.Rev was cloned into pCR4-TOPO® (Invitrogen, UK). A URA4$^+$ expression cassette was released from plasmid KS-ura4 [15] and cloned into a *Hin*dIII site occurring 423 bp behind the ATG codon in SDCB3b8.07c. The URA4-disrupted dihydroceramide desaturase was excised with *Kpn*I and *Sac*I and used to transform *S. pombe* strain NCYC2036. Recombinant clones (designated strain SGPY36) were selected on SD medium lacking uracil and the integration verified by PCR using primers: SGprom.For (5′-CGTTAAGCATCTCTTTAGAA-3′), SGterm.Rev (5′-CGAAACCCAATCATCATCAT-3′), sp5′-Ura4 (5′TGAAATACTCTAGCATCCAT-3′) and sp3′-Ura4 (5′-GCAAAGACTTTCTCAGCATTAA-3′).

3. Results

In the course of our studies on polyunsaturated fatty acid biosynthesis we noted the conserved presence of desaturase-like sequences in the genomes of many organisms (Fig. 1), though these ORFs were distinct from previously functionally characterised fatty acid desaturases [16]. To further examine these desaturase-like sequences, the yeast *S. pombe* was chosen for study. Gene-specific PCR was used amplify the entire coding sequence of the single desaturase-like ORF (which is present on chromosome II and designated SPBC3B8.07c) of 362 residues from *S. pombe* genomic DNA. This amplicon was then cloned into the *S. cerevisiae* galactose-inducible expression vector pYES2 and fully sequenced, confirming the DNA sequence given in GenBank accession number AL022244. Initial experiments in which a wide range of (C_{16}–C_{20} saturated and unsaturated) fatty acids were examined as potential substrates for SPBC3B8.07c indicated that

