Isolation of two developmentally regulated genes involved in spore wall maturation in *Saccharomyces cerevisiae*

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During sporulation of *Saccharomyces cerevisiae*, the four haploid nuclei generated by meiosis are encapsulated within multilayered spore walls. Taking advantage of the natural fluorescence imparted to yeast spores by the presence of a dityrosine-containing macromolecule in the spore wall, we identified and cloned two genes, termed *DIT1* and *DIT2*, which are required for spore wall maturation. Mutation of these genes has no effect on the efficiency of spore formation or spore viability. The mutant spores, however, fail to accumulate the spore wall-specific dityrosine and lack the outermost layer of the spore wall. The absence of this cross-linked surface layer reduces the resistance of the spores to lytic enzymes, to ether, and to elevated temperature. Expression of the *DIT* and *DIT2* genes is restricted to sporulating cells, with the *DIT1* transcripts accumulating at the time of prospore enclosure and just prior to the time of dityrosine biosynthesis. Both genes act in a spore-autonomous manner implying that at least some of the activities responsible for forming the outermost layer of the spore wall reside within the developing spore rather than in the surrounding ascus cytoplasm. As the *DIT2* gene product has significant homology with cytochrome P-450s, *DIT2* may be responsible for catalyzing the oxidation of tyrosine residues in the formation of dityrosine.

[Key Words: *Saccharomyces cerevisiae*; sporulation; spore wall; dityrosine; cytochrome P-450]

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In the yeast *Saccharomyces cerevisiae* the portion of the life cycle referred to as sporulation consists of meiosis followed by the encapsulation of the haploid nuclei within spore walls. Initiation of sporulation, induced in the laboratory by starvation of *MATa/MATα* cells for nitrogen in the presence of a nonfermentable carbon source, is under the control of the key regulatory genes *RME1*, *IME1*, and *IME2* (Kassir and Simchen 1976; Mitchell and Herskowitz 1986; Kassir et al. 1988; Smith and Mitchell 1989). A large number of genes involved in meiotic recombination and the segregation of homologous chromosomes have been characterized (Tsuboi 1983; Rockmill and Fogel 1988; Rockmill and Roeder 1988; Hollingsworth and Byers 1989; for reviews, see Esposito and Klapholz 1981; Dawes 1983). After the completion of the two meiotic divisions, spore walls are laid down around the haploid nuclei, generating an ascus containing a tetrad of haploid spores that are more resistant to various lytic enzymes, to some organic solvents, and to high temperature than are vegetative cells (Dawes and Hardie 1974; Put and De Jong 1981). The series of genetic and morphological events that occurs as cells progress through the sporulation pathway has been shown to be accompanied by the sequential activation of temporally distinct classes of sporulation-specific genes

(for review, see Magee 1987; Clancy et al. 1983; Kurtz and Lindquist 1984; Percival-Smith and Segall 1984; Weir-Thompson and Dawes 1984; Holaway et al. 1985; Yamashita and Fukui 1985; Gottlin-Ninfa and Kaback 1986; Atcheson et al. 1987; Wang et al. 1987; Law and Segall 1988; Thompson and Roeder 1989). Here, we describe the identification of two sporulation-specific genes that are required for spore wall maturation.

One of the first events in spore wall formation is the coalescence of vesicles to initiate formation of a flattened double membrane on the cytoplasmic side of the outer spindle plaque; this then expands to envelop the four nuclear lobes budding off from the parental nucleus (for review, see Byers 1981; Moens 1971). Spore wall components are then deposited between this double membrane (Lynn and Magee 1970). Electron microscopic analysis of the mature spore wall reveals four layers (Kreger-van Rij 1978). The two inner layers, consisting mainly of glucans and mannans, are similar in composition to the vegetative cell wall and often appear as a single layer (Katohda et al. 1984; Briza et al. 1988). Next is a layer containing glucosamine with 95% being polymerized into chitosan and 5% into chitin (Briza et al. 1988). This layer and the outermost layer, which is very thin and osmiophilic, are thought to confer the protec-

tive nature on the spore wall. Spores produced by glucosamine auxotrophs lack these two outermost layers of the spore wall and are sensitive to lytic enzymes such as Glusulase and zymolyase (Ballou et al. 1977).

Although detailed characterization of the molecular composition of the spore wall has been hindered by the insoluble nature of this structure, analysis of an acid hydrolysate of purified spore walls led to the discovery that dityrosine comprises 20 mole-percent of the amino acid content of the spore wall (Briza et al. 1986). The cross-linked tyrosine, which arises only during sporulation, is present in an insoluble macromolecule and exists as both *LL* and *DL* stereoisomers (Briza et al. 1990). Cross-linking of tyrosine also occurs in other insoluble structures such as the insect cuticle (Andersen 1964) and the fertilization membrane of the sea urchin egg (Foerder and Shapiro 1977). Although horseradish peroxidase will cross-link tyrosine residues in vitro and a peroxidase has been shown to be responsible for the formation of dityrosine in the fertilized sea urchin egg (Foerder and Shapiro 1977), no significant peroxidase activity can be detected during sporulation in yeast (P. Briza, unpubl.). To gain insight into the mechanism of tyrosine cross-linking in yeast and to allow further characterization of the cross-linked macromolecule, we isolated mutants that initiate spore wall formation but fail to synthesize dityrosine.

Results

Isolation of mutants defective in spore wall maturation

The deposition of a dityrosine-containing macromolecule in the outermost layers of the yeast spore wall imparts a bluish fluorescence to the spores (Briza et al. 1986). We took advantage of this fluorescence, which provides a useful tag to assess the completion of sporulation, to devise a screen aimed at identifying sporulation mutants specifically defective in spore wall maturation (see Materials and methods). To allow the identification of recessive mutations, we used the haploid strain PB2-1C (*MAT α spo13-1 sir4^{ts} trp1-1 leu2 his6*) (Briza 1987) for this screen. Although usually only diploid *MAT α /MAT α* cells are able to sporulate, PB2-1C can initiate sporulation because mutation of *SIR4* effectively leads to mating type heterozygosity due to expression of the otherwise silent *HML* and *HMR* loci (Herskowitz and Oshima 1981). This haploid strain is also able to successfully complete sporulation generating two viable haploid spores, as the presence of the *spo13-1* allele restricts meiosis to a single division (Wagstaff et al. 1982).

We first identified mutants that formed dityrosine-deficient colonies on sporulation medium as assessed by their lack of fluorescence. To eliminate those mutants that might be blocked in early events of the sporulation pathway and might therefore only indirectly be prevented from forming spore walls, we then discarded those mutants that failed to form visible asci as assessed by light microscopy. In this way, we isolated eight independent mutants that completed early events of spore

formation but generated spores that failed to emit the fluorescence diagnostic of the spore wall-specific dityrosine (Fig. 1A). High performance liquid chromatography (HPLC) fractionation of an acid hydrolysate of cells transferred to sporulation medium confirmed that the spores of all eight mutant strains contained greatly reduced amounts of dityrosine (for an example, see Fig. 1B). We have named the eight mutant alleles *dit101*–*dit108* to reflect the dityrosine-deficient nature of the mutant spores. Quantitation of the amount of residual dityrosine by fluorescence spectrophotometric analysis indicated that the level of dityrosine in strains containing the *dit101* or *dit105* alleles was ~8% of that in

Figure 1. Mutant ascospores fail to fluoresce and are deficient in dityrosine. (A) Sporulated colonies of wild-type cells (*top* and *bottom* rows) and the eight *Dit⁻* mutants (*two middle* rows) were treated as described in Materials and methods and then examined under UV light for fluorescence. Approximately 30% of the cells in each colony had formed asci. (B) Acid hydrolysates of sporulated cultures of wild-type cells (PB2-1C) and the mutant strain containing the *dit101* allele were fractionated by HPLC (see Materials and methods). The absorbance peaks diagnostic of dityrosine in the fractions from the wild-type culture are indicated by the arrows (*di-Tyr*). The peak appears as a doublet due to the presence of both *DL*- and *LL*-dityrosine. The fractions in this region were then subjected to fluorescence spectrophotometric analysis to quantitate the amount of dityrosine. The arrow in the HPLC elution profile of the hydrolysate of the mutant cells indicates the expected position for elution of dityrosine. The fractions in this region were also analyzed by fluorescence spectrophotometry to quantitate the amount of residual dityrosine.

the wild-type strain, whereas the corresponding levels were 4% in the strain containing *dit108* and 0.1–0.8% in the strains containing *dit102*, *dit103*, *dit104*, *dit106*, or *dit107*.

