CHARACTERIZATION OF SPORULATION-SPECIFIC GENES OF SACCHAROMYCES CEREVISIAE

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ABSTRACT

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In response to nutrient deprivation, **a**/α diploid strains of yeast *Saccharomyces cerevisiae* undergo meiosis and spore formation. During sporulation, several sets of sporulation-specific genes (*SPR* genes) are specifically transcribed, but their roles in sporulation are not clear. In this study, one sporulation-specific gene isolated previously by differential hybridization, *SPR9*, was characterized molecularly and physiologically. *SPR9* was transcribed 4 hours after transfer to sporulation medium and the transcript lasted at least 20 hours in the sporulation medium. The absence of *SPR9* gene products did not block ascus formation; instead the mutant spores germinated less efficiently than the wild-type spores at high temperature. The protein predicted from the nucleotide sequence of *SPR9* was 16.8 kD, and no homologous sequences have been found in a computer search. Two other abundantly transcribed *SPR* genes, *SPR1* and *SPR2*, were studied to identify their phenotypes. Both mutant strains produced viable spores, but *spr1* mutant spores were less heat-resistant than the wild-type spores. There was no difference detected between *spr2* mutant spores and wild-type spores under the conditions used in this study. The double mutants,

spr1/9 and *spr2/9*, were constructed to test for interaction among these three genes. The results indicate that the *spr1/9* double mutant spores were very delicate and were very sensitive to heat, while the *spr2/9* mutant spores were less sensitive to heat than *spr1/9* spores but more sensitive to heat than the *spr9* mutant spores. These observations suggest that the *SPR1* and *SPR9* gene products are involved in thermotolerance and germination of spores.

To my parents

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Literature Survey

1.1 A short history of the yeast *Saccharomyces cerevisiae*

1.1.1 Yeast as a model system for studying developmental processes of eukaryotic cells

In eukaryotes, the mechanisms involved in the sequential activation of genes during differentiation and development are not well understood. Among the eukaryotic systems currently in use, *S. cerevisiae* is one of the most amenable systems for this kind of study. The chromosome behavior of *S. cerevisiae* is typical of higher eukaryotic cells, while it possesses all of the technical advantages of microbial systems. The landmark events of the sporulation process of *S. cerevisiae* have been well defined genetically, morphologically, and biochemically (Esposito and Klapholz, 1981). The recovery of all four meiotic products and the presence of well-characterized mitotic recombination events provide useful probes to analyze the interactions among gene functions involved in meiotic recombination and the mitotic process. Yeast is also easy to manipulate experimentally. Site-specific mutations can be easily created by gene disruption and replacement techniques. Large numbers of single cells can be stimulated to undergo meiosis in response to a simple change in medium. Meiosis can be interrupted and viable cells recovered at various stages of development. Another important feature of using yeast as a model system is that there are many mutants of meiosis, cell cycle division, and metabolism available to be used in different studies.

1.1.2 The life cycle of yeast

S. cerevisiae can grow vegetatively by budding as a haploid cell or as a diploid cell. There are three distinct cell types of *S. cerevisiae*: **a** haploid, α haploid, and **a**/α diploid (Figure 1.1). The type of any cell is determined by alleles, **a** or α, at the mating type locus, *MAT* (Herskowitz and Oshima, 1981). This *MAT* locus controls a variety of cellular processes including agglutination, phermone production, mating, and sporulation.

The **a** haploid has the **a** allele at the *MAT* locus, whereas the α haploid has the α allele at the same locus. When haploids of opposite mating type meet, they will mate efficiently under the direction of the mating phermones, **a**-factor and α-factor, produced by **a**-haploid and α-haploid respectively. The mating phermones secreted by an **a**- or α-haploid can cause the cells of opposite mating type to arrest at the G1 stage of the cell cycle, to undergo morphological and physiological changes, to agglutinate, and then to fuse with the cells of opposite mating type to form a diploid zygote containing both **a** and α alleles at the mating type locus (Thorner, 1981). The **a**/α diploid formed can grow mitotically, undergo sporulation to produce two **a**-ascospores and two α-ascospores, but can no longer mate. Diploid cells that are homozygous at the mating type locus are asporogenous but they can still mate. Diploids homozygous and heterozygous at the *MAT* locus differ in their response to sporulation medium, providing substantial information about sporulation-specific events: **a**/**a** behaves as **a**, and α/α behaves as α; hence the gene dosage of the mating type loci is unimportant. Therefore, even higher ploidy strains can be constructed.

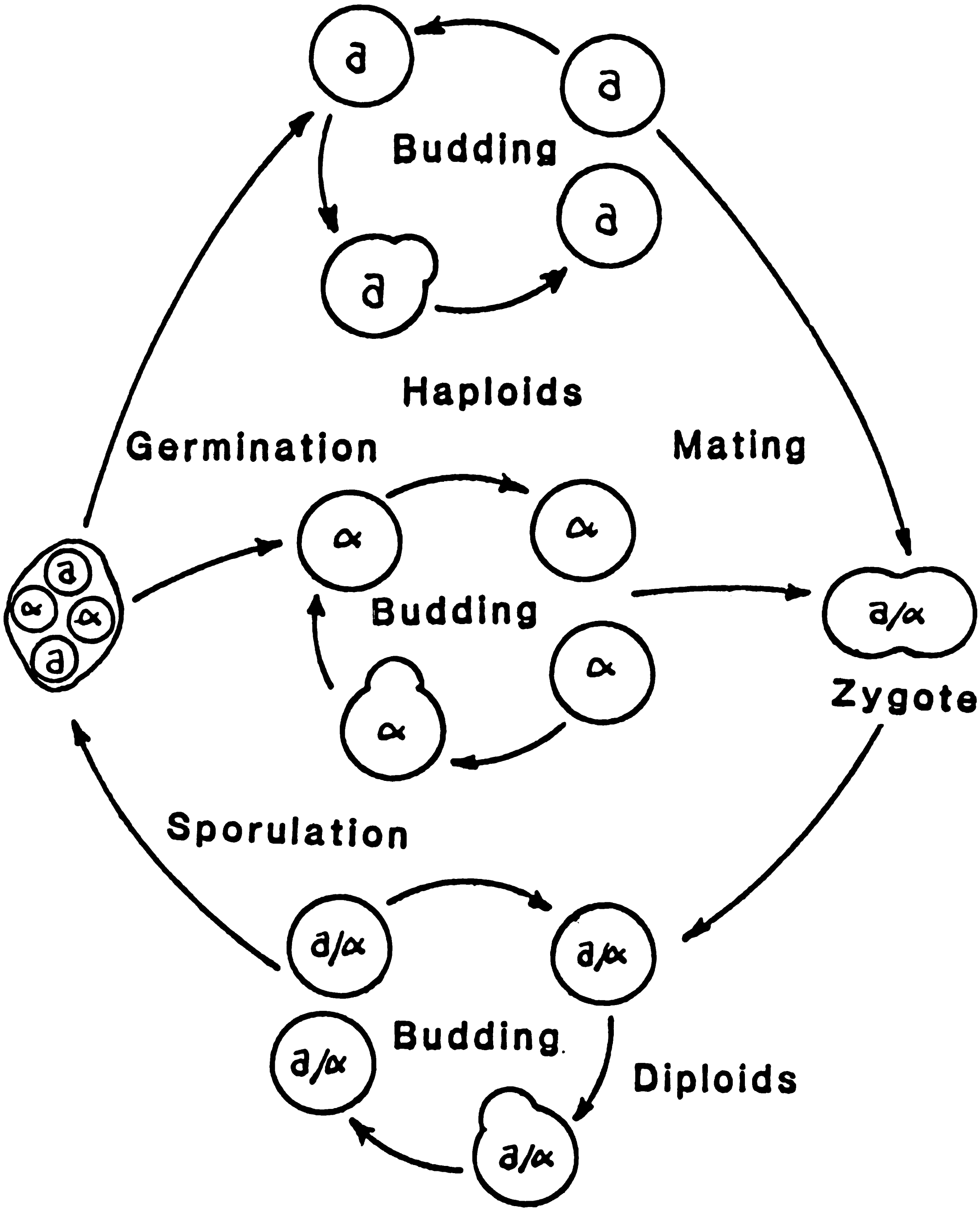


Figure 1.1: The life cycle of *S. cerevisiae.*

1.2 Events in sporulation

Meiosis and spore formation in yeast are generally triggered by deprivation of a source of nitrogen and the presence of nonfermentable carbon source (Olempska-Beer, 1987). These processes are coordinately regulated and are accompanied by major biochemical and morphological changes. During sporulation, considerable macromolecular synthesis and degradation occur (Hopper et al., 1974; Illingworth et al., 1978; Kraig and Haber, 1980). Within several hours after transfer to sporulation medium, vegetatively growing cells will complete their mitotic cell cycle and arrest in G1 phase (Esposito and Esposito, 1974) in order to initiate meiosis and spore formation.

1.2.1 Three developmental stages in sporulating cells

Three developmental stages have been described with respect to commitment to sporulation (Simchen et al., 1972). During the first stage, cells achieve "readiness" which is defined as the period when cells after exposure to sporulation medium are able to sporulate in water without further stimulus by the medium or revert to vegetative growth if transferred back to growth medium. Readiness begins 3 hours after exposure to sporulation medium and then lasts for about 4 hours. At this point, the cells are not yet irreversibly committed to sporulation. The second stage, "precommitment", is very brief and is detectable after about 7 hours in sporulation medium. During precommitment, cells exhibit readiness but are thought to lose their ability either to return to vegetative growth or to sporulate in nutrient medium. The third stage, "commitment to sporulation", begins at 7 to 8 hours incubation in sporulation medium. At this stage, cells sporulate even when challenged with growth medium. Irreversible commitment to sporulation occurs after premeiotic

DNA replication and initiation of commitment to genetic recombination, coincident with SPB (spindle pole body) separation at pachytene of meiosis.

1.2.2 Morphological development during sporulation

After arresting at G1 phase, the cells gradually change their shapes from elongated to round, increase in size, and eventually form tetranucleate asci. During sporulation, the chromosomes are replicated and recombine, and two meiotic divisions occur to yield four haploid nuclei. Toward the end of the second meiotic division, the nuclear membrane buds at each of the four poles, and a prospore wall initiates development at each nuclear lobe (Moens, 1971). The inner membrane of the prospore-wall becomes the spore plasmalemma, the outer membrane apparently contributing to the surface protein layer of the spore (Lynn and Magee, 1970; Illingworth et al., 1973; Beckett et al., 1973). At prospore-wall closure, four ascospores with nucleus are formed.

During sporulation, each cell contains only one mitochondrion,which is highly branched and extends throughout much of the cytoplasm. As prospore-wall formation begins, the single mitochondrion, which surrounds the parental nucleus (Stevens, 1978), is distributed into the ascospores in development. A significant portion of the mitochondrion, however, is not incorporated into the ascospores and remains behind in the ascus cytoplasm. The distribution of the mitochondrion into the ascospores is apparently a nonrandom process.

1.2.3 Macromolecular synthesis and breakdown during sporulation

DNA metabolism

When **a**/α diploids are transferred to sporulation medium, there is a small initial increase in incorporation of precursors into DNA during the first 2 hours as cells complete mitotic rounds of replication. The DNA content per sporulating cell then doubles during a discrete period beginning at 4–6 hours and ending at 8–10 hours (Roth and Lusnak 1970). In general, the increase in DNA content in a culture is proportional to the number of sporulating cells in the culture. The precursors for premeiotic replication may be derived primarily from the extensive turnover of RNA during sporulation (Simchen et al., 1972).

Since there are events that occur in meiosis but not in mitosis, for example chromosome pairing and genetic exchange, it is possible that there are some unique gene functions for premeiotic replication that are nonessential for mitotic replication. From studies of *cdc* and *spo* mutants, it is suggested that mitotic and meiotic DNA replication are under separate genetic control but share some functions in common (Esposito and Esposito, 1978). In addition, the association of replication with other landmark events also differs in meiosis and mitosis.

RNA metabolism

There is a general increase in RNA synthesis immediately after induction of sporulation, and all classes of RNA are made during sporulation. Most of the synthesis occurs between 4 and 10 hours, reaching a maximum at 6 hours, regardless of the sporulation regimen (Hopper et al., 1974). The RNA polymerase activities have also been reported to change during sporulation (Magee, 1974). Extensive RNA degradation was observed along with RNA synthesis (Croes, 1967; Hopper et al., 1974). A rise in the level of RNase within the first 8–12 hours after transfer to sporulation medium, specific for **a**/α sporulation-competent diploids, has also been reported (Tsuboi, 1976). Therefore, the total amount of RNA per cell appears to decline at the end of sporulation. In **a**/α diploids, approximately 50–70% of total preexisting RNA is degraded, whereas in **a**/**a** and α/α cells, about half of that amount is broken down. These data suggest that at least a portion of the RNA degradation is sporulation-specific (Hopper et al., 1974).

Protein metabolism

Protein synthesis is necessary for the occurrence of most major events of sporulation (Magee and Hopper, 1974). There is a 10–35% increase in net amount of cellular protein during the first 6–10 hours of sporulation, and the net amount of cellular protein declines thereafter (Peterson et al., 1979). The protein patterns produced by one-dimensional and two-dimensional gel analyses showed differences between the vegetative state and the sporulating state of **a**/α diploids. When the protein patterns of asporogenous **a**/**a** cells and α/α cells were compared to **a**/α cells in the same state, there was no detectable difference (Kraig and Haber ,1980). However, several proteins have been detected which have differences in activities and in abundance between vegetative and sporulating states (Ballou et al., 1977; Colonna and Magee, 1978). A number of explanations have been proposed for the failure to find sporulation-specific proteins (Hopper and Hall, 1975; Peterson et al., 1979). All suggest that only a few crucial genes that require both *MAT***a** and α for expression exist and that the products of these genes may be too few or too low in concentration to detect. Alternatively, such proteins may have poor solubility , extremely low isoelectric points, or low molecular weights, rendering them undetectable by the techniques used currently. One activity that may truly be unique to **a**/α sporulating cells is an α-1,4-glucosidase (Colonna and Magee, 1978). There is no activity in vegetative cells or in sporulating **a**/α cells prior to 8 hours and only trace levels of activity in α/α cells at any time during incubation in sporulation medium.