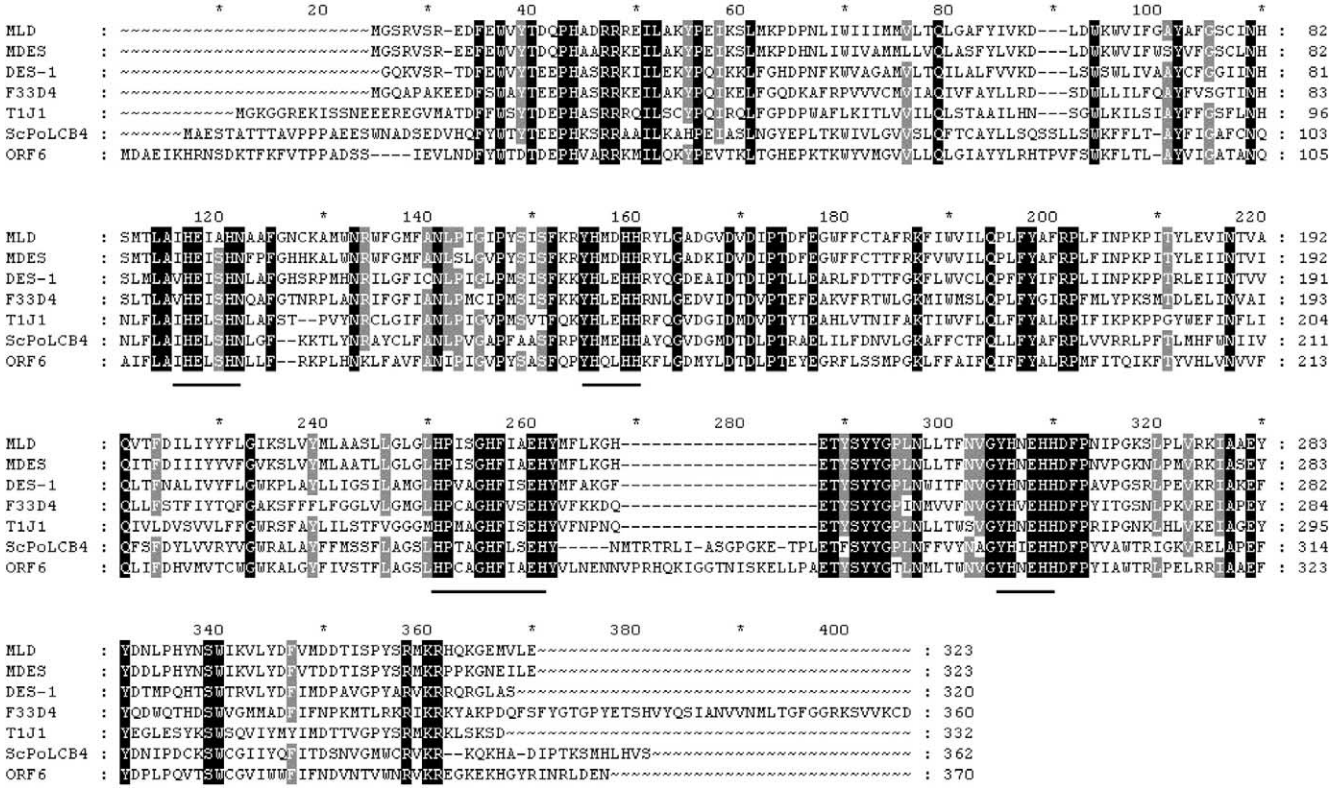

Fig. 1. Sequence comparison of the deduced amino acid sequence of desaturase-like ORFs from different species. Sequences compared are: MDES, mouse *des-1*-related sequence (Y08460)*; MLD, human membrane lipid desaturase (AF002668)*; Des-1, *Drosophila* degenerative spermatocyte gene product (X94180)*; F33D4.4, *Caenorhabditis elegans* ORF, member of an uncharacterised protein family (AAB88372.1); T1J1.1, *Arabidopsis* putative fatty acid desaturase (AF220201); ScPoD4, *S. pombe* ORF SPBC3B8.07c (AL022244), functionally characterised as dihydroxyceramide Δ^4-desaturase in this study; ORF6, *Candida albicans* (ORF 6.4414)*. Database accession numbers are given in parentheses. The conserved histidine-rich motifs are underlined. Sequences marked with an asterisk have been recently functionally characterised as sphingolipid Δ^4-desaturases [13].

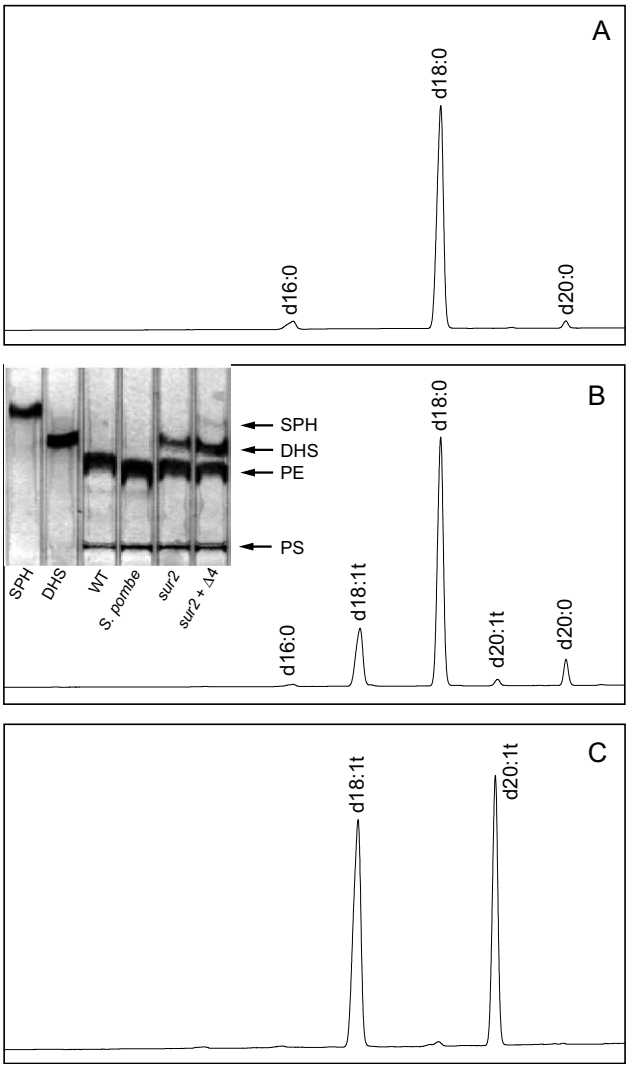

Fig. 2. Functional identification of the dihydroceramide Δ^4-desaturase by LCB analysis. Hydrolysed and derivatised LCBs were analysed by reversed-phase HPLC, with detection at 350 nm. Samples analysed were: (A) *S. cerevisiae sur2*Δ mutant; (B) *S. cerevisiae sur2*Δ mutant expressing the *S. pombe* desaturase ORF; (C) authentic C_{18} and C_{20} sphingosine (dihydroxy-Δ^4 *trans*-desaturated LCBs). Free LCBs were also analysed by TLC for the accumulation of sphingosine (B, inset). The position of this novel LCB (arrowed and labelled SPH) is shown, compared to the prevalent LCB dihydrosphingosine (DHS). Phosphatidylethanolamine (PE) and phosphatidylserine (PS) also migrate under these TLC conditions. Standards for SPH and DHS were resolved by TLC, as were free LCBs from *S. cerevisiae* (wildtype and *sur2*Δ) and *S. pombe*.

this *S. pombe* presumptive desaturase does not recognise fatty acids as substrates.