Preliminary examination of these eight mutants indicated that they behaved like wild-type cells during vegetative growth and produced viable asci as efficiently as did the parental strain upon transfer to sporulation medium. However, we discovered that the spores of all eight mutant strains were unusually sensitive to Glusulase, a mixture of lytic enzymes prepared from snail intestinal juice. Although the vegetative cell wall and the ascus cell wall are rapidly degraded by Glusulase, wild-type spore walls are extremely resistant to digestion. The observation that resuspension of the mutant asci in a solution of Glusulase led to rapid lysis of the spores (see below) supported the presumption that the mutant strains were defective in spore wall maturation.

To gain further insight into the nature of the defects in the *Dit*[−] strains, we compared the structure of the mu-

tant spore walls with that of wild-type spore walls by electron microscopy. A typical wild-type ascus is depicted in Figure 2 (upper left). The outermost surface of the spore wall, which appears as a thin and very osmiophilic layer, and the underlying, more diffuse, chitosan-containing layer are spore-specific, whereas the innermost layers resemble the vegetative cell wall. Inspection of electron micrographs of sporulated cells of the mutant strains indicated that the *dit102*–*dit108* alleles led to spores that lacked the outermost, thin, and osmiophilic layer of the spore wall, whereas mutation of the *dit101* allele led to spores lacking both outer layers of the spore wall (Fig. 2 and data not shown).

Consistent with the defects in the structure of the spore wall in the *Dit*[−] mutants as visualized in the electron micrographs, we found that primulin, a chitin-specific fluorescent dye that stains wild-type asci inefficiently, stained all the mutant asci with the exception of those produced by the strain containing the *dit101* allele (data not shown). Previous studies have shown that the

Figure 2. Electron microscopic analysis of mutant asci. (A) A typical ascus is depicted for the haploid wild-type strain PB2-1C (WT), the haploid mutant strains containing the *dit101*, *dit104*, and *dit107* alleles and the heterozygous diploid strains *+ /dit101*, *+ /dit104*, and *+ /dit107* obtained by mating the strains containing the *dit101*, *dit104*, and *dit107* alleles, respectively, with a wild-type strain (SHU32). (WT) Wild-type spore; (M) mutant spore. Note that sporulation of PB2-1C (*MAT α spo13-1 sir4 trp1-1 leu2 his6*) produces asci containing two haploid spores and that sporulation of the diploid strains (*MAT α /MAT α sir4/SIR4 dit[−]/DIT⁺ spo13/SPO13*) produces asci containing four haploid spores. The electron micrographs of the four-spored asci rarely show more than three spores within one plane as a result of the tetrahedral arrangement of the spores. Bars, 1 μ m. (B) A portion of the spore wall of each ascus is shown at increased magnification relative to A. The wild-type spore wall contains four layers (see Introduction). The outermost, thin, and very dark layer is denoted with the arrow labeled *d*. The underlying chitosan-containing layer is denoted with the arrow labeled *c*. Occasionally, this layer appears to be fragmented (see Discussion). The two inner layers, which often appear as a single layer, are denoted with the arrow labeled *i*. Because of differences in staining properties, wild-type spores sometimes appear darker than mutant spores.

outermost layer of the spore wall prevents primulin from staining the underlying chitosan layer, presumably because the dye is unable to penetrate the outermost layer (Briza et al. 1988). The absence of this outermost layer in spores mutant for the *dit102-dit108* alleles might make the chitosan layer more freely accessible to primulin. The inability to stain spores produced by the strain containing the *dit101* allele presumably reflects not only the absence of the dityrosine-containing surface layer but also the underlying chitosan layer.

Assessment of the phenotype of heterozygous diploid strains revealed that six of the *dit* alleles represented genes that behaved in a spore autonomous manner. For this analysis each mutant strain was mated with a wild-type haploid strain, and the resulting diploid strain was sporulated. Sporulation of diploid strains heterozygous for *dit102*, *dit103*, *dit104*, *dit106*, *dit107*, or *dit108* yielded asci that displayed phenotypic segregation of the spore wall defect. Thus, two spores in each ascus were Glusulase sensitive and two spores were Glusulase resistant. As assessed by electron microscopic examination, two spores in each ascus lacked the outermost layer of the spore wall, whereas two spores had wild-type spore walls (for examples, see Fig. 2). In contrast to the lack of complementation between the wild-type and mutant alleles of these spore autonomous genes, *dit101* and *dit105* behaved as recessive mutations. Sporulation of the diploid heterozygous strains *MATa dit101/MATα DIT101* and *MATa dit105/MATα DIT105* generated only phenotypically wild-type spores, all four spores present in an ascus being Glusulase resistant and containing mature spore walls as assessed by electron microscopy (for an example, see Fig. 2). In the present study, we focused on characterization of the six spore-autonomous mutations, *dit102-dit104* and *dit106-dit108*.

Cloning the *DIT1* and *DIT2* genes by complementation

Complementation analysis of the mutant alleles by standard genetic techniques was complicated by the Glusulase-sensitive nature of the mutant spores. Additionally, the spore-autonomous behavior of the alleles made it difficult to outcross the mutations. We therefore decided to compare the mutations by cloning the corresponding wild-type genes. A yeast DNA library, constructed by Nasmyth and Tatchell (1980), was screened for plasmids that would restore fluorescence to sporulated cultures of the mutant cells (see Materials and methods). The plasmid denoted pPB-10 (Fig. 3A), which was first isolated by complementation of the *dit102* mutation, was reisolated on screening for complementation of the *dit103* and *dit106* mutations. A plasmid denoted pPB-20 (Fig. 3A) with a yeast DNA insert that largely overlapped that of pPB-10 was isolated by complementation of the *dit104* mutation. We then tested these two plasmids for their ability to complement each of the six spore-autonomous mutations. Although each plasmid could complement all six mutations, we found that subclone pPB-18 could complement only the *dit103*, *dit104*, and *dit108* alleles and that subclone pPB-17 could complement only the *dit102*, *dit106*, and *dit107* alleles (Fig. 3A). This suggested that the six *dit* mutations defined two closely linked genes. We refer to these genes as *DIT1*, defining the complementation group containing the *dit103*, *dit104*, and *dit108* alleles, and *DIT2*, defining the complementation group containing the *dit102*, *dit106*, and *dit107* alleles. As the slightly smaller nonoverlapping clones pPB-12 and pPB-13 (Fig. 3A) were unable to complement any mutant allele, the *DIT1* and *DIT2* genes must be very closely linked.