The increase in protein content is not specific to **a**/α cells (Hopper et al., 1974). Extensive degradation of vegetative proteins occurs during sporulation (Betz and Weiser, 1976; Betz, 1977), and ranges from 25–70% in different studies. During sporulation, there is an increase in the activities of three proteinases: proteinase A, B, and C, and this is **a**/α specific (Klar and Halvorson, 1975; Betz and Weiser, 1976). No new proteinase activities have been observed during sporulation (Klar and Halvorson, 1975). Diploids homozygous for a mutation that results in a low level of proteinase-A activity are unable to complete sporulation (Betz, 1975). Diploids defective at the structural gene of proteinase-B can complete sporulation but form asci that are abnormal in appearance (Zubenko et al., 1979; Zubenko and Jones, 1981). However, diploids defective at the *PRC1-1* locus have no proteinase-C activity but can sporulate normally (Wolf and Fink, 1975). The increase in proteinase C is not significant during sporulation (Betz and Weiser, 1976). Zubenko and Jones (1981) have shown that proteinases B and C are responsible for approximately half of the total amount of protein degradation during sporulation. Both proteinase and ribonuclease activities have been localized to the yeast vacuole (Hasilik et al., 1974), which undergoes extensive fragmentation both in **a**/α and **a**/**a** cells (Trew et al., 1979).

Carbohydrate metabolism

Sporulation is accompanied by the extensive accumulation of intracellular carbohydrates, including trehalose and glycogen as reserve materials and mannan and glucan as the major carbohydrate components of yeast cell wall. The carbohydrates are synthesized to the same extent in both sporulating and non-sporulating cells after transfer to sporulation medium and are considered to be a response to starvation conditions (Kane and Roth, 1974); however only sporulating cells exhibit a period of glycogen degradation coinciding with the appearance of mature spores. The enzyme which degrades glycogen, glucoamylase, appears specifically during sporulation.

The accumulation of carbohydrates causes a dramatic increase (70%) in dry weight to occur early in sporulation (Croes, 1967; Esposito et al., 1969). Trehalose, which can be found in ascospores, is synthesized continuously during sporulation, rising to a level approximately tenfold higher than that in vegetative cells (Kane and Roth, 1974). At present, only trehalose accumulation has been shown to be required for ascus production, as mutants defective in its accumulation do not sporulate (Lillie and Pringle, 1980). Substantial degradation of glycogen specific to $\mathbf{a}/\alpha$ cells takes place late in sporulation, during the appearance of asci (Hopper et al., 1974; Kane and Roth, 1974). There are also other data that suggest that the activity responsible for glycogen degradation is sporulation-specific. For example, glycogen degradation depends upon new protein synthesis during sporulation (Magee and Hopper, 1974) and is inhibited by the addition of nitrogen (Opheim, 1979). During this same period in $\mathbf{a}/\alpha$ cells, a tenfold increase in specific alkaline phosphatase activity occurs, which may be involved in the metabolism of phosphorylated sugars to glucose (Fonzi et al., 1979). These two events are thought to be responsible for the accumulation of glucose during the late stage of sporulation.

Lipid metabolism

Cytological studies demonstrate the accumulation of lipid vesicles in the cytoplasm during sporulation and they play a crucial role in spore-wall development. Biochemical studies have also shown that extensive lipid synthesis takes place during sporulation (Esposito et al., 1969; Illingworth et al., 1973). There are two different periods for lipid synthesis during sporulation: the first period occurs between 0–10 hour and is not specific for **a**/α cells; the second period occurring after 20 hours in sporulation medium, lasting for 5–6 hours, coincides with the time of the first appearance of mature asci, and is specific to **a**/α cells and consists almost entirely of neutral lipid synthesis.

1.3 The regulation of sporulation

1.3.1 Control of sporulation

In the yeast *Saccharomyces cerevisiae*, there are two parallel regulatory paths that govern entry into meiosis. They are the environmental signal, starvation, heterozygosity at *MAT*, which causes arrest of the mitotic cell division cycle at G1 phase, and the genetic signal, *MAT*a1 and α2 (mating type locus), which activate the meiotic pathway (Mitchell, 1988). These two regulatory paths converge at some point to activate those sporulation-specific genes that are expressed only in **a**/α diploids during starvation. However, it should be noted that a very low, but detectable, fraction of **a**/**a** and α/α cells sporulates (Zakharov and Kozhira, 1967).

There are several genes involved in nutritional control of sporulation; they are *CYR1* (adenylate cyclase [Matsumoto et al., 1983]), *RAS1* and *RAS2* (stimulators of adenylate cyclase [Toda et al., 1985]), and genes encoding cAMP-dependent protein kinase (Toda et al., 1987). cAMP-dependent protein kinase inhibits the entry of cells into meiosis, but starvation can inhibit cAMP accumulation and thus inhibit the activity of the protein kinase (Tatchell, 1986). Glucose and other sugars repress sporulation by the suppression of gluconeogenic enzymes (Miyake et al., 1971).

The regulation of sporulation by the *MAT* locus is mediated by the *RME1* (regulator of meiosis) gene product. *RME1* is expressed in haploid cell, its product inhibits meiosis. In **a**/α diploids, **a**1-α2 negatively regulates *RME1* expression and thus leads to derepression of meiosis and spore formation (Mitchell and Herskowitz, 1986). Two sporulation-specific genes, *IME1* (inducer of meiosis) and *IME2*, are essential for meiosis and sporulation. *MAT***a**1-α2 may activate the expression of sporulation-specific genes through expression of *IME1* (Kassir et al, 1988). Smith and Mitchell (1989) recently proposed that the main role of *IME1* is to activate the expression of *IME2*, through which to induce the expression of other sporulation-specific genes.

1.3.2 Genetic control of meiosis and spore formation

All of the *cdc* mutants, other than those defective in bud emergence and cytokinesis, are also defective in meiosis. The *rad* mutants, particularly those involved in the repair of X-ray damage, are blocked in sporulation probably because of their involvement in meiotic recombination and repair. The rationale is that meiosis and mitosis require common recombination functions. The cell types determined by the mating type locus also affect sporulation. The *MAT* mutants were examined because of the requirement for sporulation of heterozygosity at the mating-type locus. There are two codominant *MAT* alleles, *MAT***a** and *MAT*α. The *MAT***a** allele produces two transcripts, but only **a**1 seems to carry out *MAT***a** functions. *MAT*α is transcribed in opposite directions to give rise to two transcripts, α1 and α2. The gene product of

MATα1 is thought to be a positive regulator of α-specific functions, while the gene product of *MAT*α2 is thought to be a negative regulator of **a**-specific functions. Since neither the α1 nor the α2 transcript are synthesized in **a**-type haploids, α-specific genes are not turned on and **a**-specific genes are not repressed. Therefore, a *MAT***a** cell is constitutive for **a**-specific functions, is able to produce **a**-factor and is able to mate with an α-type cell. Both α1 and α2 transcripts are made in α-haploids, so the α-specific genes are expressed and the **a**-specific genes are silent in these cells. The α haploid can thus produce α factor and mate with an **a**-type cell. In *MAT***a**/*MAT*α diploids, the α2 product inhibits expression of the **a**-specific genes, while the **a**1 product inhibits the transcription of α1, preventing the turn on of transcription of the α-specific genes. These cells produce neither α factor nor **a**-factor and are unable to mate. One reason that *MAT***a**1- and *MAT*α2-gene functions are necessary for sporulation is that they turn off *RME1* (Mitchell and Herskowitz, 1986).

A number of other nuclear genes are also required for sporulation. These include the numerous PET (petite) loci required for mitochondrial function, loci required for fatty acid metabolism (Keith et al., 1969), and loci required for the TCA cycle (Ogur et al., 1965). Mutations in any of these loci render the cells respiratory-deficient and, hence, incapable of sporulation. Several loci involved in amino acid biosynthesis are also required for sporulation (Lucchini et al., 1978). For example, *met2* and *met13* mutations are methionine-auxotrophic and also result in an imbalance in different rRNA species and a lack of sporulation. These defects are reversible by the addition of methionine to the sporulation medium (Wejksnora and Haber, 1974).

1.4 Sporulation-specific genes and their mRNA and proteins

1.4.1 Sporulation-regulated genes

It was established earlier that sporulation in *S. cerevisiae* is dependent upon both sporulation-specific genes and certain vegetative genes. Genetic analyses have suggested that at least 50 loci code for functions indispensable to the process of meiosis and sporulation (Espsito et al., 1972). These loci contain genes affecting both early and late events in sporulation.

Mills (1980) reported the first evidence for a sporulation-specific mRNA population, about 7% of total mRNA. Recently, sporulation-specific genes have been isolated by selecting sequences that are preferentially transcribed during sporulation. They were named *SPR* genes (Clancy et al., 1983), *SPS* genes (Percival-Smith and Segall., 1984), and *SIT* genes (Gottlin-Ninfa and Kaback., 1986) by different groups. The *SPR* genes were isolated from a Lambda Charon 28 library of yeast genomic DNA by using two cDNA probes; one complementary to polyA(+) RNA isolated from an **a**/α diploid incubated in sporulation medium, and the other complementary to poly(A)+ RNA of an α/α diploid under same situation. The Lambda plaques that hybridized to the **a**/α probe, but not the α/α probe were considered to contain sporulation-specific DNA. Twenty four different *SPR* genes were found to be represented by the 46 clones identified. In another differential hybridization screen, Percival-Smith and Segall isolated 15 different sequences, from a pBR322-yeast genomic DNA library, that represented 14 different sporulation-specific genes (*SPS* genes). Gottlin-Ninfa and Kaback isolated genes, again from a gene-size insert-Lambda recombinant DNA library, which are complementary to **a**/α-dependent sporulation-induced transcripts.

The regulation of gene expression during sporulation could occur at the level of transcript accumulation or posttranscription. Each sporulation-specific expressed sequence may have a function in sporulating cells, or these transcripts may be merely the result of a generalized relaxation of control in which many transcribable DNA sequences are expressed. The former case is similar to some other developing systems characterized by the appearance of specific transcripts, e.g., globin transcripts in reticulocytes. In the latter case, specific transcripts would not always have distinct or even definable functions, e.g., the lampbrush-chromosome stage found in oocytes of amphibians and other higher organisms(Davidson, 1976). In *Aspergillus nidulans*, some of the sporulation-specific RNAs are also present in hyphae but in a nonpolyadenylated form which later becomes polyadenylated during sporulation (Timberlake, 1987). These results suggest that posttranscriptional control mechanisms may play a role in the development regulation of gene expression.

Kaback and Feldberg (1986) suggested the existence of a developmentally regulated program of gene expression for sporulation of *S. cerevisiae*. Various transcripts appeared and disappeared with different kinetics throughout this process. These patterns were not observed in asporogenous **a**- and α/α cells incubated in sporulation medium where all transcripts appeared to have similar kinetics. Those transcripts that became most abundant at 1 to 3 hours in the sporulating cells behaved almost identically in the asporogenous cells. Accordingly, this early behavior may not be part of the **a**/α-dependent sporulation-specific program. In contrast, only sporulating cells had the transcripts most abundant at later times. Notably, all cloned sporulation-specific transcribed sequences so far investigated also appear to be most highly expressed at these later times (Clancy et al., 1983; Percival-Smith and Segall, 1984). Consequently, the period after 3 hours would represent the predominant period during which any developmental program would be operating.

The accumulation of *SPR* transcripts in yeast cells during sporulation was examined by Holaway et al. (1987). They determined the transcription rates of three *SPR* genes, *SPR1*, *SPR2*, and *SPR3*, using a filter binding assay. *SPR1* and *SPR3* mRNA abundance increased from less than 0.7 copies per cell in vegetative and early sporulating cells to 130 and 90 copies per cell respectively 8 hours after transfer to sporulation medium. Similarly, the level of fusion protein of *SPR3-LacZ* increased at least 700-fold. The accumulation of these *SPR* mRNAs may be due to the enhanced transcription of the corresponding genes. It is also possible that the *SPR* mRNAs accumulate because of their increased stability. The average half-life of mRNA (20 min) and the proportion of polyA(+) RNA (1.3–1.4%) has been shown to be similar in vegetative and sporulating cells (Kraig and Haber, 1980). However, the results of Holaway et al. could not distinguish between these two possibilities.

1.4.2 Identification of sporulation-specific proteins

One of the disadvantages of using yeast to study meiosis is the impermeability of sporulating cells to radiolabeled precursors. As cells progress in the sporulation process, they become more impermeable. In the past, attempts to elucidate the changes in sporulating cells at the protein level have not been very successful, although these earlier studies did identify changes in the electrophoretic pattern of proteins in both sporulating cells and asporogenous cells incubated in sporulation medium (Hopper et al., 1974; Peterson et al., 1979; Trew et al., 1979). If the changes in protein pattern observed are associated with changes in gene expression rather than modification of existing proteins, it should be possible to show that the mRNA content of the cells alters during meiosis and sporulation. Earlier studies could not distinguish between those two possibilities. By using an *in vitro* translation system, one can prove that specific mRNAs can be translated at certain periods. Weir-Thompson and Dawes (1984) first applied this system to study protein changes during sporulation in *S. cerevisiae.* Developmentally related changes do occur in the translatable mRNA population of *S. cerevisiae* during sporulation. Extensive degradation of total cellular RNA and a concomitant increase in RNase activity specific to sporulating cells is reflected in a specific decline in the functional mRNA population during the later stages of spore development. The availability of functional mRNA increased by about 25% during the first 4 hours after transfer of either sporulating or non-sporulating diploids to sporulation medium. Thereafter functional mRNA decreased such that in the **a**/α strain after 24 hours there was only about 50% of the amount in vegetative cells; a less marked decrease was observed in **a**/**a** and α/α strains. A significant number of sporulation-specific changes in individual mRNA species was found to occur during meiosis and spore formation, although it is interesting that only four were found to involve completely new transcripts; the remainder were due to marked changes in the abundance of the mRNA species.

Kurtz and Lindquist (1984) also observed changing patterns of gene expression during sporulation in yeast. They isolated RNAs from **a**/α sporulating cells and asporogenous **a**/**a** and α/α cells and determined their protein-coding capacities by translation in cell-free lysates. The ability of an RNA sample to stimulate incorporation of labeled amino acids into protein in a cell-free translation system was taken to represent the availability of functional mRNA sequences in the sample. They also found that there is a rapid increase in the amount of RNAs encoding a broad range of polypeptides 2 hours after transfer to sporulation medium in both **a**/α and **a**/**a** cells. But two sets of abundant sporulation-specific mRNAs are induced in a strict temporal order later in sporulation. The first appeared after 6 hours and encoded polypeptides of 17, 20, 25, 31, 38, 50, 65, and 68.5 kd. These RNAs were maximally induced between 8 and 14 hour and then began to disappear. Their pattern of synthesis is similar to that of the four new transcripts seen by Weir-Thompson and Dawes (1984). The second set of RNAs, encoding 21.5 kd and 34 kd polypeptides, was induced after 16 hours. These two RNAs continued to accumulate late into sporulation and were packaged into spores in translatable forms. These results are also in good agreement with the results of Clancy et al. (1983). These 10 sporulation-specific proteins were considered an absolute minimum, since this system can only detect mRNAs that accumulate in substantial quantities.