Therefore, sphingolipid constituents (in particular LCBs) were also examined as potential substrates for SPBC3B8.07c. Total yeast LCBs were extracted by alkaline hydrolysis, derivatised with DNP and characterised by reversed-phase HPLC and MS. This experimental approach was used in preference to the analysis of *N*-acylated LCBs (i.e. ceramides). Expression of the *S. pombe* ORF in wildtype yeast did not reveal any modification to endogenous *S. cerevisiae* sphingolipid LCBs, with the predominant components being C_{18} and C_{20} phytosphingosine (data not shown). Based on these data, it is clear that SPBC3B8.07c does not modify trihydroxylated LCBs.

However, when SPBC3B8.07c was expressed in the *S. cerevisiae sur2*Δ mutant which lacks the Sur2p C-4 hydroxylase and only contains dihydroxy-LCBs (Fig. 2A), two additional products were observed (Fig. 2B). Identification of these two novel peaks was facilitated by their identical retention times to authentic C_{18} and C_{20} sphingosine standards derivatised by the same protocol (Fig. 2C). Free (i.e. unacylated) LCBs were also extracted from *sur2*Δ yeast expressing the *S. pombe* ORF. These were analysed by TLC and an additional product was observed co-migrating with authentic sphingosine standards in this recombinant strain (Fig. 2B, inset). Definitive identification of these two new LCB peaks was provided by LC-MS, with analysis of these DNP derivatives revealing actual masses for the molecular ions of 465 and 493, 2 mass units smaller than that determined for d18:0 and d20:0 dihydrosphingosine standards (467 and 495, respectively), indicating that they had undergone desaturation (i.e. loss of two hydrogens). Further confirmation of the identity of these novel LCBs as C_{18} and C_{20} sphingosine was provided by tandem MS. This revealed identical patterns of daughter ions for the authentic standards and experimentally derived samples. Specifically, lower-mass daughter ions of *m/z* 162, 178, 210, common to both C_{18} and C_{20} compounds, as well as higher-mass daughters of *m/z* 435 for C_{18} and 463 for C_{20}, which relate to the differences in carbon chain lengths, were present in both sets of samples. Based on these mass spectrometry data, and the co-migration with authentic sphingosine standards in two distinct chromatographic systems, it is clear that the enzyme encoded by the *S. pombe* ORF SPBC3B8.07c is the dihydroceramide Δ^4-desaturase.

Gene targeting has previously been shown to serve as an efficient tool for insertional inactivation of *S. pombe* genes [15]. We therefore used homologous recombination to disrupt the *S. pombe* dihydroceramide Δ^4-desaturase gene SPBC3B8.07c, to assess the importance of sphingosine synthesis and bioactive metabolites (such as S-1-P) [17] in this organism. A URA4$^+$ selectable marker cassette was cloned into an endogenous *Hin*dIII site present in the desaturase ORF, and used to transform the ura$^-$ *S. pombe* strain 2036 (h− ura4$^-$ D18). Uracil auxotrophs were then characterised by genomic PCR to confirm the disruption of the dihydroceramide Δ^4-desaturase gene, using primers 5′ and 3′ to the actual ORF (Fig. 3A). This indicated the expected homologous recombination and replacement of the endogenous desaturase gene with the URA4$^+$-disrupted version, as indicated by the presence of a larger amplification product compared with (parental) wildtype (Fig. 3A). PCR-positive *S. pombe* cells were then further characterised, with the DNP-derivatised LCBs analysed by LC-MS and compared with similar samples prepared from the wildtype strain. Analysis of total extracted LCBs from this latter strain indicated that the predominant sphingoid base in *S. pombe* is C_{20} phytosphingosine (t20:0), with C_{20} sphingosine (d20:1^4) accounting for approximately 20% and C_{20} dihydrosphingosine ~2%. However, in the case of the mutant strain, the levels of C_{20} dihydrosphingosine were greatly elevated (from ~2% to >20% of total sphingolipids). Concomitantly, only basal levels of C_{20} sphingosine were detectable in the mutant (<2% of the total LCBs, compared with 23% in the wildtype). There was no alteration in levels of C_{20} phytosphingosine, nor the appearance of any

additional products (Fig. 3B). This indicates that disruption of the *S. pombe* dihydroceramide Δ^4-desaturase gene resulted in almost complete loss of sphingosine and an increase in the levels of the precursor dihydrosphingosine; this is consistent with the failure to identify any other obvious orthologues in the *S. pombe* genome. However, in spite of these changes in the LCB profile, the deletion mutant is clearly viable (Fig. 3C). Thus, high endogenous levels of sphingosine are therefore not essential for normal growth or function of *S. pombe*.