The presence of the two contiguous *DIT* genes was

Figure 3. Isolation of the *DIT1* and *DIT2* genes. (A) Plasmids pPB-10 and pPB-20 were isolated by screening a yeast DNA library for plasmids that could complement the nonfluorescent defect of various *Dit*⁻ mutants (see Results). Portions of the yeast DNA insert of pPB-10 were subcloned into YEp13 generating pPB-11, pPB-12, pPB-13, pPB-17, pPB-18, and pPB-30. The yeast DNA fragment present in each of these plasmids is denoted beneath the map of the *DIT1-DIT2* region. The arrows on this map denote the open reading frames corresponding to *DIT1* and *DIT2* (Fig. 7). Each of these plasmids was tested for its ability to complement the *dit102-dit104* and *dit106-dit108* alleles by transforming the replicating plasmids into the various mutants and then testing sporulated colonies of the transformed cells for fluorescence. The results of this analysis are indicated in the table denoted COMPLEMENTATION. (B) Poly(A)⁺ RNA isolated from *MATa/MATα* cells at 12 hr after transfer to sporulation medium was separated on a 1.5% agarose gel and transferred to a nylon membrane. Adjacent strips cut from this filter were hybridized with radioactively labeled pEMBL19 derivatives containing the yeast DNA insert of pPB-10 (lane 1), pPB-13 (lane 2), and pPB-12 (lane 3). The filter of lane 3 was exposed four times longer than the filters of lanes 1 and 2 to compensate for the lower level of *DIT2* transcripts.

confirmed by Northern analysis. RNA isolated from wild-type sporulating cells was separated by gel electrophoresis and transferred to a nylon membrane. A radioactively labeled pEMBL19 derivative containing the yeast DNA insert of pPB-10 hybridized with two transcripts, a relatively abundant 1.9-kb transcript and a less abundant 1.6-kb transcript (Fig. 3B, lane 1). Hybridization with radioactively labeled probes prepared with subcloned DNA sequences indicated that the *DIT1* gene encodes the 1.9-kb transcript and that the *DIT2* gene encodes the less abundant 1.6-kb transcript (Fig. 3B, lanes 2 and 3).

As the yeast DNA library had been prepared in a high-copy 2 μ -based vector, we subcloned the yeast DNA insert of pPB-10 into a low-copy centromere-containing plasmid. We found that the low-copy plasmid also complemented the *dit* alleles, suggesting that the putative cloning of *DIT* DNA did not result simply from the suppression of the *dit* mutations by an increased dosage of an alternative gene product.

Mutational analysis of the *DIT1* and *DIT2* genes

To exclude the possibility that the cloned DNA represented an extragenic suppressor, we then verified that the *DIT* DNA contained the wild-type alleles of the original *dit* mutations. An approach frequently used for such verification is to use the cloned DNA to direct integration of a selectable marker at the chromosomal locus corresponding to the cloned sequence and to test for linkage between the selectable marker and the locus defined by mutation. However, this approach was not feasible with the *DIT* DNA as the Glusulase-sensitive nature of the *dit* spores rendered standard tetrad analysis difficult. We therefore decided to base our analysis on the phenotypes of cells containing disruptions of the *DIT* genes and the frequencies of recombination between these disrupted genes and the original mutations.

Mutations were introduced into the chromosomal *DIT1* and *DIT2* genes as follows. First, a yeast DNA fragment containing the *URA3* gene was inserted into the coding region of the cloned *DIT1* gene, and a linear DNA fragment containing the disrupted gene was used to transform *MATa ura3* and *MAT α ura3* cells to Ura⁺ (see Materials and methods). *MATa dit1 :: URA3* and *MAT α dit1 :: URA3* transformants were then mated to obtain a homozygous mutant diploid strain. The same procedure was used to obtain a *MATa dit2 :: URA3/MAT α dit2 :: URA3* strain. Southern blot analysis of genomic DNA confirmed that the chromosomal copies of the genes had been replaced with the corresponding disrupted copies (data not shown). We refer to these mutant alleles of *DIT1* and *DIT2* as *dit1-1* and *dit2-1*, respectively. We then sporulated the disruption homozygotes and examined the phenotype of the resulting asci. As expected, the spores produced by both mutant strains were sensitive to Glusulase and were deficient in dityrosine, containing <0.05% of the amount present in wild-type spores, as assessed by spectrofluorometric analysis of HPLC-purified dityrosine (data not shown). Electron microscopic examination revealed that all four spores

present within asci of either strain lacked the outermost layer of the spore wall (Fig. 4). As expected from the spore autonomous behavior of the original *dit* alleles, heterozygous *MATa DIT1/MAT α dit1-1* and *MATa DIT2/MAT α dit2-1* strains produced asci containing two wild-type spores and two spores lacking the outermost surface layer of the spore wall (Fig. 4). Consistent with the visualization of the chitosan-containing layer in electron micrographs of the mutant spores (Fig. 4B), we found that these spores could be stained readily with primulin (Fig. 4C). As a further test for the presence of the chitosan layer in the mutant spores, we measured the glucosamine content of hydrolysates of sporulated cultures. We found that the glucosamine content of sporulated cells of both homozygous mutants, *MATa dit1-1/MAT α dit1-1* and *MATa dit2-1/MAT α dit2-1*, was comparable to that of sporulated wild-type cells, increasing approximately fourfold during sporulation (Table 1). Most of the glucosamine was present in the insoluble fraction of a lysate of sporulated cells, suggesting that it was polymeric and associated with the spore walls (Table 1). The observation that the glucosamine was degraded by HNO₂ confirmed that the glucosamine was present mostly as chitosan rather than as chitin (Table 1).

We then mated the original strains containing *dit103*, *dit104*, and *dit108*, presumptive alleles of *DIT1*, with the *MATa dit1-1* strain. The original mutant strains containing *dit102*, *dit106*, and *dit107*, presumptive alleles of *DIT2*, were mated with the *MATa dit2-1* strain. We found that sporulated cultures of each of the six resulting diploid strains contained only trace amounts of dityrosine (data not shown). Moreover, electron microscopic examination of >500 asci from both the *MATa dit1-1/MAT α dit104* and the *MATa dit2-1/MAT α dit106* strains failed to reveal any spores containing the outermost layer of the spore wall (data not shown). Because the phenotype of the mutant strains constructed using the cloned *DIT* genes was indistinguishable from that of the original mutants and because no recombination was observed between the original *dit* alleles and the disruption mutations, we concluded that the *DIT* genes isolated by complementation represent the wild-type alleles of the original mutations.

Mutation of *DIT1* or *DIT2* leads to less hardy spores

Because the *MATa dit1-1/MAT α dit1-1* and *MATa dit2-1/MAT α dit2-1* strains sporulated more efficiently than did the original PB2-1C-derived mutant strains, we took advantage of these newly constructed homozygotes to examine the phenotype of the *Dit*⁻ mutants in more detail. First, we confirmed our qualitative observations that mutation of either the *DIT1* gene or the *DIT2* gene led to Glusulase sensitivity of the mutant spores. Greater than 90% of *MATa dit1-1/MAT α dit1-1* spores and *MATa dit2-1/MAT α dit2-1* spores lysed after a 30-min exposure to Glusulase (Fig. 5A). Wild-type spores, treated in the same way, remained intact. We then examined the viability of the mutant spores. As the Glusulase-sensitive nature of the mutant spores made te-

Figure 4. Electron microscopy and fluorescence microscopy of asci of strains containing the *dit1-1* and *dit2-1* alleles. (A) An electron micrograph of a typical ascus is shown for the wild-type diploid strain LP-HT (WT), the homozygous diploid *MATa dit1-1/MATα dit1-1* (*dit1-1/dit1-1*) and *MATa dit2-1/MATα dit2-1* (*dit2-1/dit2-1*) strains, and the heterozygous diploid *MATa DIT1/MATα dit1-1* (*+/dit1-1*) and *MATa DIT2/MATα dit2-1* (*+/dit2-1*) strains. The layers of the spore wall are denoted as in Fig. 2. Bars, 1 μ m. (B) A portion of the spore wall from each ascus is shown at increased magnification relative to A. Fluorescent (C) and phase-contrast (D) microscopic analyses of primulin-treated sporulated cultures. Efficient primulin staining of the chitosan-containing layer of the spore wall occurs only if the outermost layer of the spore wall is absent. Nonfluorescent wild-type spores in heterozygous asci are indicated with an arrow.

trad dissection difficult, we estimated spore viability by determining the plating efficiency of sporulated cultures of the homozygous mutant strains 72 hr after transfer to sporulation medium. The plating efficiencies of mutant and wild-type cells were indistinguishable (85–90%).