Kurtz and Lindquist (1986) related the pattern of expression of those two sets of mRNAs to the morphological landmarks of sporulation to get some information about the functions encoded by these mRNAs. The induction of the first set of sporulation-specific mRNAs coincides with the initial events of nuclear budding and spore wall formation which occur in 8 hour sporulating cells. During the period when these messages are detected, spore walls increase dramatically in thickness (10 hours). By 16 hours, the spores have reached their mature size and the walls appear fully synthesized. At this point, the first set of sporulation-specific RNAs is rapidly degraded. The first set of mRNAs was only found in ascal cytoplasm, and the later set of mRNAs was only found inside spores. The limitation of location of these two sets of mRNAs may be due to the degradation of RNAs in the external compartment when spore wall synthesis is complete. Thus, what appears to be selective degradation of these messages midway through spore development may be a simple consequence of their location in a compartment where degradation predominates.

The first set of sporulation-specific RNAs encodes at least 8 polypeptides. The authors suggest that these polypeptides are involved in spore wall assembly based on several observations:

- The intensity of RNA translation *in vitro* indicates that the corresponding messages are very abundant and that they most likely encode structural proteins.
- These messages appear at the tetranucleate stage of meiosis, and they are degraded a few hours later. Their appearance is coincident with the initiation of spore wall synthesis.
- Components of the wall must cross the membrane in order to be deposited within the double membrane.

The translation behavior of the first set of mRNAs *in vitro* suggests that their translation is associated with membranes. The second set of sporulation-specific RNAs which is found only in spore compartment encodes two polypeptides. The authors suggested that the functions encoded by these RNAs may be involved in establishing the final stages of dormancy or may be stored for use during spore outgrowth.

There are also some proteins with known functions that are sporulation-specific. The enzyme which degrades glycogen, glucoamylase, appears specifically in sporulating cells (Colonna and Magee, 1978). The expression of the sporulation-specific glucoamylase gene (SGA) is positively regulated by the *MAT* locus, both *MAT*a1 and *MAT*α2; and the control of SGA is exerted at the level of transcription. Expression of SGA and the consequent degradation of glycogen to glucose in sporulating cells is not required for meiosis or sporulation, since **a**/α diploid cells homozygous for an insertion mutation at SGA still formed four viable ascospores. Diploid cells carrying the mutations in *MAT*a1 and *MAT*α2 could neither undergo sporulation nor produce glucoamylase activity, while the *mat*a2 and *mat*α1 mutations affected neither sporulation nor enzyme activity. These results indicate that both **a**1 and α2 are essential for this enzyme production (Yamashita and Fukui, 1985). Another enzyme required for glucosamine synthesis, 2-amino-2-deoxy-D-glucose-6-phosphate keto-1-isomerase, is also sporulation-specific (Ballou et al., 1977). The isomerase enzyme activity is detected at the tetranucleate stage of meiosis. Mutation at this locus produces structurally abnormal, but viable, asci.

1.5 Phenotypes of sporulation-specific genes

Aspergillus nidulans is another fungus that has been used as a model system to study development. Timberlake (1987) placed mutations that affect development in *A. nidulans* into two classes. The first class includes those mutants that prevent or alter the timing of initiation of conidiation. Genes in this class, regulating conversion from vegetative growth to conidiation, have been termed strategic by Clutterbuck (1969). The second class, termed auxiliary genes by Clutterbuck, are those whose mutations directly affect the formation of conidiophores or conidia, but mutant strains can initiate conidiophore development normally. These mutations can be further divided according to their functions. They include: genes that directly determine the structure and specialized physiological activities of conidiophores, genes that result in the formation of morphologically abnormal conidiophores or conidia, and genes that are expressed specifically during conidiation, but which have unknown physiological functions or little or no effect on the morphology or the viability of conidiophores or spores.

The number of genes that are expressed preferentially during conidiation in *A. nidulans* has been estimated directly by hybridization of an excess of polyA(+) RNA from conidiating cultures with sporulation-specific and spore-specific cDNA probes (Zimmermann et al., 1980). The resulting data suggested that about 1200 putative mRNAs accumulate during conidiation and are absent from or present at low levels in hyphae. Of those 1200 mRNAs, approximately 1000 accumulate in conidiating cultures only. The remaining 200 accumulate specifically in mature conidia. It is likely that these RNAs encode enzymes or structural proteins that have specific functions in spore differentiation or spore germination. The same rules can be used to classify developmental genes of *S. cerevisiae.* Surprisingly, most sporulation-specific genes of *A. nidulans* and *S. cerevisiae* that have been cloned and characterized show no effect on sporulation and spore germination in strains containing these mutations.

Segall's group has worked on five *SPS* genes, *SPS1*, *SPS2*, *SPS3* (Percival-Smith and Segall, 1986), *SPS4* (Garber and Segall, 1986), and *SPS100* (Law and Segall, 1988). Only two *SPS* genes show alternate phenotypes when disrupted. *SPS1* is indispensable for completion of sporulation, and *SPS100* is required for spore wall formation and ascus maturation. Three *SPS* genes, *SPS1*, *SPS2*, and *SPS3*, have been shown to be closely linked and they encode 1.9 kb, 1.6 kb, and 1.75 kb transcripts, respectively.

Although *SPS2* expression is not essential for sporulation, Percival-Smith and Segall (1987) showed that increased copy number of the 5′ end of the *SPS2* sequence inhibits sporulation of *S. cerevisiae.* In other systems, the introduction of multiple copies of a DNA regulatory sequence into a cell may result in the *in vivo* titration of transcriptional regulatory factors. Such studies can thus delineate control sequences and has demonstrated that common factors are involved in the expression of distinct genes. However, in the case of *SPS2*, the reduction in ascus formation required the presence in the plasmid not only of the 5′ end sequences of the *SPS2* gene but also of the initial coding sequence of the gene. This observation suggests that the inhibition of sporulation by increased copy number of the 5′ end of the *SPS2* gene is not simply due to the competition for the regulatory factors.

The authors concluded that the accumulation of a fusion protein containing the amino-terminal portion of the *SPS2* gene product, synthesized from the transcripts derived from the truncated *SPS2* genes present in the high-copy-number plasmid, has an adverse affect on the progression of sporulation-specific events. The asporogenous phenotype is dependent on the synthesis of plasmid-derived *SPS2* transcripts, and hence requires that a promoter be present. There is not a stringent requirement for the *SPS2* promoter itself. The phenotype results from the overproduction of a protein containing the amino-terminal portion of the *SPS2* gene product. It is possible that this either leads to the accumulation of an aberrant sporulation-specific structure or prevents the assembly of a sporulation-specific structure, which in the former case inhibits and in the latter case is required for the continuation of the temporal progression of sporulation-specific events. Although the *SPS2* gene product may be dispensable, it nonetheless contributes to a sporulation-specific event. The cell can compensate for the absence of this gene product, but the putative overproduction of its amino-terminal portion is deleterious and prevents the completion of sporulation. The effect of overproduction of the entire protein has not been determined.

The *SPS4* gene encodes one of the prominent sporulation-specific proteins observed among the *in vitro* translation products of RNA from sporulating cells. From the nucleotide sequence of the *SPS4* gene, the putative product inferred is 38.6 kd with an isoelectric pH of 8.2 which is similar to the properties of one protein identified by Kurtz and Lindquist (1986). The gene product of *SPS4* is not essential for sporulation, since **a**/α diploids homozygous for a partial deletion of the *SPS4* gene appeared to be unaffected in their ability to sporulate and to form viable ascospores. The relative ease with which plasmids containing the *SPS4* gene were identified suggested the possibility that this gene encodes a relatively abundant sporulation-specific mRNA. Alternatively, it may reflect the fact that the gene is present in several copies in the yeast genome.

The *SPS100* gene (Law and Segall, 1988) is activated late in the sporulation process. It is first expressed at 12 hours of sporulation and remains unchanged from 15 to 35 hour. Several lines of evidence suggest that the gene product of *SPS100* may be a spore wall protein and have a protective function for the spores. The level of transcripts of the *SPS100* gene remains steady between 15 and 35 hours, a time coincident with spore wall maturation. The putative 34.2 kd protein encoded by *SPS100* contains a potential signal sequence and cleavage site. The resistance to ether treatment of spores of homozygous mutants of *SPS100* was delayed by 5 h relative to the wild-type strain.

Gottlin-Ninfa and Kaback (1986) also tried to determine the functions of three of the *SIT* genes, *SIT2*, *SIT3*, and *SIT4*. All three genes proved to be dispensable for sporulation and spore germination based on a gene disruption experiment. One *SIT2* disruption contained a *HIS3* insertion in one part of the gene and a second had a *LEU2* insertion into the site of a 0.3 kb deletion in another part of the gene. The authors reported the creation of a fusion transcript between a *HIS3* RNA molecule and a 300bp long *SIT2* RNA molecule, giving a very low but finite possibility of *SIT2* activity. The other two genes, *SIT3* and *SIT4*, were disrupted by simple insertion mutations, and thus might still possess some gene activity. Therefore, further experiments are required to create strains completely lacking *SIT3* or *SIT4* message for phenotype exploration.

In this lab, we have worked on the *SPR1* and *SPR2* genes which are both abundantly transcribed late genes (Primerano et al., unpublished data). Disruption of *SPR1* and *SPR2* separately does not affect the sporulation process; the disruptants form four viable spores. The results from the DNA sequence of the *SPR1* gene and an *SPR1-LacZ* fusion suggested that the *SPR1* gene product may be a membrane protein and that the gene product is localized to a particulate fraction of the developing spores. These data suggest that the *SPR1* gene product may have a role in spore wall maturation.

The results obtained from three different groups suggest that not all sporulation-induced genes are essential for sporulation and spore germination. On the other hand, since examination of ascus formation and spore viability only monitors the most significant meiotic and morphological events of sporulation, a more careful analysis of spores and their formation may be required to determine the functions of these genes.

1.6 Focus of this study

In the first part of this study, one sporulation-specific gene, *SPR9*, was analyzed molecularly and genetically. It was also compared to other previously studied sporulation-specific genes for a generalized view of all sporulation-specific genes. In the second part, the *SPR9* gene was disrupted and the phenotypes of three *SPR* genes, *SPR1*, *SPR2*, and *SPR9*, were analyzed from several different aspects. Two double mutants containing two defective sporulation-specific genes were also constructed to gain insight into the relationship among these three genes.

CHAPTER II

Materials and Methods

2.1 Strains and media

All strains used in this study and their genotypes are described in Table 2.1. All the media used are listed in Table 2.2.

The media were prepared in either liquid form or in solid form. The solid form was made by adding 20 g Bactoagar per liter of media. For growing strains auxotrophic for different kinds of amino acids, media were supplemented with those amino acids required for growth at a final concentration of 40 mg per liter.

2.2 Culture conditions

For cell growth and maintenance, the rich medium YEPD was normally used. For auxotrophic phenotype examination, a minimal medium (MIN) with or without various supplements was used. For testing respiratory-competence, YEPGlycerol was used. For regeneration of spheroplasts, solid MIN and YEPD both containing 1 M sorbitol were used.

Sporulation in liquid medium was done at 30°C with vigorous aeration. Cells were inoculated into YEPD medium to grow overnight. The following day cells were transferred to presporulation medium (PSP2) supplemented with the auxotrophic

Table 2.1: List of strains.

Strains	Genotype	Source
W66-8A	**a**/α *(HO ade2-1 leu1 trp5-2 ura3-1 met4-1 lys2-1)*	Rothstein and Sherman, 1980
W7	same as W66-8A, except *spr9::URA3/SPR9*	This study
W7a	same as W66-8A, except *spr9::URA3/spr9::URA3*	This study
W7a-1	same as W7a, except *LEU1*	This study
WSRB1	same as W66-8A, except *spr1-1::URA3/SPR1*	This lab
W61A	same as W66-8A (sister spore to W61C)	This lab
W61C	same as W66-8A, except *spr1-1::URA3/spr1-1::URA3*	This lab
SCMS7-1	**a**/α *(HO adeX his4-519 leu2-3,112*	This lab
SPB1	same as SCMS7-1, except *spr1-2::LEU2/SPR1*	This lab
S52A	same as SCMS7-1, except *spr1-2::LEU2/spr1-2::LEU2*	This lab
S52B	same as SCMS7-1 (sister spore to S52A)	This lab
AKAS2	same as SCMS7-1, except *spr2::LEU2/SPR2*	This lab
T4C	same as SCMS7-1, except *spr2::LEU2/spr2::LEU2*	This lab
BS1	**a**/α *(HO ADE2-1/ade2-1 ADEX/adeX TRP5-2/trp5-2 URA3-1/ura3-1 MET4-1/met4-1 LYS2-1/lys2-1 HIS4-519/his4-519 LEU2-3,112/leu2-3,112 spr1-2::LEU2/SPR1 spr9::URA3/SPR9)*	This study
CS1	same as BS1	This study
4-8-a	**a**/α *(HO adeX met4-1 lys2-1 his4-519 spr1-2::LEU2 spr9::URA3)*	This study
CT2	same as BS1, except *spr2::LEU2/SPR2*	This study
CT3	same as CT2	This study
5-4-d	**a**/α *(HO lys2-1 spr2::LEU2 spr9::URA3)*	This study
AP1 **a**/α	A364a x α131-20	Hopper et al., 1974
AP1α/α	derived from AP1 **a**/α	Hopper et al., 1974
AP3**a**/**a**	derived from AP1 **a**/α	Hopper et al., 1974
YPH149		Hieter, unpub. 1989

requirements, to preadapt to respiratory metabolism. When the cell density reached 2–5 × 10^7 cells/ml, cells were collected and washed twice in sterile, distilled water, and then transferred to sporulation medium (SP2). Cells began to sporulate after 12 hours of incubation in SP2 medium. For examining the ability to sporulate of a large number of samples, sporulation was performed on solid medium at 30°C. Cells were patched onto YEPD and grown overnight. Patches were then replicated onto PSP2 and incubated 48 hours. Each patch was then transferred to SP2 by toothpicks and incubated for three days.