4. Discussion

In this study, we report the functional characterisation of the *S. pombe* dihydroceramide Δ^4-desaturase, the enzyme responsible for the synthesis of sphingosine. The deduced amino acid sequence of this *S. pombe* desaturase shares a number of common motifs with the presumptive orthologues present in mammals, invertebrates, plants and fungi (Fig. 1) and several of these ORFs have now been functionally characterised [13,18]. Sequence analysis of these dihydroceramide desaturase orthologues confirm the presence of several histidine-rich motifs though these do not conform to a previously defined consensus and, as such, define a new class of lipid desaturase [13,18]. Hydrophobicity analysis of the *S. pombe* sphingolipid Δ^4-desaturase indicates that it contains several domains capable of spanning a phospholipid bilayer. However, the ORF lacks any obvious topogenic signals (such as endoplasmic reticulum retention motifs), differing from other characterised examples of fatty acid desaturases that often contain C-terminal di-lysine motifs for endoplasmic reticulum recycling [16].

In higher plants, sphingolipid trihydroxy-LCBs are desaturated by a stereo-unselective Δ^8-desaturase [7]. That enzyme is a member of the cytochrome b_5-fusion class of desaturases, containing an N-terminal domain with homology to the electron donor cytochrome b_5 [19]. This N-terminal cytochrome b_5 domain is also present in the C-2 ceramide hydroxylase Fah1p [20]. However, it is clear that the dihydroceramide Δ^4-desaturase identified in this study does not contain a cytochrome b_5 domain, as it lacks the diagnostic 'H-P-G-G' haem-binding motif [19]. This observation is in agreement with a previous demonstration that microsomal cytochrome b_5 was required for ceramide synthesis [4].

One surprising observation of this current study is that *S. pombe* is viable when the dihydroceramide Δ^4-desaturase gene has been insertionally disrupted, resulting in the dramatic reduction in the endogenous levels of sphingosine present in this yeast strain. Several of the recently functionally characterised orthologues [13] of the *S. pombe* Δ^4-LCB desaturase had previously been characterised genetically and biochemically. In *Drosophila*, a male-sterile mutant blocked at the initiation of meiosis during spermatogenesis was found to be due to the transposon inactivation of a transmembrane desaturase-like protein [21]. This *Drosophila* gene, designated *des-1* (for de-