We then tested whether the loss of the outermost layer of the spore wall affected the resistance of spores to ether or to elevated temperature. As shown in Figure 5B, asci of the mutant strains *MATa dit1-1/MATα dit1-1* and *MATa dit2-1/MATα dit2-1* were 100-fold more sensitive to an 8-min exposure to ether than were asci of the wild-type strain. The mutant asci were almost as sensitive to ether as stationary-phase cells were. The thermotolerance of the mutant spores was examined by exposing cultures of sporulated cells to 55.5°C for various periods of time. We found that asci of the mutant strains

MATa dit1-1/MATα dit1-1 and *MATa dit2-1/MATα dit2-1* were significantly more sensitive to elevated temperature than wild-type asci were (Fig. 5C). Survival of the mutant asci was, however, higher than stationary-phase cells treated in the same manner (Fig. 5C). Correlation of these phenotypic effects of the *dit* mutations with the effect of the mutations on the structure of the spore wall suggests that the outermost cross-linked layer of the spore wall is responsible for the resistance of the spore to Glusulase and to ether and contributes to the heat resistance of spores.

Sporulation-specific expression of the DIT1 and DIT2 genes

Several studies have revealed that expression of many

Table 1. Glucosamine content of wild-type and mutant spore walls

Strain	D-Glucosamine			
	$\mu\text{mole}/10^{10}$ cells ^a		percent in cell and spore walls ^b	percent in chitosan ^c
	veg	spo		
WT	8.5	30.5	85	81
<i>dit1-1/dit1-1</i>	7.5	27.5	84	83
<i>dit2-1/dit2-1</i>	11.2	36.0	88	70

^aGlucosamine was determined in total cell hydrolysates, using a modified Morgan–Elson procedure as described by Ballou et al. (1977).

^bCells of a sporulated culture were disrupted by agitation with glass beads, and soluble and cell wall fractions, obtained by low-speed centrifugation, were assayed for glucosamine.

^cThe sensitivity of chitosan to degradation by HNO_2 was used to distinguish between chitin and chitosan. The glucosamine content of the HNO_2 insoluble fraction of the cell walls was subtracted from the total glucosamine content of the cell walls to estimate the percentage of glucosamine present in chitosan (Briza et al. 1988).

genes encoding sporulation-specific functions is developmentally regulated. To examine the developmental specificity of expression of the *DIT* genes, filters containing poly(A)⁺ RNA purified from *MATa/MATa*, *MATa/MAT α* , and *MAT α /MAT α* cells during vegetative growth and at various times after transfer to sporulation medium were hybridized with radioactively labeled *DIT* DNA. We found that the *DIT* transcripts accumulated only in *MATa/MAT α* cells in sporulation medium (Fig. 6A). Transcripts were not detected in RNA from vegetatively growing cells (Fig. 6B, and data not shown) nor in RNA extracted from the asporogenous *MATa/MATa* and *MAT α /MAT α* cells at various times after transfer to sporulation medium (Fig. 6A, and data not shown). Consistent with the presumed involvement of the *DIT* genes in spore wall maturation, transcripts corresponding to the *DIT1* and *DIT2* genes began to accumulate at 8–10 hr after the transfer of cells to sporulation medium, just prior to the time at which dityrosine began to accumulate (Fig. 6C). The time of maximal *DIT* transcript accumulation, 12–14 hr of sporulation, corresponded to the time span at which immature asci could be detected by light microscopy and maximal dityrosine synthesis occurred (Fig. 6C). The *DIT* transcripts disappeared from sporulating cells as mature spores began to appear (Fig. 6C).

Previously, we characterized two temporally distinct classes of sporulation-specific (*SPS*) genes, referred to as middle and late genes, which are expressed during the later phases of the sporulation program (Percival-Smith and Segall 1984; Law and Segall 1988). Transcripts corresponding to middle *SPS* genes, represented in Figure 6B by the *SPS1* and *SPS2* genes, can be detected between 8 and 14 hr after transfer of cells to sporulation medium. Transcripts corresponding to the late *SPS* genes, represented in Figure 6B by the *SPS100* gene, begin to accumulate at 14 hr of sporulation and then remain at a constant level. Comparison of the temporal pattern of ex-

pression of the *DIT* genes with the expression of the *SPS* genes revealed that the *DIT* genes were first expressed after the middle *SPS* genes had been turned on but prior to the time of late *SPS* gene transcription (Fig. 6B). Thus, the *DIT* genes define a new temporal class of sporulation-specific genes. It is interesting to note that the time of activation of these *SPS* genes correlates with the time of action of their gene products. Mutation of the *SPS1* gene blocks sporulation after the completion of meiosis such that mutant cells become tetranucleate but fail to form spore walls (Percival-Smith and Segall 1986). As described here, strains with a mutation of either the *DIT1* gene or the *DIT2* gene are able to complete prospore enclosure but fail to deposit the outermost layer of the spore wall. Mutation of the *SPS100* gene delays, but does not prevent, spore wall maturation (Law and Segall 1988).

Nucleotide sequence of the *DIT1* and *DIT2* genes

As a first step in elucidating the function of the *DIT* genes, we determined the nucleotide sequence of the yeast DNA insert present in plasmid pPB-10. As expected, this revealed the presence of two open reading frames (ORF) (Figs. 3A and 7). The two ORFs are sepa-

Figure 5. Mutation of the *DIT1* or the *DIT2* gene leads to less hardy spores. A sporulated culture of *MATa/MAT α* cells ($\square$), a sporulated culture of *MATa dit1-1/MAT α dit1-1* cells ($\blacksquare$), a sporulated culture of *MATa dit2-1/MAT α dit2-1* cells ($\bullet$), and a stationary-phase culture of *MATa/MAT α* cell ($\circ$) were tested for cell viability after exposure to Glusulase (A), ether (B) and 55.5°C (C) for the indicated times (see Materials and methods). The efficiency of ascus formation in the sporulated cultures was >80%.