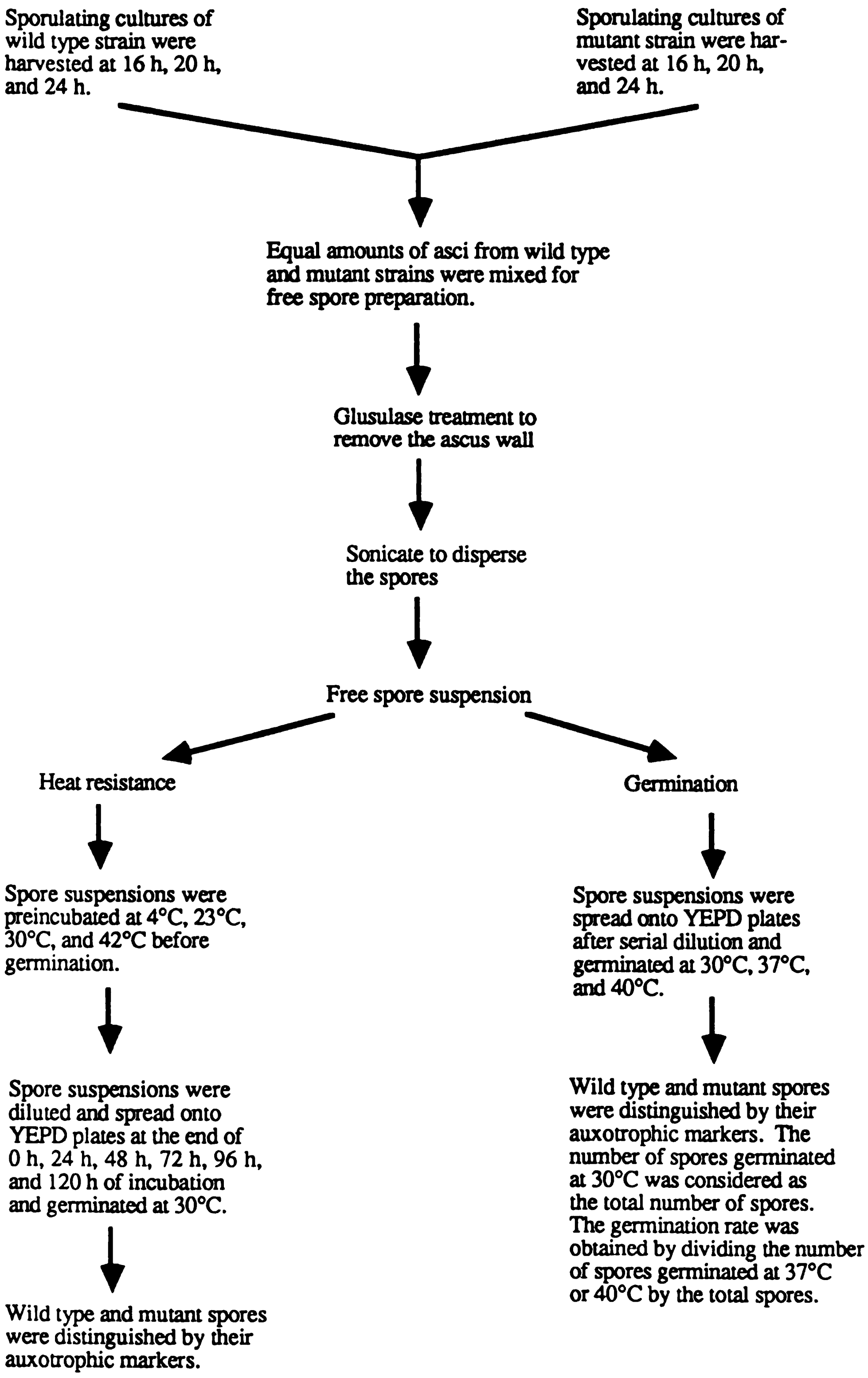
Heat shock was done as follows: Samples were grown in YEPD medium at 30°C until they reached log phase. Before harvesting, cells were transferred to 39°C for heat shock for 60 min. Cells were then used for further preparation.

2.3 Testing of free spores for thermoresistance and germination

For testing ascospore thermoresistance, strains were induced to sporulate in 10–20 ml of SP2 medium as described before. For homozygous strains, equal numbers of asci (1 × 10^8 asci/ml) from wild-type and mutant strains were mixed together. By mixing asci from different strains, we could minimize variations in treatment between wild-type and mutant strains that might occur during the preparation of free spores or during the experimental stress. For heterozygous strains, there were two wild-type and two mutant spores produced from one ascus; therefore no mixing between wild-type and mutant strains was needed. The asci were harvested at 16, 20, and 24 hour and washed three times with sterile, distilled water. Free spores were obtained by treating the ascus wall with β-mercaptoethanol at 23°C for 30 min followed by glusulase (Endo Laboratories) treatment at 37°C until more than 90%

broken asci were observed in 5% SDS (Sherman et al., 1981). B-mercaptoethanol and glusulase were removed by washing three times in sterile, distilled water. The spores were resuspended in 10 ml of sterile water and then disrupted by three twenty second bursts of sonication at 150 mA. This converted 99% of all tetrad clusters to single spores. At this point, the numbers of total spores in each preparation were determined by plate count. Spore preparations were then distributed into individual vials and incubated at 4°C, 23°C, 30°C, 37°C, 42°C, and 45°C in various experiments. At several times (0, 24, 48, 72, 96, and 120 hour) spore viability was determined by plating diluted samples onto YEPD plates and incubating at 30°C for 48 hours. The colony counts from YEPD plates were used as the total survivors after heat treatment. Two hundred colonies were picked onto minimal plates with and without the auxotrophic supplements in order to determine the genotype of the spore colony and the percentages of mutant spores among the total survivors. Therefore, the number of wild-type and mutant survivors after heat treatment could be deduced by multiplying the number of total survivors by the percentage of wild-type or mutant spores (Figure 2.1).

For testing the germination ability of mutant spores, free spores were prepared as described above. Instead of preincubation for heat treatment, diluted spore suspensions were plated immediately onto YEPD plates to germinate at different temperatures. Plates were incubated at 23°C, 30°C, 37°C, and 40°C for 48 hours or longer depending on their growth conditions. The numbers of colonies formed when incubated at temperature T in the experiments were used as total spores. The genotypes of two hundred spore colonies were also determined as before and used to calculate the percentages of wild-type and mutant spores in the experiments. The number of total spores obtained by germinating at 30°C was considered as the starting



Sporulating cultures of wild type strain were harvested at 16 h, 20 h, and 24 h.

Sporulating cultures of mutant strain were harvested at 16 h, 20 h, and 24 h.

Equal amounts of asci from wild type and mutant strains were mixed for free spore preparation.

Glusulase treatment to remove the ascus wall

Sonicate to disperse the spores

Free spore suspension

Heat resistance

Spore suspensions were preincubated at 4°C, 23°C, 30°C, and 42°C before germination.

Spore suspensions were diluted and spread onto YEPD plates at the end of 0 h, 24 h, 48 h, 72 h, 96 h, and 120 h of incubation and germinated at 30°C.

Wild type and mutant spores were distinguished by their auxotrophic markers.

Germination

Spore suspensions were spread onto YEPD plates after serial dilution and germinated at 30°C, 37°C, and 40°C.

Wild type and mutant spores were distinguished by their auxotrophic markers. The number of spores germinated at 30°C was considered as the total number of spores. The germination rate was obtained by dividing the number of spores germinated at 37°C or 40°C by the total spores.

Figure 2.1: Free spore preparation for thermotolerance and germination.

number of spores before treatment. The number of wild-type spores and mutant spores germinated at 30°C were considered to be the expected numbers, since we supposed that all spores could germinate at 30°C. Therefore,

- The number of mutant spores germinated at temperature T = (the number of total spores at temperature T) *times* (% of mutant spores at temperature T)
- The number of wild-type spores germinated at temperature T = (the number of total spores at temperature T) *times* (% of wild-type spores at temperature T)
- % of germinated mutants at temperature T = (the number of mutant spores germinated at temperature T) / (the number of mutant spores germinated at 30°C) *times* 100%
- % of germinated wild-types at temperature T = (the number of wild-type spores germinated at temperature T) / (the number of wild-type spores germinated at 30°C) *times* 100%

2.4 Construction of plasmids

Restriction endonucleases and other DNA modifying enzymes were obtained from Bethesda Research Laboratories or Boehringer Mannheim and were used under the supplier's conditions.

A 5.8 kb ClaI fragment carrying the *SPR9* gene from λsp104 was cloned into the ClaI site of pBR322 to give rise to p803. The 1.6 kb SalI-XhoI fragment of p803 was further removed to generate p803s/x and more unique restriction sites. For RNase protection, a 2.2 kb ClaI-SstI fragment containing the *SPR9* gene was forced-cloned into a GEM3 and a GEM4 plasmid (Promega company) at AccI and

SacI sites to form G3S and G4S. All four plasmids constructed for this study are shown in Figure 2.2. To produce the mutant allele for one-step gene disruption, the 3.0 kb HindIII fragment containing the *SPR9* gene of p803s/x was replaced by a 1.1 kb *URA3*-containing HindIII fragment as shown in Figure 4.2.

2.5 Yeast transformation

Yeast transformation was done as described in Sherman et al (1987). Recipient strains were grown in 200–300 ml of YEPD until they reached 2–4 × 10^7 cells/ml. Cells were harvested by centrifugation in the GSA rotor for 5 min at 8 K. Cells were then resuspended in 20 ml SED (1 M sorbitol, 25 mM EDTA, pH 8.0; 50 mM dithiothreitol (DTT)) and incubated at 30°C for 10 min. After incubation, cells were washed once in 20 ml 1 M sorbitol and resuspended in 20 ml of SCE (1 M sorbitol, 0.1 M sodium citrate, pH 5.8; 10 mM EDTA). Then, 0.25–0.5 ml glusulase (Endo) were added to the suspension which was incubated at 30°C for 30–60 min with occasional shaking. Spheroplasting was assayed by checking the degree of lysis of cells in 5% sodium dodecyl sulfate under microscope. Because spheroplasts are fragile, cells were handled very carefully in subsequent steps. When spheroplasting had reached 90%, the suspension was centrifuged and the pellet was washed twice in 1 M sorbitol and once in STC (1 M sorbitol, 10 mM calcium chloride, 10 mM Tris-HCl, pH 7.5). Cells were then resuspended in 1.0 ml of STC. Sheared salmon sperm DNA at a final concentration of 50 μg/ml was added to the spheroplast suspension as carrier. The suspension was then dispensed into 10 aliquots. DNA (0.1–5 μg in 1–10 μl) was then added and the tubes incubated at 23°C for 10 min. 1 ml of PEG (20% polyethylene glycol 4,000, 10 mM calcium chloride, 10 mM Tris-HCl, pH 7.5) was added to each tube. After 10 more min at 23°C, the cells were pelleted

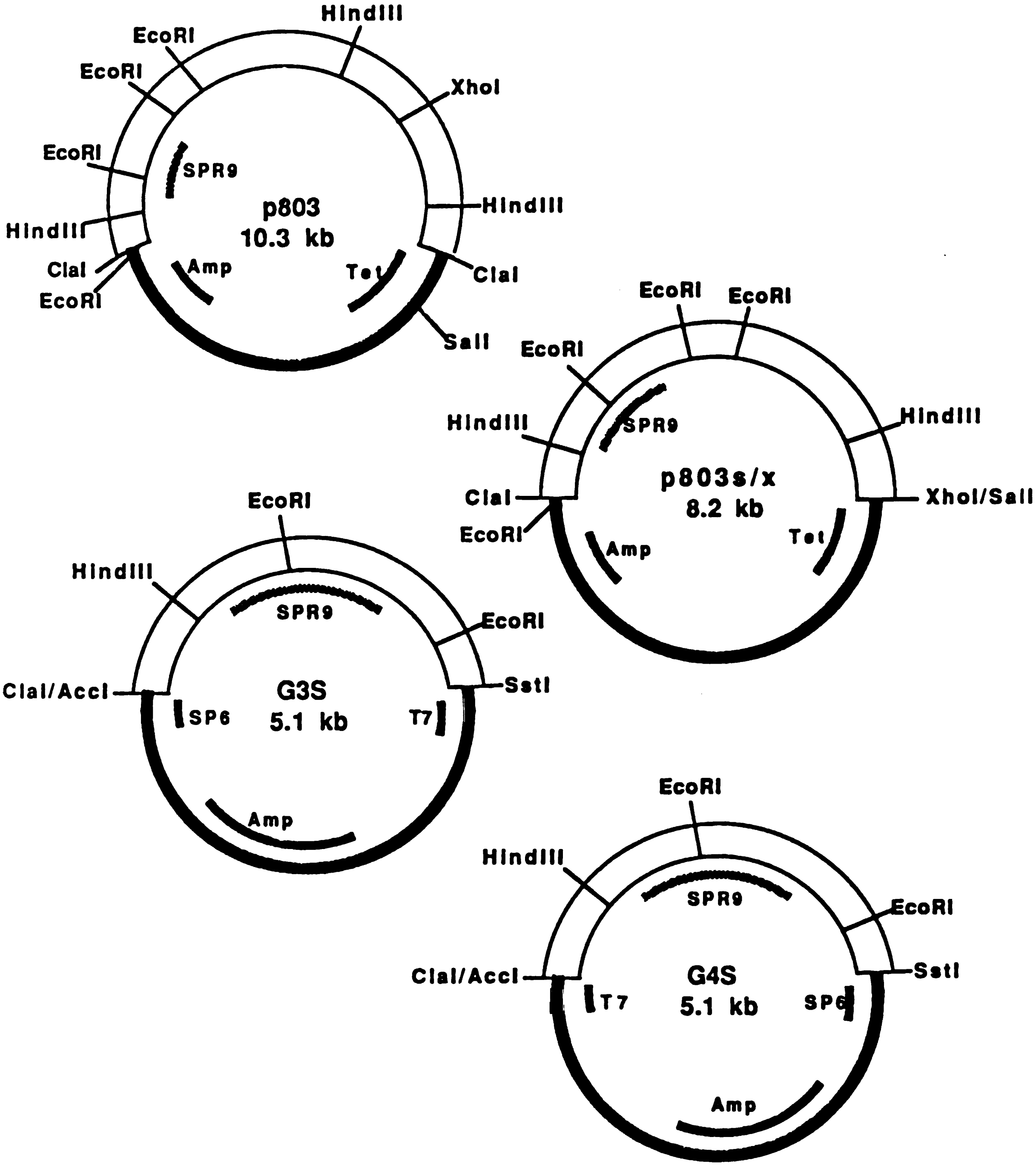
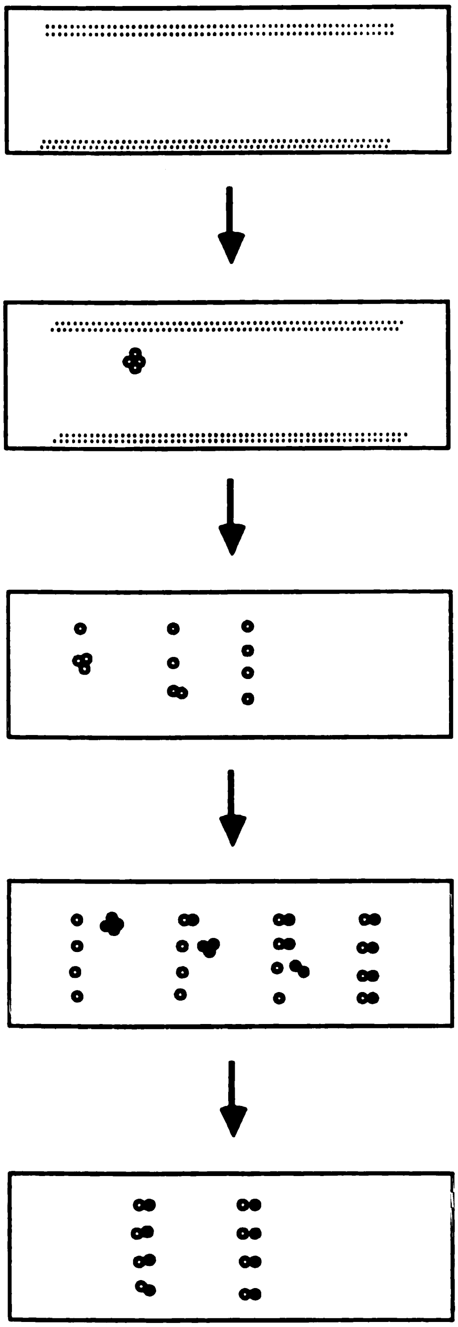


Figure 2.2: Restriction maps of four plasmids.

carefully, resuspended in 150 μl of SOS (10 ml 2 M sorbitol, 6.7 ml YEPD, 0.1 ml 1.2 M calcium chloride), and incubated at 30°C for 20 min. Cells were plated out by adding 6 ml of top agar to each tube and immediately pouring onto plates. The plates were incubated at 30°C until the transformants had appeared (usually two days).