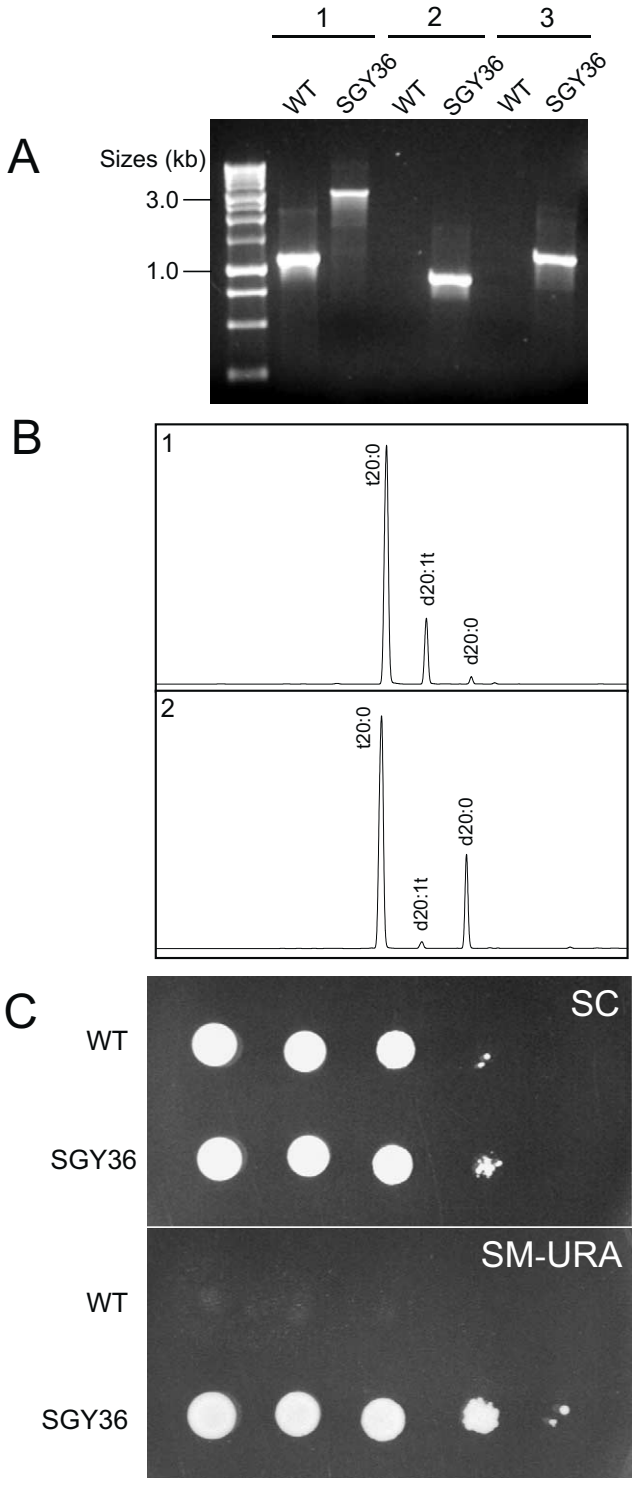

Fig. 3. Insertional inactivation of the *S. pombe* dihydroceramide desaturase. A cassette encoding the uracil-selectable marker was used to disrupt the coding sequence of the *S. pombe* dihydroceramide desaturase. Uracil auxotrophs were characterised by genomic PCR (A). Gene-specific primers (1) for the dihydroceramide desaturase amplified a much larger product in the insertion mutant (designated SGY36) due to the presence of the URA4 cassette. When primers designed to the 5′ (2) or 3′ (3) region of the dihydroceramide desaturase were used in conjunction with primers specific for the URA4 gene, amplification products are only seen in the SGY36 disruption strain. B: Total LCB analysis of sphingolipids from (parental) wildtype *S. pombe* (1) and SGY36 disruption strain (2). The predominant LCBs in *S. pombe* are C_{20}. In the case of the wildtype, C_{20} sphingosine accumulates to $>20\%$ of total. C: Disruption of the dihydroceramide desaturase does not alter cell growth or viability of *S. pombe*. Growth of wildtype or disruption strain SGY36 is comparable on complete (SC) medium. Growth on medium lacking uracil is only observed in SGY36 due to the presence of the (disrupting) URA4 cassette. Cultures were spotted onto plates after five-fold serial dilution.

generative spermatocyte), is now known to be a functional dihydroceramide Δ^4-desaturase [13]. These observations have led to speculation that sphingosine and metabolites such as S-1-P play a (gametophytic-specific) role in meiosis and cell cycle transition in *Drosophila* and *Caenorhabditis elegans* [13]. However, our characterisation of the role of sphingosine in *S. pombe* (which serves as an excellent model for cell cycle studies [22]) would indicate that this LCB is only required in very small quantities, if at all. This is clearly unlike the situation observed in the well-characterised mammalian systems, where sphingosine is a predominant component of sphingolipids.

The presence of low levels of sphingosine still present in the dihydroceramide desaturase disruption mutant is unlikely to result from activities derived from a related desaturase-like ORF, as the *S. pombe* genome lacks any obvious candidate orthologues. It is therefore possible that this apparent functional redundancy may result from the limited activity of a (structurally unrelated) lipid-modifying enzyme towards the substrate. Such metabolic plasticity has previously been observed for other enzyme activities involved in sphingolipid biosynthesis [23].

The role of LCBs and their phosphorylated metabolites is a topic of considerable current interest, with S-1-P now known to have important functions as both an extra- and intracellular signal in mammalian cells. *S. pombe* is considered to be a useful simple model for more sophisticated multicellular eukaryotic organisms [22]. In that respect, the observation that *S. pombe* sphingolipids contains Δ^4- (but not Δ^8-) desaturated LCBs is analogous to the modifications present in animal cells. However, reduction of sphingosine has no impact on the viability of the *S. pombe* yeast cells. This is in contrast to the semi-lethality observed in both male and female *Drosophila* homozygous for the *des* mutation (see above); insertional inactivation of the *des-1* dihydroceramide desaturase gene resulted in 20–50% embryo lethality in this organism [13,21]. Recent data have also implicated sphingosine metabolites in the response of higher plants to biotic and abiotic stress [10,24]. It will be of great interest to determine the precise role of sphingosine in plant and fungal processes, as these may be distinct from the previously well-defined roles in animal cells.

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