Figure 6. Developmental-specific expression of the *DIT1* and *DIT2* genes. (A) The *DIT1* and *DIT2* genes are not expressed in asporogenous *MATa/MATa* and *MAT α /MAT α* cells in sporulation medium. Poly(A)⁺ RNA purified from *MATa/MATa*, *MATa/MAT α* , and *MAT α /MAT α* cells at 12 hr after transfer to sporulation medium was separated by electrophoresis through an agarose gel and transferred to a nylon membrane. The RNA blot was hybridized with a radiolabeled pEMBL19 derivative containing the yeast DNA insert of pPB-10 (Fig. 3A). The control transcript represents hybridization with radioactively labeled pC4 (Law and Segall 1988). (B) The *DIT* genes define a temporally distinct class of sporulation-specific genes. Poly(A)⁺ RNA purified from *MATa/MATa* cells growing vegetatively (VEG) and from cells at the indicated times after transfer to sporulation medium (SPO) was separated on an agarose gel and transferred to a nylon membrane. The blots were hybridized with radioactively labeled probes containing the *SPS1* and *SPS2* genes (p18; Percival-Smith and Segall 1986), the *DIT1* gene (a pEMBL19 derivative containing the yeast DNA insert of pPB-13), the *DIT2* gene (a pEMBL19 derivative containing the yeast DNA insert of pPB-12), the *SPS100* gene (pE18-B8a; Law and Segall 1988), and a control gene (pC4). Only the portions of the blots hybridizing with the probes are shown. (C) The *DIT* genes are expressed at the time of prospore enclosure and just prior to the time of ditryosine accumulation. *MATa/MATa* cells were examined at various times after transfer to sporulation medium. Appearance of immature (○) and mature asci (□) was monitored by light microscopy with phase-contrast optics. Poly(A)⁺ RNA was purified from aliquots of cells, and a blot was probed for *DIT2* transcripts as described above. The relative level of *DIT2* transcripts (●) was determined by densitometric scanning of an autoradiograph of the blot. Acid hydrolysates of aliquots of cells were analyzed by thin layer chromatography, and relative levels of ditryosine (■) were determined by densitometric scanning of a photograph of the chromatogram taken under UV illumination.

rated by 899 nucleotides. A potential TATA box consensus sequence is found 114 and 107 nucleotides upstream of the translational start codons of the *DIT1* gene

and the *DIT2* gene, respectively. We then mapped the transcriptional start sites by primer extension. The 5' ends of *DIT1* transcripts mapped to multiple sites 18–50 nucleotides upstream of the translational start codon; the 5' ends of *DIT2* transcripts mapped to sites 22 and 31 nucleotides upstream of the translational start codon.

The ORF corresponding to *DIT1* encodes an acidic 61.3-kD protein. The ORF corresponding to *DIT2* encodes a 56-kD protein with a hydrophobic amino terminus. We note that neither of the predicted proteins has a high content of tyrosine. This makes it unlikely that either of the *DIT* genes encodes the putative precursor to the ditryosine-containing macromolecule of the spore wall.

DIT2 is a member of the cytochrome P-450 superfamily

Comparison of the deduced amino acid sequences of *DIT1* and *DIT2* with the NBRF/PIR and SWISS-PROT data bases revealed that the *DIT2* gene product had significant homology with several members of the cytochrome P-450 superfamily. The highest homology noted for the predicted *DIT2* gene product was 24% identity with the bovine cytochrome P-450 XVIIA1 (17 α -hydroxylase/17,20 lyase; Zuber et al. 1986). Identity with the rat cytochrome P-450 IIIA1 [pregnenolone 16 α -carbonitrile-inducible (pcn1); Gonzalez et al. 1985], rat P-450 IIIA2 [(pcn2); Gonzalez et al. 1986], and human P-450 XIA1 [cholesterol side-chain cleavage (scc); Chung et al. 1986] was ~20%, whereas identity with the only previous known cytochrome P-450 of *S. cerevisiae* (P-450 LI, lanosterol 14 α -demethylase; Kalb et al. 1987) was only 12%.

Analysis of >70 cytochrome P-450s led to the grouping of these proteins into 15 distinct families (Nebert et al. 1989). Although the sequence homology between families is low, Kalb and Loper (1988) identified a region of ~170 amino acids conserved among all cytochromes. This region, present in the carboxy-terminal portion of the proteins, is divided into four domains of sequence similarity that are separated by short stretches of variable length. The consensus sequence derived for this region, which includes invariant, conserved, and variable amino acids, is presented in Figure 8. The high degree of identity observed between this sequence and the carboxy-terminal domain of the *DIT2* gene product (53%) confirmed that *DIT2* is indeed a member of the cytochrome P-450 superfamily (Fig. 8). The highest percentage of invariant residues among the P-450s occurs in the proximal cysteine-containing heme-binding region. The invariant cysteine of this domain, as well as several neighboring invariant residues, is present in the *DIT2* gene product (Phe-428, Cys-435, Gly-431, Gly-437). Although the invariant threonine residue of the distal heme-binding region, as well as the adjacent serine/threonine-rich region, is not found in the *DIT2* gene product, several other conserved amino acids of this region, in particular an invariant glycine (Gly-294), are present in *DIT2*. We propose that the *DIT2* gene defines a novel cytochrome P-450 that could be involved in cross-