2.6 Tetrad analysis and micromanipulation

Yeast cells were incubated in SP2 medium until more than 30% of cells formed four-spore asci. The time required differs for different strains, but 16 hours is the usual minimum. The cells were harvested by centrifugation at 4000 rpm for 5 min, then washed with sterile distilled water three times. The samples were resuspended in 5 ml of sterile distilled water and 0.5 ml of glusulase (Endo) was added to the cell suspension. The suspension was incubated at 30°C until free ascospores were visible under the light microscope in a drop of 5% sodium dodecyl sulfate(SDS), usually 30 min. The glusulase was removed immediately by 3 washes in sterile water. The sample was resuspended in a small amount of sterile water, and this suspension was streaked along one edge of a slice of very thin YEPD agar. Individual intact asci containing four spores were picked, moved far from others, and dissected into four separate spores by micromanipulation as shown in Figure 2.3c. After they grew to form colonies, they were streaked onto a YEPD plate to give isolated colonies. They were subsequently checked for auxotrophic requirements.



a. Streak one spore suspension on the edge of one side of agar slice, and another suspension on the other side.

b. Move one four-spored ascus to one spot far from all other asci with the needle of micromanipulator.

c. Separate the four spores to a line on the agar.

d. One four-spored ascus from the other sample was separated to four single spores in the same way.

e. Two spores from different strains were pushed side by side to mate.

Figure 2.3: Micromanipulation of tetrads and spore-spore matings.

2.7 Nucleic acid preparation

2.7.1 Preparation of plasmid DNA

Large amounts of plasmid DNA were prepared by a procedure described in Krieg and Melton (1984). The plasmids were transformed into appropriate *E. coli* strains. A single colony carrying the plasmid of interest was picked and inoculated into 10 ml of LB containing the appropriate antibiotic. The culture was incubated at 37°C overnight with vigorous shaking. One ml of the overnight culture was inoculated into 250 ml of the same medium and grown for 12 hours. The cells were harvested by centrifugation at 5,000 rpm, 4°C for 15 min. The pellet was resuspended in 6 ml of freshly prepared lysis solution (25 mM Tris-HCl, pH 7.5; 10 mM EDTA, 15% sucrose, 2 mg/ml lysozyme (Sigma)) by pipetting up and down with a 10 ml pipette. The suspension was incubated in ice water for 20 min. Then, 12 ml of 0.2 M NaOH, 1% SDS were added to the tube. The tube was carefully inverted several times to mix thoroughly, and incubated in ice water for 10 min. Then 7.5 ml of 3 M sodium acetate, pH 4.6 were added. The tube was carefully inverted several times until mixed thoroughly, and incubated in ice water for another 20 min. Then, the tube was centrifuged at 15,000 rpm for 15 min. The supernatant was removed to another tube, and 50 μl of RNase A (1 mg/ml)was added to remove cellular RNA. After 20 min of incubation with RNase A at 37°C, the supernatant was extracted twice with an equal volume of phenol:chloroform (1:1). Then, two volumes of 95% ethanol were added to precipitate the DNA. The DNA was pelleted by centrifugation at 10,000 rpm, 4°C for 15 min. The pellet was dissolved in 1.6 ml of sterile distilled water. 0.4 ml of 4 M NaCl were added to the tube and mixed, and followed by 2 ml of 13% polyethylene glycol. The tube was then incubated in ice water for 60 min.

The suspension was centrifuged at 10,000 rpm for 10 min after 60 min of incubation. The DNA pellet was washed with 70% ethanol, and then dissolved in an appropriate volume of sterile distilled water.

2.7.2 Preparation of yeast genomic DNA

Yeast genomic DNA was prepared as described in Sherman et al. (1987). 10 ml of cells were grown overnight in YEPD. Cells were harvested at stationary phase and washed in 1 ml of 1 M sorbitol. Cells were spheroplasted by resuspending in 1 ml of spheroplasting buffer (1 M sorbitol pH 7.5, 50 mM potassium phosphate, 0.1% β-mercaptoethanol, and 1 mg/ml Zymolase 20,000 (Kirin Brewery, Japan)) at 37°C. After 30 min of incubation, cells were centrifuged in an Eppendorf microfuge tube to remove the solution. The cells were then lysed in 0.5 ml of lysing buffer (50 mM Na EDTA, pH 8.5; 1 mg sodium dodecyl sulfate, 3 μl diethylpyrocarbonate (DEPC)) at 70°C for 30 min. 50 μl of 5 M potassium acetate were added at the end of incubation. After mixing the lysates and 50 μl of 5 M potassium acetate by rocking the tubes, they were incubated at 0°C for 30 min. The lysates were then centrifuged to get a clear suspension and the supernatant decanted into 1 ml of 95% ethanol. The tubes were gently rocked to mix the supernatant and ethanol. After centrifugation, the pellet was washed with 70% ethanol. Then the pellet was resuspended in 0.2 ml of TE and 0.1 μl of 10 mg/ml RNase A at 37°C for 30 min to remove RNA. The DNA was reprecipitated by adding 200 μl of isopropyl alcohol, centrifuged, washed with 70% ethanol, and dried. Finally, the pellet was resuspended in 50 μl of TE (10 mM Tris-Cl, 1 mM EDTA) and was ready for restriction enzyme digestion. 10 μg of enzyme digested genomic DNA of each strain was loaded onto a 0.7% agarose gel. Southern blotting was performed as described by Maniatis et al. (1980). Radiolabeled probes for filter hybridization were prepared by nick-translation of gel-purified restriction fragments (Maniatis et al., 1980).

2.7.3 Preparation of RNA

The RNA was prepared in RNase-free glassware which had been baked at 250°C for 4 or more hours. Sterile, disposable plasticware was used without pretreatment for the preparation and storage of RNA. All solutions except those containing Tris were prepared by RNase-free techniques, treated with 0.1% diethylpyrocarbonate for at least 12 hours, and autoclaved.

Total RNA was prepared from vegetative or sporulating yeast cells by the method described in Zitomer et al. (1981). 500 ml of cells grown in YEPD were harvested when the culture had reached 4×10^7 cells/ml. For cells grown in SP2, they were harvested at different time points. After harvesting, the cells were washed once with sterile distilled water and the cell pellet resuspended in 0.5 ml of RNA lysis buffer (0.1 M Tris-Cl, pH 7.4; 0.1 M LiCl, 1 mM EDTA). One ml buffered-saturated phenol and 1/3 volume of glass beads (0.45 mm diameter) were added to the suspension. Cells were broken by vortexing 20 sec and chilling 20 sec for 10 cycles. The lysate was centrifuged to separate phases. The top, aqueous phase was transferred to a new tube. 0.5 ml lysis buffer, 0.5 ml buffer-saturated phenol, and 0.2 ml 10% Sarkosyl were added to the original tube. It was vortexed for another 3 cycles and centrifuged to separate phases. The aqueous phase was added to the original one. Usually four extractions were needed for completion of cell breakage and nucleic acid extraction. The pooled aqueous phases were then extracted two times with phenol:chloroform (1:1). The purified aqueous phase was precipitated by 1/10 volume of 3 M sodium acetate, pH 5.4 and 1.5 volumes of isopropanol at −20°C for at least 1 hour. The

RNA was pelleted after precipitation and resuspended in 3.5 ml of DEPC-treated water for preparing an RNA/CsCl solution for CsCl density centrifugation. An equal volume of 10% Sarkosyl was added to the tube after the RNA had completely dissolved. Then, 0.25 g/ml of CsCl was added to the RNA solution, and finally 4 ml of 5.7 M CsCl, 1 mM EDTA solution was layered on top. The RNA/CsCl solution was centrifuged for 15 hours at 15°C in an SW41 rotor at 25000 rpm or an SW27 rotor at 20000 rpm. After centrifugation, the white RNA pellet could be seen at the bottom. The supernatant was removed with a Pasteur pipette. The RNA pellet was resuspended in DEPC-treated sterile water and its concentration was determined.

PolyA(+) RNA was isolated by the method described in Harper et al. (1980). Type II oligo(dT) cellulose (Collaborative Research, Inc.), which bound 1.2 mg to 4 mg of polyA(+) RNA per gram of resin (dry weight), was used to enrich for the polyadenylated fraction of total RNA. There is usually 2-4% polyA(+) RNA in total RNA of yeast, therefore 1 gram of oligo(dT) resin is sufficient to process at least 30 mg total RNA. Total RNA was dissolved in DEPC-treated water, and KCl and Tris-HCl were then added to final concentrations of 0.5 M and 0.01 M, respectively. The RNA solution was heat-denatured at 50°C for 10 min. Then, the RNA solution was applied to the oligo(dT) column with A buffer (0.5 M KCl, 0.01 M Tris). The polyA(+) RNA was allowed to adsorb to the resin for 15 min, after which the column was washed with 10 volumes of A buffer. The polyA(+) RNA was then eluted with DEPC-treated water, and the first 1 ml was collected. The polyA(+) RNA was precipitated with ethanol in the presence of 0.3 M sodium acetate, pH 5.4, pelleted at 10,000 rpm in a microfuge, and resuspended in DEPC-treated water for measuring its concentration.

For Northern blots, 25 μg of either total or polyA(+) RNA was fractionated on

1% denaturing agarose gels (2.2 M formaldehyde), blotted onto nitrocellulose filters, baked at 80°C for 2 hours, and finally hybridized to radiolabeled probes according to procedures described by Thomas (1980). After hybridization, filters were washed twice with 5 × SSC (0.75 M NaCl, 0.25 M sodium citrate), 0.1% SDS for 15 min at 23°C, and twice with 0.2 × SSC (0.03 M NaCl, 0.01 M sodium citrate), 0.1% SDS for 15 min at 45°C. Filters were then air-dried and exposed to Kodak X-ray films for a period of time according to the intensity of the signals. For slot blot analysis, 5 to 10 μg of polyA(+) RNA was dissolved in 100 μl of DEPC-treated water followed by adding 60 μl of 6 × SSC and 40 μl of formaldehyde. The RNA solution was denatured at 65°C for 15 min. At the end of denaturation, the tubes were placed in ice and 100 μl of ice-cold 5 M NaCl was added. The RNA samples were then loaded onto nitrocellulose filters. The filters were then handled as for Northern blot analysis.

2.8 Contour-clamped homogeneous electric field gel electrophoresis

Intact yeast chromosomes were prepared from strain YPH149 by the agarose embedding method of Carle and Olson (1985). Cells were grown in 50 ml YEPD until late log phase. The cells were then centrifuged and washed twice with 50 mM EDTA, pH 7.5; and one time with 10 ml of SCE (1 M sorbitol, 0.1 M sodium citrate, pH 5.8; 0.01 M EDTA, pH 7.5). Cells were then resuspended in 3.25 ml of SCE and 3.0 ml of the cell suspension was mixed with 1 ml SCE, 50 μl β-mercaptoethanol, 2.0 mg Zymolase 20,000 (Kirin Brewery, Japan), and 5 ml of 1% low melting agarose in 0.125 M EDTA, pH 7.5 at 42°C. The agarose mixture was poured into a 6 cm petri plate immediately and allowed to gel at 23°C. After solidification, 5 ml of 0.45 M

EDTA (pH 9.0), 0.01 M Tris-Cl (pH 8.0), and 7.5% β-mercaptoethanol were poured on the gel. The petri plate was then sealed in a bag and incubated at 37°C overnight. After incubation, the solution was replaced by 0.45 M EDTA (pH 9.0), 0.01 M Tris-Cl (pH 8.0), 1% Sarkosyl, and 1–5 mg proteinase K. The plate was sealed in a bag again and incubated at 50°C overnight or until the agarose was clear. The solution was then replaced with 5 ml of 0.5 M EDTA, pH 9.0. At this point, the agarose was ready to be used for electrophoresis or stored at 4°C.

Yeast chromosomes were resolved by contour-clamped homogeneous electric fields (CHEF) gel electrophoresis for 20 hours in 1.2% agarose gels at 300 Volts with a switching time of sixty seconds (Chu et al., 1986). Chromosomes were then transferred to nitrocellulose filters according to Carle and Olson (1985). Hybridization with radiolabeled probes and autoradiography were performed as for Southern blotting.

2.9 RNase protection

Plasmids containing the gene of interest were linearized at a restriction site within the gene or close to the end of the gene that was far from the phage promoter used for transcription. After linearization of the plasmid, 5 μl of 20 μg/μl proteinase K was added to the reaction mixtures which were then incubated at 37°C for 1 hour to remove restriction enzymes and RNases. Phenol:chloroform (1:1) purification was performed and DNA was precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol. These linearized plasmids were used as templates to prepare radioactive RNA probes. 1 μg of plasmid DNA was resuspended in 1 μl of DEPC-treated sterile water. 5 μl of 5× reaction buffer, 0.5 μl of 1 M DTT, 0.5 μl of 40 units/μl RNasin (Promega), 2.5 μl of a 1 mM/ml solution of all four nucleotides, 10 μl of sterile distilled water, 0.5 μl of T7 or SP6 polymerase, and 5 μl of ^{32}P-UTP were added and incubated at 37°C for 1 hour. After the RNA probe was synthesized, 225 μl of DNase buffer (40 mM Tris-HCl, pH 7.9, 10 mM NaCl, 6 mM magnesium chloride) and 1 μl of RNase-free DNase (1 mg/ml) were added and incubated at 37°C for 15 min to remove the template DNA. Phenol:chloroform (1:1) extraction and ethanol precipitations were performed, and the ethanol suspension was left at −20°C for 1 hour. The probe (100,000 cpm) was then resuspended in 30 μl of sterile distilled water. The hybridization procedure was performed by coprecipitation of 20 μg of isolated yeast RNA and 1 μl of probe. The RNA mixture was resuspended in 30 μl of hybridization buffer (80% formamide, 0.4 M NaCl, 0.04 M PIPES) and incubated at 90°C for denaturation and then transferred to 55°C overnight. After overnight incubation, 300 μl of TE (pH 8.0), 1.2 μl of 10 mg/ml RNaseA, and 0.6 μl of 1.1 mg/ml of RNaseT1 were added and the solution was incubated at 37°C for 1 hour to remove unhybridized and single-stranded RNA. The enzymes were then removed by treatment with 2.5 μl of proteinase K (20 mg/ml) and 20 μl of 10% SDS at 37° for 15 min. The suspension was then phenol:chloroform (1:1)-extracted and ethanol-precipitated. The RNA pellet was collected and washed with 70% ethanol. The pellet was then resuspended in 3 μl of loading buffer (90% formamide, 10 mM EDTA, dye). The suspension was denatured at 90°C for 2 min and loaded onto a 6% urea-polyacrylamide gel immediately. The gel was run at 30 W for 4 hours, then autoradiographed.