Genes required for spore wall maturation

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 GGCGCCTTAA GGGACCTTCT ATGACAAATA TGGTGAAGTA TGCAACCTCA ATGAAGAGCA ATGAGAAAT
 -395
 TTAAGGGGTA AGTAAGCATC GGAATTTGTT GTTCTCTAAC AAATTTGCTA ATTACTACAA TAATATCAGG
 -325
 AGAATTGATC GAAAAAGCA AACAGGAAC CCTCACAA TAAGGAACA TAAAGTAAT GCTGCTCTT
 -255
 ACATACATGG CACTCAATCC CAGACGTGCG GTGCTAAAAA TCTTTATATT ATTGGCCOCT CAGGAGTTTA
 -185
 TTGAAATTTT GATTGCAATT CTTCAGTGG ACAGTATATC ATAAATTTTG CAAGGCGATA GTGCTGCCCC
 -115
 TACGATGTTG TAAACAATTT TCTGAAATA GTTTCAGMAT CAAAAATGAT GTATAATAT TGAAATAAAT
 -45
 TTTCACATAA ATTGTGCTCC TCCGCAAGT CTGACTAA TAAACATTT GTTAATATCC TAAITCGGTA
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 AAGCTTTGCT GAGACATTA CAAA
 51/1
 ATG ACA TTT ACT AGC AAC TTG CCC TCA AGC TCC GAG CAG TCA ATA TCA CCA CCT GCC TCT
 met-thr-phe-thr-ser-asn-leu-pro-ser-ser-ser-glu-gln-ser-ile-ser-pro-ala-ser
 111/21
 TCA TTT TCT TCA TCG ACT GAT ACG TTG AAA GAT ATT GAT ATT CCC CAT AAT GGG GCC GAT
 ser-phe-ser-ser-ser-thr-asp-thr-leu-lys-asp-ile-asp-ile-pro-his-asn-gly-ala-asp
 171/41
 TTG TCT ACA TAT AGT AAA TTT CTG GCC CTG TAT TCC AGA AGC GAC AAA TGT GAC GAT TTT
 leu-ser-thr-tyr-ser-lys-phe-leu-ala-leu-tyr-cys-arg-ser-asp-lys-cys-asp-asp-phe
 231/61
 TAC TCT TTA GAG GAA AAA CAG AAT TGT AAA TTT GGA GAC CAA TGG CTC GAT TTT ATC AAC
 tyr-ser-leu-glu-glu-lys-gln-asn-cys-lys-phe-gly-asp-gln-trp-leu-asp-phe-ile-asn
 291/81
 ACT ATT CAC AAT CTG GAC TTT TCT GAA TCC GAA GTA AGT GGA CGG GTT TCT GAA GAA ATT
 thr-ile-thr-tyr-ser-lys-phe-leu-ala-leu-tyr-cys-arg-ser-asp-lys-cys-asp-asp-phe
 351/101
 TTG CCA GCT TCT TTA CCA AAT AAA TTT ACC AAT AAT TTA GGA GTG CCA ATA AAG ATT TCT
 leu-pro-ala-ser-leu-ala-asn-lys-phe-thr-asn-asn-leu-gly-val-ala-ile-lys-ile-ser
 411/121
 GAA TAT ACA CGC GAT GAC GAA CGC CAG ATT CGT GGG TGT GTT ACA ACA GTT GAG AAT GAA
 glu-tyr-thr-arg-asp-asp-phe-ser-gln-ile-arg-lys-cys-val-thr-thr-val-glu-asn-glu
 471/141
 AAT TCT TTC AAT AAC TGG TTC GTA TAT CAT ATT TTA GAC CAA TCT CAA TTA TCT CTA AGT
 asn-ser-phe-asn-asn-trp-phe-val-tyr-his-ile-leu-asp-gln-ser-gln-leu-ser-leu-ser
 531/161
 GAA CAT CCA ATT GTA ACC AAA GAA GTT AAG TAT CAC GAA TTA TTT GCA GAT TTT TTT GAG
 glu-his-pro-ile-val-thr-lys-glu-val-lys-tyr-his-glu-leu-phe-ala-asp-phe-thr-glu
 591/181
 AAA AAT TTG AAA AAC ACA ATA GTT AAT GAT CAA TGG AAT TTT GGT GGC GGT GAT TAT TTT
 lys-asn-leu-lys-asn-thr-ile-val-asn-asp-gln-trp-asn-phe-gly-gly-arg-asp-tyr-phe
 651/201
 ATT GAA CGT TCA AGA TAT TTT ACC GAT CGA TAT TTG AGA ATT GAA TGC ATC TTG CCA CGG
 ile-glu-arg-ser-arg-tyr-phe-thr-asp-arg-tyr-leu-arg-ile-glu-cys-ile-leu-pro-ala
 711/221
 TTT CCA TGT AAG TCA TCT AAT GAG CAA AAA GTG TAC GGT TCC GTT CTT GAC AAA GGC GAA
 phe-pro-cys-lys-ser-ser-asn-glu-gln-lys-val-tyr-gly-ser-val-pro-asp-lys-gly-glu
 771/241
 GAA CTC GCT TTG AAA AGA TTA ATA AAA GGC ACA CAA GAC CTT GTC AAG ATA TAT CCA CCG
 glu-leu-ala-leu-lys-ile-lys-ala-thr-gln-asn-leu-val-lys-ile-tyr-pro-pro
 831/261
 GGT ACT AAA ATT TGG ATA GTT AGT GAT GGC CAT GTT TTC TCC GAT TGT ATT GGG GTC GAT
 gly-met-lys-ile-trp-ile-val-ser-asp-gly-his-val-phe-ser-asp-cys-ile-gly-val-asp
 891/281
 GAT GAC GTC GTG AGT ACT TAC ACG ACC AAA TTG CAC GAA CTG TAT AAA AGA GTG GCT ATA
 asp-asp-val-lys-ser-thr-tyr-thr-lys-leu-his-glu-leu-tyr-lys-arg-val-ala-ile
 951/301
 CCT GGT GTT GAC GCC ATT GGC TTT GTT GGA TTT GAT TTT TTT GAC GGT GCA CAC TAC ACA GCT
 pro-gly-val-asp-ala-ile-gly-phe-cys-gly-leu-asn-asp-leu-phe-phe-ser-gly-ala-ala
 1011/321
 AGT AAA GTT TTC GAT CCA AAG TGG GTT AGT GAT GTT GAA GTT GCA CAC TAC ACA GCT
 ser-lys-val-phe-asp-pro-lys-tyr-trp-val-ser-asp-val-glu-val-ala-his-tyr-thr-gly-thr
 1071/341
 CAA ACT TGT CCT AAG TCC GAT TTT TGG AGA GAT TTT GAT AAA GGC TGT GAT CAA GCT
 gln-ile-cys-pro-lys-ser-asp-leu-ser-arg-gln-ile-leu-met-lys-gly-cys-asp-thr-asp
 1131/361
 GCA GGT GGT TTG AGA AAG CAG ATT CCA ATA GAA GGA CAT CCA AGA TTG CAT CTG TAT AGG
 ala-gly-arg-leu-arg-lys-gln-ile-ala-ile-glu-gly-his-pro-arg-leu-his-leu-tyr-arg
 1191/381
 GGC TTT TCA GGT TTT ATG ATG GAA GAT TTA TCT CTA CTG GAA CAT TTT CAA GTT TAT TCC
 gly-phe-ser-arg-phe-met-met-glu-asp-leu-ser-leu-leu-glu-his-phe-gln-ser-tyr-ser
 1251/401
 AGA AAA AAA TTC AAG AAA ATC ATT TCA ATG ATC GCT TTT AAC ATG ATT AAG AGA AAT GAC
 arg-lys-lys-phe-lys-lys-ile-ile-ser-met-ile-ala-phe-asn-met-ile-lys-arg-asn-asp
 1311/421
 GCG TAT TGG AAC TTA GTG GAA TTG ATA TTC CCT CAT TTG AGA ATT TCT ATT CAT CCG
 ala-tyr-ser-asn-leu-val-glu-leu-ile-phe-pro-his-his-leu-arg-ile-ser-ile-his-ala
 1371/441
 CAC ACT AAC AGC GCG CCC AAA TTT GGT ATA AAA GTA ATC TCC AAC GAA CAG TGT TCT ATT
 his-thr-asn-ser-gly-pro-lys-phe-gly-ile-lys-val-ile-ser-asn-glu-gln-cys-ser-ile
 1431/461
 GGT AGT TGG TTA GAA GAC CTT GAT GAA CCC AAA TTT GAA GAT TTT TTA CAT ATT CCC ACA
 val-ser-leu-leu-glu-asp-leu-asp-glu-pro-lys-phe-glu-asp-phe-leu-his-ile-pro-thr
 1491/481
 CCT TGG CAT AAT TGT GTC GTG AAG GTT GAG GAT GAA AAG GAG AAA TAC TTT TTG ACA AAA
 pro-trp-his-asn-cys-val-val-lys-val-glu-asp-glu-lys-glu-lys-tyr-phe-leu-thr-lys
 1551/501
 TCA AAA GTA GTC AAG GAG GCT CTC GAA AAG GGT ATG TAT GAT GGT GTA TGG AAA GAT ACT
 ser-lys-val-val-lys-glu-ala-leu-gly-met-tyr-asp-gly-val-trp-lys-asp-thr
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 CGT TTC GAT ATT GGA GAA GGA GGA CAT TTC GTT ATC AAG AAA ATC TCT TAA
 arg-phe-asp-ile-gly-glu-gly-gly-his-phe-val-ile-lys-lys-ile-ser-STOP
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Expression of the *DIT1* and *DIT2* genes at the time of prospore enclosure could readily account for the spore-autonomous behavior of these genes. The lack of complementation between sister spores would result from synthesis of the *DIT* gene products within the maturing spore at a time when free exchange of macromolecules between spores is restricted. This suggestion of synthesis of spore wall components within the developing spore is consistent with the observation of Lynn and Magee (1970) that endoplasmic reticulum is found apposed to the prospore wall within the immature spore. Kurtz and Lindquist (1986) noted that some sporulation-specific transcripts that are synthesized during ascus formation and are presumed to encode spore wall proteins are selectively localized to the ascus cytoplasm. As genes encoding such transcripts would not be expected to behave in a spore autonomous manner, we presume

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that the transcripts encoded by the *DIT1* and *DIT2* genes do not belong to this class of RNAs. On the other hand, transcripts encoded by the genes represented by *dit101* and *dit105*, which behave as recessive mutations, may belong to this class. Alternatively, the transcripts may accumulate prior to enclosure of the newly formed nuclei by the prospore wall, thereby allowing complementation between sister spores.

The spore autonomous behavior of the *DIT2* gene, which we postulate encodes an enzyme involved in the cross-linking of tyrosine residues (see below), is somewhat surprising as it is unlikely that the cross-linking would occur within the prospore prior to deposition of the presumptive tyrosine-rich macromolecule on the spore surface. As the predicted *DIT2* gene product has a hydrophobic amino-terminal domain, it is possible that the enzyme is present on the surface of the spore but remains membrane-bound such that its activity is restricted to the spore of origin. Most cytochrome P-450s are membrane-bound, being localized to the endoplasmic reticulum or mitochondria.