2.10 DNA sequencing

The region of the SPR9 locus between ClaI and SstI was sequenced by the dideoxy method of Sanger et al. (1977). Three strategies were used to generate the subclones for sequencing. First, several well-characterized restriction fragments were subcloned into replicative forms of M13mp18 and M13mp19 (Boehringer Mannheim). Second, the HindIII-SstI fragment was digested with Sau3A and subcloning the generated fragments into both phage vectors at appropriate sites. Third, specific oligomers were synthesized and used as primers to fill in the remaining gaps in the sequence. Single stranded templates were prepared from insert containing M13mp18 or M13mp19 phage vectors by the procedure of Messing and Vieira (1982). The sequencing reactions were prepared by using the Sequenase 2.0 version kit (United States Biochemical Corp.) under the supplier's conditions.

Table 2.2: List of media.

Media	Ingredients	Reference
YEPD	1% yeast extract, 2% peptone, 1% dextrose	Fink, 1970
MIN	1% dextrose, 0.67% yeast nitrogen base w/o amino acids	Sherman et al., 1981
YEPGlycerol	1% yeast extract, 2% peptone, 1% Glycerol	Sherman et al., 1981
PSP2	1% KOAc, 0.67% yeast nitrogen base w/o amino acids, 5% phthalic acid, pH 5.5	Roth and Halvorson, 1969
SP2	1% KOAc, 0.2% raffinose	Hopper et al., 1974
LB	1% peptone, 0.5% yeast extract, 1% NaCl	Maniatis et al., 1982

CHAPTER III

Analysis of the Sporulation-Specific Gene, *SPR9*

3.1 Cloning and mapping of *SPR9*

The *SPR* genes were isolated from a genomic library of *Saccharomyces cerevisiae* by Clancy et al. (1983). These SPR genes were identified by their ability to be transcribed preferentially during sporulation. The Lambda phage clone 104 carries the *SPR9* sequence whose transcript lies within a 5.7 kb ClaI fragment. This ClaI fragment was cloned into a pBR322 vector to give plasmid p803 (Figure 2.2) which was then mapped with several restriction enzymes (Figure 3.1).

3.2 Developmental regulation of *SPR9*

Some genes may be turned on during sporulation as a physiological response to the change in conditions that are needed to induce sporulation, and they may not be involved in meiosis or spore formation. These genes would be transcribed in nonsporulating cells in the same changing conditions. On the other hand, there are other genes that are essential for sporulation to occur, and they may be sporulation-specific or nonsporulation-specific genes. For example, many genes that control the cell division cycle, mating type, radiation sensitivity, etc. have been shown to be essential for sporulation (Dawes,1983). It is important to distinguish genes expressed

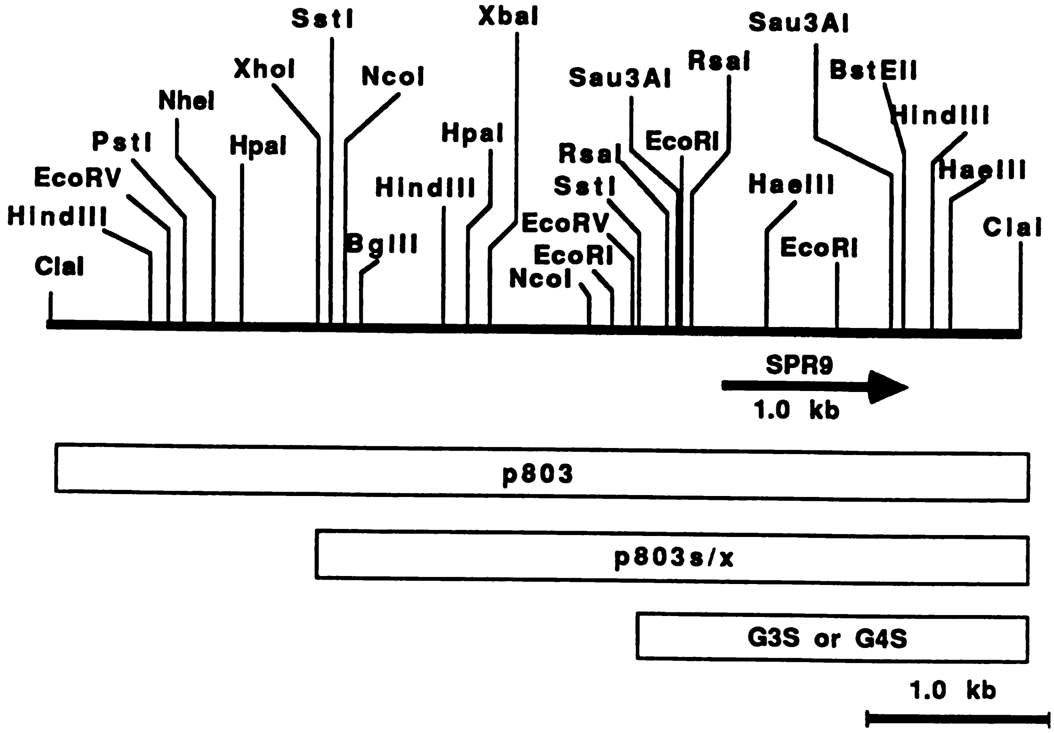


Figure 3.1: The restriction map of the *SPR9* locus.

The 5.8 kb ClaI fragment was cloned into pBR322 to produce plasmid p803. p803 was then digested with enzymes XhoI and SalI to produce p803s/x (SalI site is on pBR322). The arrow represents the transcript of *SPR9* gene.

during sporulation from those induced by sporulation conditions or by shock such as the heat shock gene, *HSP26* (Petko and Lindquist, 1986).

To prove that *SPR9* gene is not turned on solely due to environmental stress but only during sporulation, we used the following approach. First, polyA(+) RNA was prepared for slot blot analysis from strain W66-8A (**a**/α) grown at 23°C in rich medium with and without shifting to 39°C for one hour of heat shock. PolyA(+) RNA from vegetative cells (grown in PSP2) and 10 hour sporulating cells (grown in SP2) was also prepared for slot blot analysis to show that *HSP26* was turned on by sporulation. The same filter was probed sequentially with three different radiolabeled plasmids: p803 for detecting *SPR9* message, pHSP26 for detecting *HSP26* message, and pGAP for detecting the message of glyceraldehyde-3-phosphate dehydrogenase gene as a positive control. The result shows that both the *SPR9* and *HSP26* genes are turned on by sporulation as predicted, and trace *SPR9* transcripts are detected after heat shock treatment (Figure 3.2a).

To induce sporulation, a nitrogen-source is limiting in the medium. Therefore, genes that are turned on by nitrogen-starvation are also expressed in sporulation medium (Primerano, unpublished data). Since these genes will also be expressed in asporogenous diploid strains grown in sporulation medium, polyA(+) RNA was prepared from both vegetatively grown and sporulation-induced cells of strains AP1 α/α, and AP3 **a/a** for slot blot analysis. Radiolabeled p803 and pGAP were used as probes. No *SPR9* message was detected from those two strains under vegetative growth or sporulation induction (Figure 3.2b). Therefore, we conclude that the *SPR9* gene is not responding solely to environment changes or nitrogen starvation but is a developmentally regulated gene.

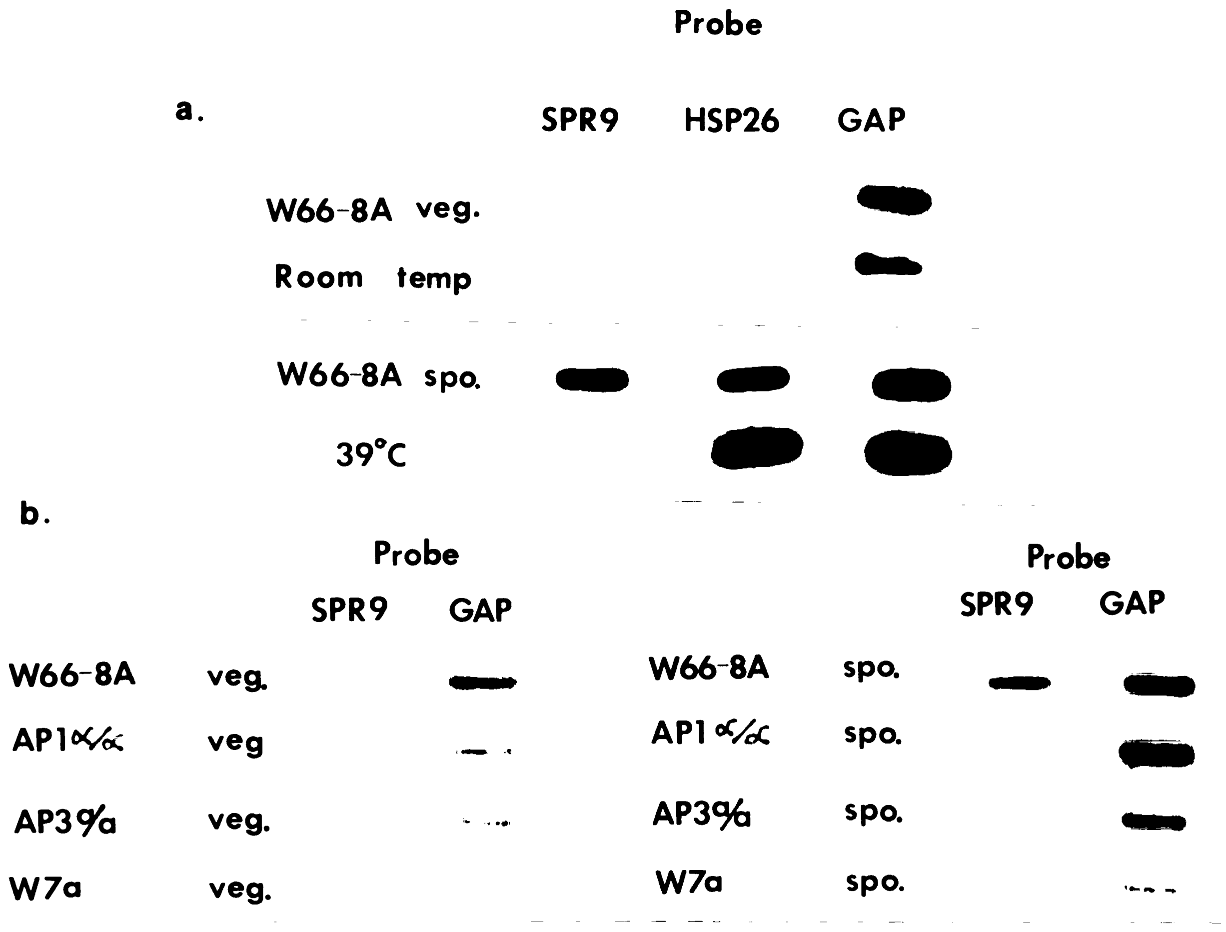


Figure 3.2: Slot blot analysis of the *SPR9* transcripts.

a. The response of the *SPR9* gene to heat shock at 39°C. A diploid strain W66-8A (a/α) was grown at 23°C in YEPD until stationary phase. For heat shock treatment, part of the culture at stationary phase was grown at 39°C for another hour. PolyA(+) RNA was prepared from both cultures for slot blot analysis. PolyA(+) RNA was also prepared from the same strain grown in presporulation medium (veg.) and sporulation medium (spo.). Radiolabeled p803, pHSP26, and pGAP were used as probes. b. The response of the *SPR9* gene to nitrogen starvation. PolyA(+) RNA was prepared from three diploid strains W66-8A (a/α), AP1(α/α), and AP3 (a/a) grown in presporulation (veg.) and sporulation medium (spo.) for dot blot analysis. Radiolabeled p803 and pGAP were used as probes. W7a (a/α) is a diploid strain which is defective at both *SPR9* alleles.

3.3 Kinetics of the transcription of *SPR9*

The dot blot analysis of Holaway et al. (1985) suggests that *SPR9* is a middle gene which is turned on at about the time of DNA synthesis, maximally transcribed at meiosis II, and the mRNA present for at least the next 3 h. Since it is impossible to tell by dot blotting if the message is intact or degraded, we analyzed the RNA by hybridization of Northern blots. In this study, polyA(+) RNA was prepared from vegetative cells (0 h) and sporulating cells of an **a**/α diploid strain, W66-8A, at different times (2 h, 4 h, 6 h, 8 h, 10 h, 15 h, and 20 h cells in sporulation medium). In order to be certain that the extracted RNA was from sporulating cells, the percentage of asci in the cultures was determined at the time of extraction by counting 200 cells under a light microscope. Before RNA extraction, a 5 ml culture of each time point was saved and further incubated to determine the sporulation efficiency after 24 h incubation in sporulation medium (Figure 3.3). The RNA and polyA(+) RNA were prepared as described in Materials and Methods. Both the total RNA and polyA(+) RNA were loaded onto a 1% denaturing gel. The filters were first hybridized to a 0.9 kb EcoRI fragment containing part of the *SPR9* coding sequence to show the pattern of transcripts (Figure 3.3). A 1.0 kb transcript could be detected by this probe. Transcripts of the *SPR9* gene begin to appear 4 h after transfer to sporulation medium; they increase in abundance as sporulation proceeds. They remain abundant after 20 h in sporulation medium. The same filter was then probed with pGAP as a control to show that equal amounts of RNA sample were loaded in each lane of the gel (Figure 3.3). A Northern blot of total RNA was prepared using RNA extracted from the same cultures used for preparing polyA(+) RNA. This blot was hybridized to the radiolabeled 0.9 kb EcoRI fragment. There was no detectable difference in the patterns of transcript abundance whether total RNA or polyA(+) RNA was used (data not shown).

3.4 Chromosomal location of *SPR9*

The chromosomal location of the *SPR9* gene was determined by CHEF (Chu et al., 1986). The chromosomes of strain YPH149 were separated and blotted as described in Materials and Methods. The filter was then cut into three strips, and these strips were hybridized to different probes: radiolabeled plasmid YIp333 which carries a chromosome II marker, *LYS2*; a radiolabeled 0.9 kb EcoRI fragment containing part of the *SPR9*; and both *LYS2* and *SPR9*. The result is depicted in Figure 3.4; the picture of the CHEF gel is at the top of the figure and the picture of the filter is at the bottom. Both probes hybridized to chromosome II despite the fact that YIp333 also hybridized to two other chromosomal bands, $11a'$ and $11a''$. To make YPH149 useful for CHEF, chromosome VII was separated into two fragments (chromosomal bands $11a'$ and $11a''$) (Hieter, 1989, unpublished data) by attaching telomere sequences to the broken ends of chromosome fragments, which resolve from chromosome XV (chromosome band 11b). Both chromosome fragments also contained part of the pBR322 sequences from the plasmid carrying the telomere sequence during the construction process, so when YIp333, a pBR322 derivative, was used as a probe both bands hybridized.

3.5 Determination of the sense strand of *SPR9*

The orientation of the transcription of the *SPR9* gene was determined by RNase protection by hybridizing the polyA(+) RNA from vegetative cells (grown in PSP2 medium) and sporulating cells (10 hours in SP2 medium) of strain W66-8A to two

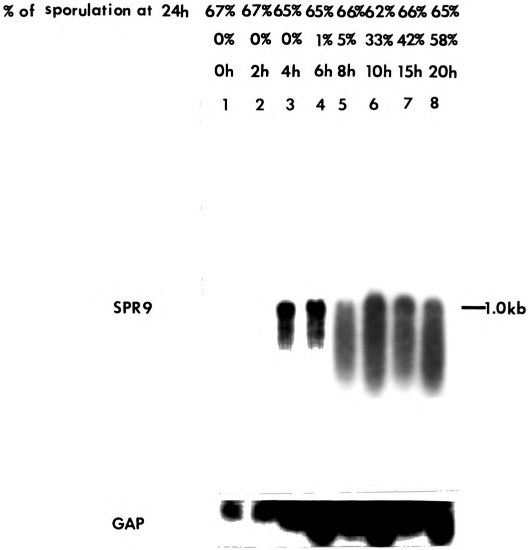


Figure 3.3: The kinetics of the appearance of *SPR9*.

PolyA(+) RNA was prepared from vegetative cells (0 h) and sporulating cells of an **a**/α diploid strain, W66-8A, at different times (2 h, 4 h, 6 h, 8 h, 10 h, 15 h, and 20 h cells in sporulation medium) for Northern blot analysis. Sporulation efficiency of cultures used to prepare RNA was examined at the time of extraction and 24 h after transfer to sporulation medium. The filter was first hybridized to a radiolabeled 0.9 kb EcoRI fragment containing part of the *SPR9* coding sequence, then to radiolabeled pGAP.

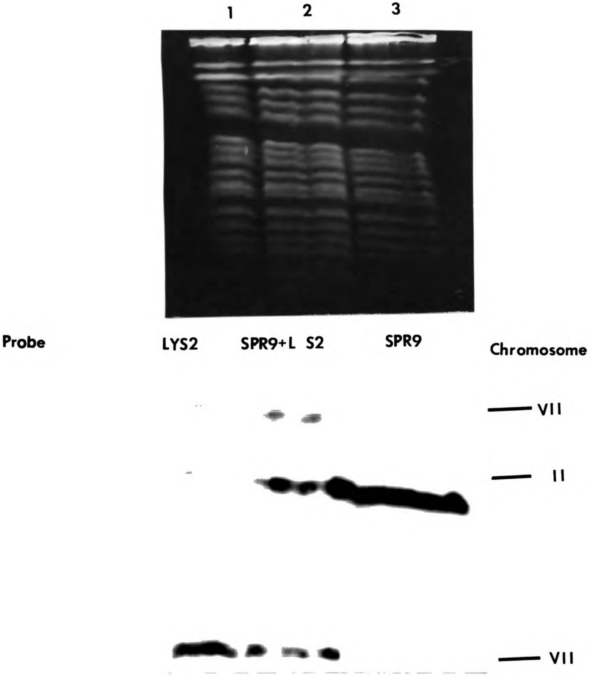


Figure 3.4: The chromosomal location of the *SPR9* locus.

The chromosomes of strain YPH149 were separated by CHEF (contour-clamped homogeneous electric field gel electrophoresis) and then blotted onto a filter. The picture of the CHEF gel is at the top of the figure. The filter was cut into three strips which in turn were hybridized to different radiolabeled probes: YIp333, mixtures of YIp333 and 0.9 kb EcoRI fragment, and 0.9 kb EcoRI fragment alone.

riboprobes. The two riboprobes were made from the two complementary strands of a region encoding the *SPR9* transcript. A 2.2 kb ClaI-SstI fragment was force-cloned into a GEM3 or a GEM4 plasmid (from Promega company) at AccI and SstI sites. The GEM plasmids contain two viral promoters, T7 and SP6, that can produce two complementary run-off transcripts from the DNA fragment inserted. The resulting G3S plasmid was linearized at the HindIII site for the T7 promoter and at the SstI site for the SP6 promoter. The transcripts from the T7 and SP6 promoters are 1800 and 2200 bases long, respectively. The template region of G3S and the transcripts from the SP6 and the T7 promoters are shown in Figure 3.5a. After hybridization, the RNA-RNA hybrids were treated with RNaseA and RNaseH to remove all single stranded RNA. The samples were analyzed on a 6% urea-polyacrylamide gel following RNase treatment. The result is shown in Figure 3.5b. The marker DNA was at lane 1. Lanes 2 and 3, which showed no protection of *SPR9* transcripts, were hybridized to the riboprobe from the T7 promoter. Lanes 4 and 5 were hybridized to SP6 transcripts, and only the RNA from sporulating cells but not vegetative cells was protected from RNase digestion. The protected band is about 1.0 kb in size, and the RNA sample shows some degradation. This result indicates that the strand used for *SPR9* transcription is the one complementary to the transcript made from SP6 promoter. The data also confirm that the *SPR9* transcripts are made only during sporulation.

3.6 DNA sequencing of *SPR9* locus

The *SPR9* gene was sequenced by M13 dideoxy-sequencing to gain information about its protein product. The strategies used are illustrated in Figure 3.6. For the first strategy, plasmid 803 was digested with EcoRI restriction enzyme to generate

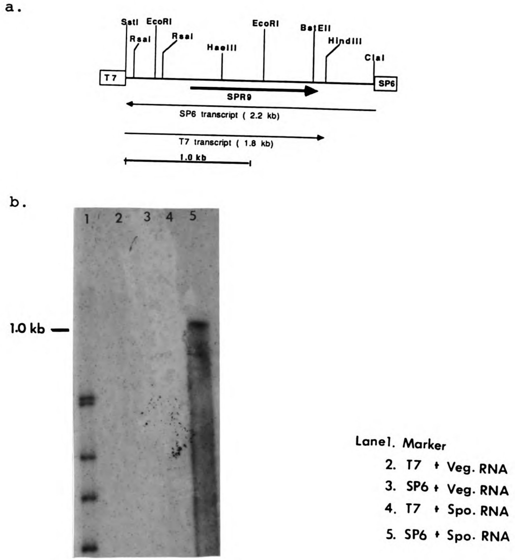


Figure 3.5: Determination of the sense strand of *SPR9*.

a. The template region of G3S and the transcripts made from the SP6 and the T7 promoters. b. The result of RNase protection analysis of *SPR9* transcript on a 6% urea-polyacrylamide gel. Lane 1 is the radiolabeled marker DNA. Lanes 2 and 3 are the hybridization mixture of vegetative RNA from W66-8A (**a**/α) and RNA transcripts from T7 and SP6, respectively. Lanes 4 and 5 are the sporulating RNA of W66-8A hybridized to the RNA transcripts from T7 and SP6, respectively.

four fragments. They are 0.4 kb, 0.9 kb, 1.0 kb, together with a 8.0 kb fragment containing pBR322 sequences. The 0.4 kb, 0.9 kb, and 1.0 kb fragments were ligated to both M13mp18 and M13mp19 linearized at the EcoRI site. For the second strategy, a 3.0 kb HindIII fragment containing the *SPR9* gene from plasmid p803 was isolated and further digested with SstI restriction enzyme. The resulting fragments were either ligated to the HindIII-SstI linearized M13mp18 and M13mp19 or further digested with Sau3A, and then cloned into the two M13 vectors. For the gap that could not be sequenced by the strategies described above, two 17-mers (-TGGAGTCCTTCTTCCCC-) and (-GCAAGAGCCTTCCCCCA-), were synthesized and used as primers to obtain the entire sequence of the *SPR9* gene.

The nucleotide sequence and the deduced open reading frame (ORF) are shown in Figure 3.7. Within this 2.5 kb sequence, one long ORF from position +1 to +435, encoding a 16.8 kD protein is found. Two possible TATA boxes (TATAAA) are found 93 bp and 174 bp upstream from the putative AUG initiation site, and there is another degenerate possible TATA box, TATAAC (5/6 matches) at position -45. When the upstream sequence was compared to that of other sporulation-specific genes, no unique consensus sequence was found for all the *SPR* genes sequenced. However, pairwise homology in different upstream regions between *SPR1* and *SPR2*, and *SPR2* and *SPR9* was observed (Figure 3.8). There also has been no significant homology found to any DNA sequence in the GENBANK library (release 54) or to any protein sequence in the PIR15 library (release 15). The sequence, AATAAA or AATATA, suggested by Fitzgerald and Shenk (1981) for the addition of polyadenylic acid tracts appeared twice after the termination codon. Both of them, +563 to +568 and +681 to +686, would produce transcripts that were much shorter than 1.0 kb. The predicted protein is composed mostly of hydrophilic amino acids.