We presume that the protective nature of the spore wall derives both from the resistance of the highly cross-linked macromolecular network present on the surface of the spore to proteolytic digestion (Briza et al. 1990) and to the impermeable nature of this surface layer, as exemplified by exclusion of primulin and ether. Previous studies have indicated that treatment of purified spore walls with Glusulase leads to digestion of the inner glucan- and mannan-containing layers with both surface layers remaining intact (Briza et al. 1986). Our data now establish that it is the outermost layer of the spore wall that is responsible for the resistance of spores to Glusulase. The observation that the spore wall is also responsible for ether resistance supports the previous suggestion that the *SPS100* gene encodes a protein involved in spore wall maturation (Law and Segall 1988). It is unclear why the absence of the outermost layer of the spore wall would reduce the heat resistance of the spores. We speculate that a reduction in stability of the spore walls resulting from the absence of the cross-linked surface layer, as well as an increase in permeability of the spores, could lead indirectly to reduced heat resistance.

The correlation of expression of the *DIT1* and *DIT2* genes with dityrosine synthesis, as well as the fact that the homozygous mutants formed spores that were totally devoid of dityrosine, suggests that the *DIT1* and *DIT2* genes may be directly involved in dityrosine synthesis. The only known mechanism for the cross-linking of tyrosine residues is by a peroxidative reaction (Foerder and Shapiro 1977). Because *DIT2* was found to have significant homology with members of the cytochrome P-450 superfamily, we propose that cross-linking of the tyrosine residues in the yeast spore walls occurs via a novel cytochrome P-450-catalyzed oxidation. The cytochrome P-450 family is a group of >100 heme-containing monooxygenases that act predominantly as hydroxylases on a large variety of substrates. The greatest homology among cytochrome P-450s is found within the

region encompassing the two α -helices involved in heme binding. *DIT2* contains all of the highly conserved amino acids suggested to be directly involved in heme binding (Poulos et al. 1985). However, a threonine residue that is conserved in the distal heme-binding helix of all but one P-450 (Kalb and Loper 1988) is replaced by an asparagine residue in *DIT2* (Asn-297) and is followed by a proline residue (Pro-298). The threonine residue plays a crucial role in P-450 activity, distorting the distal helix above the heme (Poulos et al. 1985) and contributing to substrate binding (Imai and Nakamura 1988) and probably to the scission of the oxygen-oxygen bond (Imai et al. 1989). Comparison of the secondary structures of *DIT2*, rat P-450pcn1, rat P-450pcn2, and human P-450scc, as predicted by the algorithm of Garnier et al. (1978), suggests that Pro-298 in *DIT2* could serve a role similar to that of the conserved threonine residue in destabilizing the distal helix. The Asn-297 residue in *DIT2* may reflect adaptation of the cytochrome to a novel enzymatic activity. Interestingly, P-450s with the highest overall homology to *DIT2* have somewhat unusual activities. P-450scc and P-450 17 α hydroxylase/17,20 lyase have carbon-carbon bond cleavage activity in addition to hydroxylation activity (Ortiz de Montellano 1986), and P-450pcn1 and P-450pcn2 catalyze the dehydrogenation of 1,4-dihydropyridines. The suggested mechanisms of action for these P-450s can be readily adapted for dityrosine synthesis. Oxidation of 1,4-dihydropyridines involves radical formation at the heteroatom and subsequent rearrangement of covalent bonds leading to the formation of the final reaction products (Guengerich and Böcker 1988). A similar mechanism has been proposed for cholesterol side-chain cleavage by P-450scc (Ortiz de Montellano 1986). These studies indicate that P-450s can perform reactions of the type suggested by Gross and Sizer (1959) for the formation of dityrosine by a peroxidative reaction. Although, by definition, peroxidases do not use dioxygen directly, a cytochrome P-450 could produce the same phenoxy radical that is generated by a peroxidase. Ortiz de Montellano (1986) also suggested an alternative mechanism for the side-chain cleavage reaction of cholesterol that can also be easily adapted to dityrosine synthesis, which involves the addition of a hydroxyl group to the activated oxygen.

Consistent with the absence of the chitosan layer in spores of the strain containing the *dit101* mutation, recent experiments have indicated that this strain lacks chitin synthase III (C. Bulawa, M. Pammer, and M. Breitenbach, unpubl.). Chitin is thought to be the precursor to the spore wall chitosan (Briza et al. 1988). We presume that the absence of the chitosan layer in spores of the strain containing the *dit101* mutation and in spores of glucosamine auxotrophs (Ballou et al. 1977; Briza et al. 1988) indirectly prevents the deposition of the outermost dityrosine-containing layer. These mutants accumulate a low level of dityrosine in their spores (Briza et al. 1990). We do not yet know the function of the *DIT1* gene or the gene represented by the *dit105* allele. Potential roles for *Dit* gene products include a reductase that acts with *DIT2* in cross-linking tyrosine, a

racemase involved in catalyzing the formation of the D-tyrosine present in the spore wall dityrosine, products involved in establishing putative intermolecular interactions between the chitosan layer and the surface layer of the spore wall, and a putative tyrosine-rich precursor to the dityrosine-containing macromolecule. As the dityrosine-containing macromolecule has properties similar to a bacterial peptidoglycan (Briza et al. 1990), it is also possible that a *Dit* gene encodes an enzyme responsible for the nonribosomal incorporation of tyrosine into a peptidic structure. It will be interesting to determine whether a soluble or spore surface-bound and tyrosine-rich polypeptide accumulates in the *Dit*⁻ mutants.

Materials and methods

Strains and culture conditions

The haploid meiosis strain PB2-1C (*MATα spo13-1 sir4^{ts} trp1-1 leu2 his6*) (Briza 1987) was used for the isolation of mutants. SHU32 (*MATα leu2 trp1 ura3*) was used to mate with strains containing the original *dit* alleles to obtain diploids for the initial electron microscopic analyses of heterozygotes (Fig. 2). The strains used for the construction of disruption alleles of *DIT1* and *DIT2* were W3031A-H (*MATα can1-100 leu2-3,112 trp1-1 ura3-1 ade2-1*) and W3031B-T (*MATα can1-100 his3-11 leu2-3,112 ura3-1 ade2-1*) (Law and Segall 1988). Cells were sporulated on plates containing 1% potassium acetate, 0.1% yeast extract, and 0.025% glucose after growth on YEPD plates (1% yeast extract, 2% peptone, 2% glucose) or in liquid medium (1% potassium acetate with appropriate supplements) after growth in YEPA medium (1% yeast extract, 2% peptone, 1% potassium acetate) as described (Law and Segall 1988). The sporulation temperature was 23°C for PB2-1C and 30°C for all other strains. Ascus formation was monitored by light microscopy, using phase-contrast optics. The efficiency of sporulation of PB2-1C and the diploid strain LP-HT, obtained by mating W3031A-H and W3031B-T, was ~25% and 70%, respectively.

Screen for spore wall mutants

Cells of the strain PB2-1C mutagenized by exposure to ethyl methanesulfonate (EMS) (reducing viability to 6.5%) or UV irradiation (reducing viability to 12.5%) were plated on YEPD. After the survivors had grown up at 30°C, colonies were patched onto YEPD plates and grown for 1 day at 30°C. The colonies were replica-plated onto sporulation medium (master SPO plate) and onto nitrocellulose filters on YEPD plates. After 1 day of incubation at 30°C, the nitrocellulose filters were transferred to sporulation medium and incubated for 3–4 days at 23°C. The sporulated colonies were then examined for dityrosine fluorescence as follows. First, as visualization of the fluorescence of the dityrosine present in spore walls requires prior removal of the outer ascus sac, the colonies were treated with Glusulase by transferring each filter to a 9-cm petri dish containing a mixture of 200 μl of H₂O, 70 μl of Glusulase, and 15 μl of β-mercaptoethanol. After 5 hr at 30°C, each filter was transferred to a dish containing 300 μl of 30% NH₃. The NH₃ solution provides the basic pH required for the fluorescence of dityrosine. The nitrocellulose filters were then exposed to UV light (302 nm) and photographed with Polaroid 667 film and a blue filter (Kodak Wratten filter, 390–450 nm) to obtain a record of nonfluorescent colonies. Approximately 10–15% of the colonies were nonfluorescent. The appropriate colonies on the master SPO plates were then examined microscopically by

using phase-contrast optics to determine which of the non-fluorescent mutants had formed spores. Most of the nonfluorescent colonies were asporogenous. Acid hydrolysates of those mutants that appeared to form nonfluorescent spores were then analyzed by thin layer chromatography to qualitatively assess the amount of dityrosine. If no dityrosine was detected despite ascus formation, colonies were resporulated and the dityrosine content was measured by fluorescence spectroscopy of HPLC-purified material [see below]. In this way, seven mutant strains (containing the mutations denoted *dit101–dit105*, *dit107*, and *dit108*) that formed dityrosine-deficient ascospores were obtained on examination of 10⁴ EMS-treated cells. Each of these mutants was derived from an independently mutagenized pool of cells. One putative spore wall mutant (containing the mutation denoted *dit106*) was obtained on examination of 3 × 10³ UV-treated cells.

Determination of dityrosine

Detection of dityrosine by thin layer chromatography and HPLC was as described (Briza et al. 1986). To quantitate the amount of dityrosine, 10⁷ cells of a sporulated culture were subjected to acid hydrolysis. These cells had not been treated with Glusulase. The hydrolysate was subjected to HPLC fractionation (Polygosil C₁₈ column, acetonitrile gradient in 0.01 M trifluoroacetic acid), and the fractions presumed to contain dityrosine were analyzed by fluorescence spectrophotometry as follows. After dilution of the samples 1 : 1 with 30% ammonia, fluorescence excitation spectra were recorded on a Hitachi F-3000 fluorescence spectrophotometer. The emission wavelength was 420 nm, and the excitation wavelength was from 270 to 370 nm. The spectra identified dityrosine-containing fractions and allowed quantitation of the amount of dityrosine. The putative dityrosine-containing fractions in the HPLC-derived fractions of the hydrolysates of mutant cells were identified by comparison with the elution position of dityrosine in the hydrolysate of wild-type cells.

Cloning DIT genes by complementation

Strains containing the *dit102*, *dit103*, *dit104*, or *dit106* alleles were transformed with a yeast DNA library, consisting of a partial *Sau3A* digest of genomic DNA cloned in YEpl3, constructed by Nasmyth and Tatchell (1980). Transformation of spheroplasts generated by Glusulase treatment was as described by Sherman et al. (1986). After the transformants had grown up on regeneration plates, the cells from ~3000 colonies were pooled and sporulated on plates as a lawn. After sporulation, cells were recovered from the plate and incubated at 30°C for 12 hr in 5 ml 0.1 M sodium phosphate (pH 5) containing 50 μl Glusulase and 20 μl β-mercaptoethanol. This step leads to lysis of most vegetative cells and spores of the *Dit*⁻ mutants. Survivors were then grown up on selective medium, sporulated, and tested for dityrosine fluorescence, as outlined above in the mutant screening procedure.

Nucleic acid procedures

RNA was purified from yeast cells, and Northern analyses were performed as described (Percival-Smith and Segall 1984). Standard DNA manipulations were carried out by procedures described previously (Maniatis et al. 1982). Genomic and plasmid DNAs were isolated from yeast according to Sherman et al. (1986). Yeast DNA fragments to be tested for complementation were subcloned in YEpl3. For sequencing, various restriction

fragments from the 6-kb yeast DNA insert of pPB-10 were cloned into pEMBL18 and pEMBL19. Unidirectional overlapping deletions were generated by using exonuclease III and S1 nuclease (Henikoff 1984). The sequence of both strands of the *DIT1-DIT2* region was determined by the method of Sanger et al. (1977) with Sequenase [U.S. Biochemical Corp.]. We compared the deduced amino acid sequences of *DIT1* and *DIT2* with the NBRF/PIR and SWISS-PROT data bases, using the FASTA program of Pearson and Lipman (1988) and the BIONET network (National Institutes of Health grant P41RR01685).

The one-step gene-disruption procedure of Rothstein (1983) was used to introduce mutations into the *DIT1* and *DIT2* genes as follows. First, the yeast DNA inserts of pPB-13 and pPB-12 (Fig. 3A) were subcloned into pEMBL19, generating pPB-15E and pPB-22E, respectively. A 1.2-kb *HindIII* fragment containing the *URA3* gene was isolated; after the ends had been filled in with DNA polymerase I, the fragment was cloned into the *BalI* site present within the coding region of the *DIT1* gene present in pPB-15E, generating pPB-43E. pPB-43E DNA digested with *MluI* and *HindIII* was used to transform the yeast strains W3031A-H and W3031B-T to *Ura*⁺. The *URA3* gene was similarly cloned into the *BalI* site present in the coding region of the *DIT2* gene, which was present in pPB-22E, generating pPB-42E. pPB-42E DNA digested with *HindIII* and *EcoRI* was used to transform W3031A-H and W3031B-T. The appropriate *MATa* and *MATα* strains containing the *dit1 :: URA3* alleles or *dit2 :: URA3* alleles were mated to obtain homozygous mutant diploid strains.

The 5' ends of the *DIT1* and *DIT2* transcripts were determined by primer extension, using the protocol described by Fouser and Friesen (1986). RNA isolated from *MATa/MATα* cells at 12 hr after transfer to sporulation medium was hybridized with synthetic oligonucleotides corresponding to +93 to +117 of the *DIT1* sequence and to +168 to +191 of the *DIT2* sequence (Fig. 7). The extended primers were run on sequencing gels alongside dideoxy chain-termination sequencing reactions that had been performed using the same primers hybridized to the appropriate DNA fragment.

Electron microscopy and primulin staining

Cells were prepared for electron microscopic examination by fixation and staining with glutaraldehyde, osmium tetroxide, and uranyl acetate as described in Briza et al. (1988). These procedures were optimized to allow visualization of the multi-layered spore walls. Cells were treated with primulin and examined by fluorescence microscopy as described (Briza et al. 1988).

Assays for resistance of spores to Glusulase, ether, and heat

Resistance was determined for stationary phase cells that had been in YEPD medium for 36 hr and for cells 72 hr after transfer to sporulation medium. Glusulase resistance was assessed by resuspension of cells in undiluted Glusulase (Sigma type H-2) at room temperature in a hemocytometer chamber and monitoring of cell lysis by light microscopic examination. The number of surviving asci and spores was counted at various times. Resistance of cells to diethyl ether was assessed as described in Law and Segall (1988). To determine heat resistance, cell cultures were kept at 4°C for 12 hr and diluted to a density of 3×10^6 cells/ml water. One milliliter of the cell suspension was added to an Eppendorf tube, which was then immersed in a 55.5°C circulating water bath. At various times, aliquots were taken and plated on YEPD plates.

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Note added in proof

Sequence data described in this paper have been submitted to the EMBL Data Library. The P450 Gene Nomenclature Committee has proposed that *CYP56* be the official designation for *DIT2*.

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