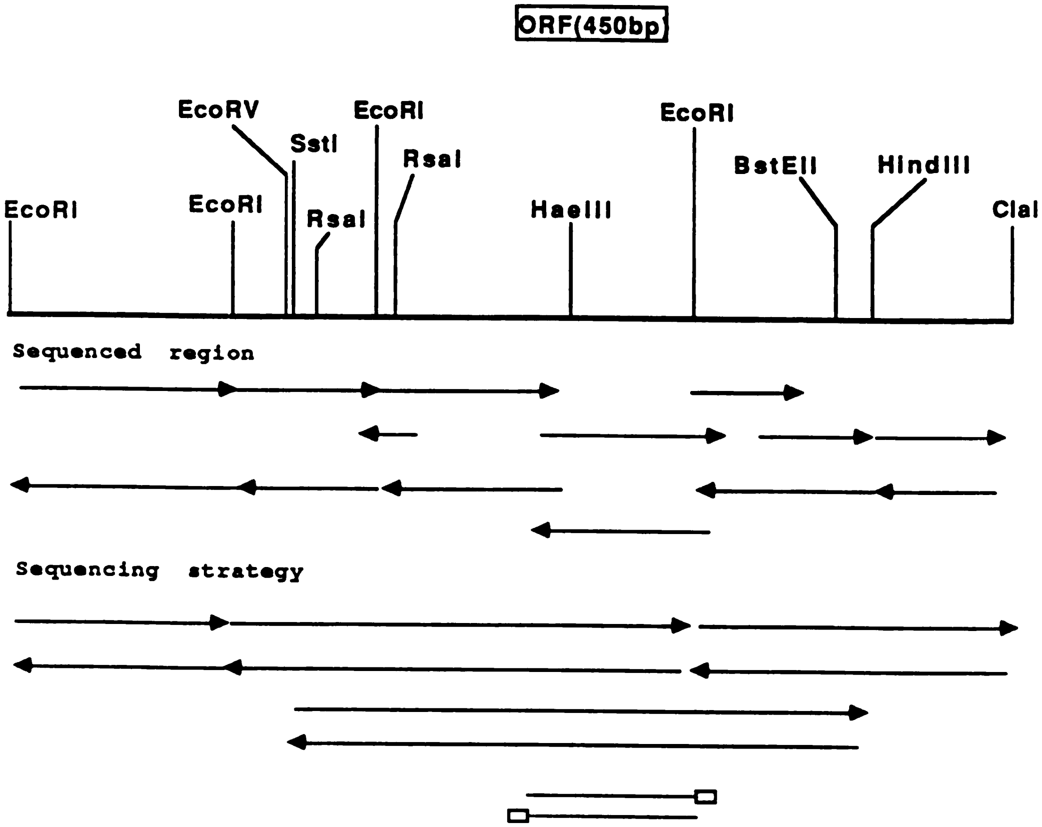


Figure 3.6: The sequencing strategies for the *SPR9* locus and flanking regions. ORF is the potential open reading frame for *SPR9* protein. The open bars are the synthetic primers.

```
-688  GCCCTCAAAG  CCTTATAAGA  AGAACCCTTT  CATGAAGTTT  TTTGAAATTG
-638  GGTTCAAGAT  ATCGCTGAGC  TCAGAATCTA  TCTCTACAAC  AATTCGTATA
-588  CAAAGAACCC  ATTATAGAAG  AGTATAGTGT  AGCGGCTAGT  ATATACCGTT
-538  TACATTCAGC  AGATTTATGC  GAACTTCTAA  GAAACAGTGT  CATAACCAGT
-488  GGATTTTCAA  GTACCCTAAA  GAACAAATGG  CTCGAACAGT  TTCTTAGCCA
-438  GCCATGACTA  CTTCGTGGAA  AACACTGGGT  TTATAGACAA  GTGGTATGAT
-388  TGTAAACCAA  ATACAAGTTT  AGAGCATAAC  GTTCCGATCA  TCAGACGTCA
-338  ATATCGCAGT  AGCACTCTCG  CAGGGGAGTG  GCGACTCATA  ATTGCGTGAA
-288  TTCGCTCATG  TTTCTTCGAG  GGGCGCATAT  TGTGACGCTA  ATATTATGTA
-238  CCGCCCTTCC  CCACACACAC  AAAAAAAAAA  GGTTAAGGAT  AAAGATAAGT
-188  AAACTAATGG  AATATATAAA  CAAATAAGTA  AGTAGATACT  GTAAATAGAA
-138  CACACGTTTT  TTAATTTATT  TTTTTTTTTA  CATATCTTAA  TTTAATATAA
 -88  ATCAAAAAGC  CCAAGCCTCA  AACAAGAGTC  TTATCACTCC  ATTTATAACA
 -38  TAACAATTCG  AAATCTCTAA  GCTCGCTTAT  AGTTGACA

   1  ATG ACC ATA TTT TCT AGG TTC TCA TAT TTT GAT AGT CTA TTC TCC
   1  Met Thr Ile Phe Ser Arg Phe Ser Tyr Phe Asp Ser Leu Phe Ser
  46  TTT AAA AAG CAA GAG CCT TCC CCC ATT GAA ATA ATC TAT TGC AAT
  16  Phe Lys Lys Gln Glu Pro Ser Pro Ile Glu Ile Ile Tyr Cys Asn
  91  GAA AAC AAT GGT TTC ATA AAC ATA AAA TCC TTA GAA AGC CCC ACT
  31  Glu Asn Asn Gly Phe Ile Asn Ile Lys Ser Leu Glu Ser Pro Thr
 136  GAC GAT AGC ATG GAG GCG GAC ATT TCA GAT AGA GAA ATG GCT ACA
  46  Asp Asp Ser Met Glu Ala Asp Ile Ser Asp Arg Glu Met Ala Thr
 181  ATC CTG ACT AGA AAC AGA AAC AAT TTG GGT AAG GTT GCC ATT GAT
  61  Ile Leu Thr Arg Asn Arg Asn Asn Leu Gly Lys Val Ala Ile Asp
 226  AAG AAA GGC GTT AAT AAT CAT TGC ATA GAT TTG AAT GAG CTG AAA
  76  Lys Lys Gly Val Asn Asn His Cys Ile Asp Leu Asn Glu Leu Lys
 271  AAG GGC CTT GTC GCC AAT GAA CAC AAA TTG GAT AAC GAT AAC TCA
  91  Lys Gly Leu Val Ala Asn Glu His Lys Leu Asp Asn Asp Asn Ser
 316  ACA CGG CAC CAG AAC ACA TAT TCA CCA GAG GAT TCC GTG GAA TTT
 106  Thr Arg His Gln Asn Thr Tyr Ser Pro Glu Asp Ser Val Glu Phe
 361  GAT AGG TTC GAT GAT AAA CAA AGC AGA ATA CTA AAG TGT AGC ACT
 121  Asp Arg Phe Asp Asp Lys Gln Ser Arg Ile Leu Lys Cys Ser Thr
 406  AGA AGG AGC TAC TTG AGA TAC AAG AAA TAA
 136  Arg Arg Ser Tyr Leu Arg Tyr Lys Lys ***

 437  GACAATTGAG  CTGCTTCATT  ATATATCTTG  ATATAATCAC  CTTTCTTATC
 487  GTCAGAAAGA  GTGTTCTCTA  TGTTTTTGTG  CTTCCATAAA  TATATAGTTC
 537  AAAAAGTAAT  ATCATATACT  ATTAATAATA  AAAGTTATAC  TGAATATATT
 587  GATTAGTTCT  ATTAATTACT  ATTGACACAC  TTTGAATTCC  CTAAATTAAA
 637  CCTCAGGAAG  AAGGGGTTCT  TAGGAAAATT  TTTTCTTTTT  TAATATAAAA
 687  GCGAGGAACA  ATGGATGGGA  ACAAAACCAT  GTTCAGCCTT  CAACAATTGG
 737  TAGAAGAAGC  CAAAAAAAAA  AAAAACAGAA  ACGCCAACCA  TGCACTTTTC
 787  TTTGAAGCAA  TTGCGTGTCT  CGGTTTACGC  TACCAATTTA  GCTCTGCCTA
 837  TGTCATTCCT  CAATTCTTCC  AGGAAGCCTT  TCAACAAGAA  GAGCCAATTG
 887  AGAATTATCT  TCCGCAATTA  AACGACGATG  ATAGCTCTGC  CGTTGCTGCC
 937  AACATTCCGA  AGCCACATAT  TCCTTACTTC  ATGAAACCAC  ATGTAGAAAG
 987  TGAAAAATTG  CAAGATAAAA  TCAAAGTTGA  CGATTTGAAT  GCTACTGCTT
```

Figure 3.7: The nucleotide and deduced amino acid sequence of the *SPR9* locus.

Underlined are the potential TATA sequences. ... are possible polyadenylation sites. *** is the termination codon for translation.

```
       SPR1

-671   ACTTTCTTGGGACATACCACAATTAC
       ***** **   ***** **** ****
-333   ACTTTGTTAATACATATCACAGTTAC

       SPR2


       SPR9

-158   TTTTTTTTTACATATCTTAATTTAATATAA
       *** **  ******** * **  **** *
-331   TTTGTTAATACATATCACAGTTACATATTA

       SPR2
```

Figure 3.8: The homologous sequences in the upstream regions of the *SPR* genes. The homologous regions are found in the upstream regions between the *SPR1* and *SPR9* genes and between the *SPR2* and *SPR9* genes. * represents the matching nucleotides for the two sequences.

CHAPTER IV

Phenotypic Analysis of Three *SPR* Genes, *SPR1*, *SPR2*, and *SPR9*

4.1 Disruptions of *SPR1, 2,* and *9*

In order to get insight into the functions of the *SPR* genes during sporulation, the *SPR* genes were mutated by one-step gene disruption which replaces part of the gene of interest with a marker gene (Rothstein, 1983). In this technique, the DNA fragment containing the disrupted gene is transformed into a diploid *HO* yeast strain deficient for the marker gene. Since the DNA fragments can recombine with homologous sequences in the genome, one copy of the wild-type chromosomal sequence of the gene of interest can be replaced by the disrupted sequence and the transformants can be selected by complementation of their auxotrophic phenotype by the marker gene (Figure 4.1). The transformants thus produced are heterozygous at the disrupted locus and can sporulate to produce two wild-type spores and two spores mutant at that locus. Each spore will diploidize due to the presence of the *HO* gene (Herskowitz and Oshima, 1981), and the ability of the resulting $\mathbf{a}/\alpha$ homozygous *spr* mutant strains to sporulate can be tested. If they do sporulate, their spores can be isolated for further examination.

The *SPR1* and *SPR2* loci were mutated by Primerano et al. (1990 and

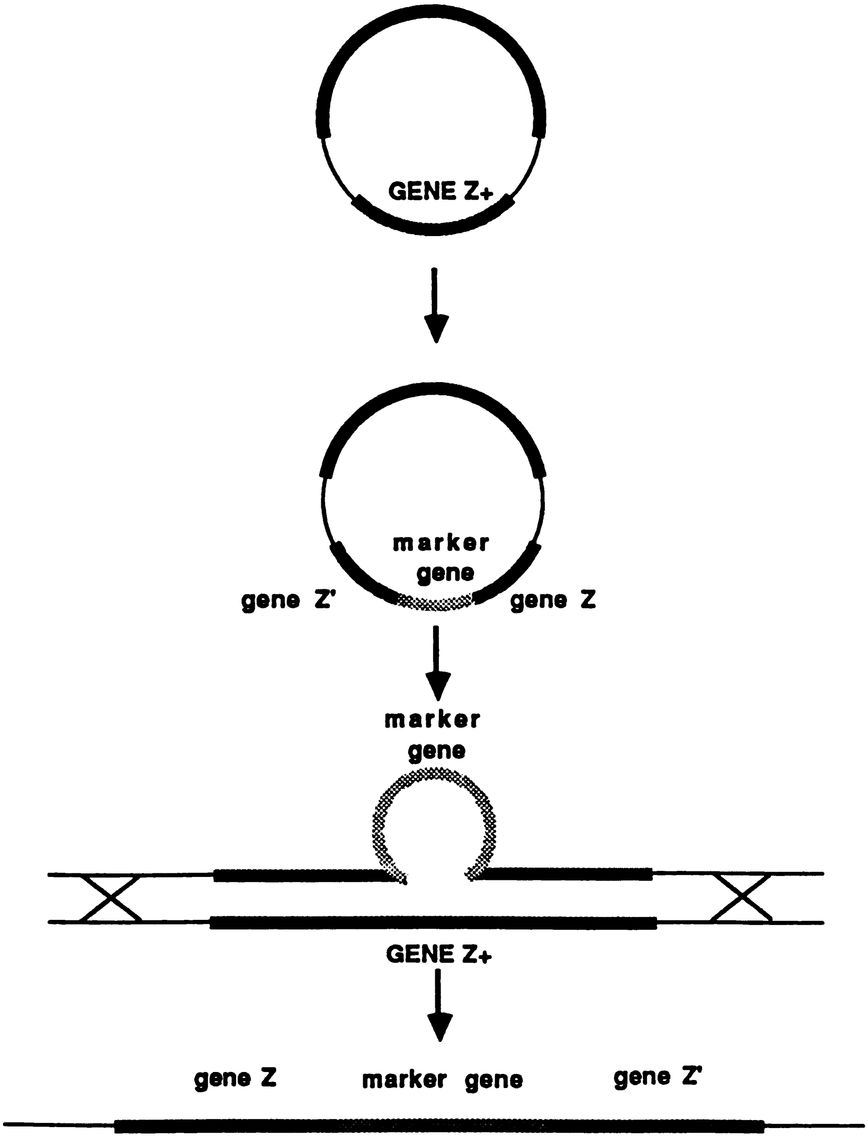


Figure 4.1: One step gene disruption.

The cloned fragment containing Gene Z+ is digested with a restriction enzyme that cleaves within the coding region of the gene z. A fragment containing a selectable yeast gene (marker gene) is cloned into the site. The fragment containing the disrupted gene Z is liberated from the plasmid, and the liberated fragment retains homology to the Gene Z region on both sides of the insert. Transformation of the yeast cells with the linear fragment results in the substitution of the linear disrupted sequence for the resident chromosomal sequence. (Rothstein, 1983) unpublished data), and these transplacements are shown in Figure 4.2. For the *SPR9* gene replacement, the 3.0 kb region between two HindIII sites was replaced by a 1.1 kb HindIII fragment carrying the *URA3* gene to create the *spr9* null mutant allele. The 3.5 kb ClaI fragment with the missing *SPR9* locus was then introduced into W66-8A, an *HO ura3* diploid strain (Figure 4.2c). Transformants carrying the *spr9*::*URA3* allele were selected on minimal medium containing all the required supplements except uracil. Southern blot analysis of their genomic DNA confirmed that the gene was replaced by the marker gene. The heterozygous *SPR9* /*spr9* transformant was sporulated and the asci dissected into individual spores by micromanipulation. The *URA3* spore grew into homozygous *spr9* diploids. The genotype of homozygous *spr9* diploids was also confirmed by Southern blot analysis. Figure 4.3a shows the genomic DNA patterns of the *SPR9* locus in the wild-type (W66-8A), the heterozygous (W7), and homozygous mutant strains (W7a). The wild-type *SPR9* locus gives rise to a 5.8 kb ClaI fragment, while the *spr9*::*URA3* mutant ClaI fragment is 3.5 kb when probed with a nick-translated 0.9 kb EcoRI fragment containing the *SPR9* gene.

The gene replacement for *SPR1* and *SPR2* genes was done by Primerano et al. (1990, and unpublished data). For the *SPR1* locus, the genomic DNA was digested with XhoI restriction enzyme and detected by nick-translated pCG33 probe (carrying the wild-type *SPR1* and flanking sequence). The wild-type *SPR1* locus lies on a 4.4 kb XhoI fragment, while the disrupted *spr1-2*::*LEU2* allele gives a 6.6 kb XhoI fragment. Therefore, the wild-type strain SCMS-7 should show only the 4.4 kb band, the mutant strain (S52B) should show a 6.6 kb band, and the heterozygous strain (SPB1) should show both bands (Figure 4.3b). For the *SPR2* locus, the genomic DNA pattern was generated by HindIII restriction enzyme and probed with nick-translated pΔCI (carrying the wild-type *SPR2* and flanking sequences). The

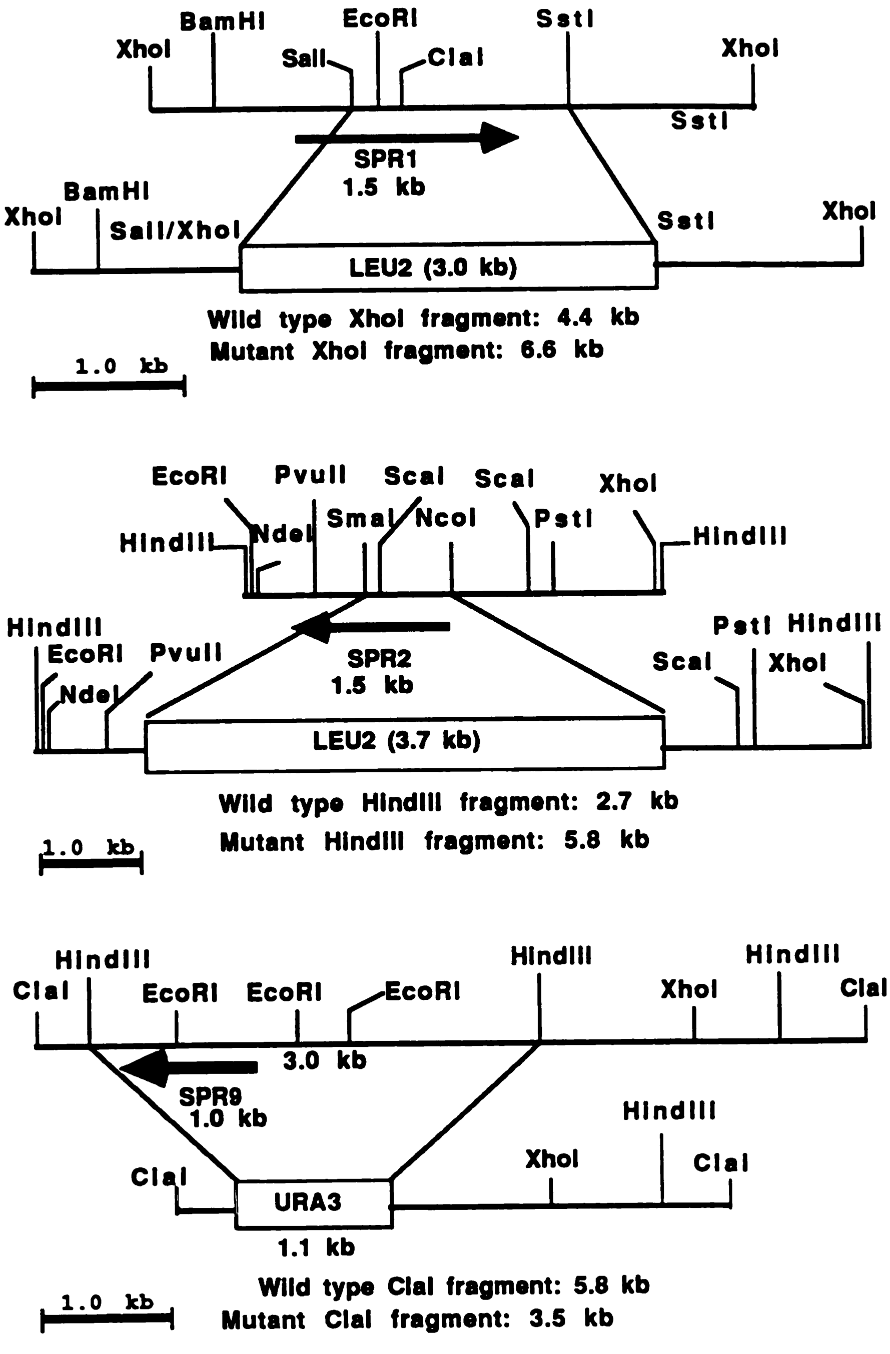


Figure 4.2: Disruption of the three *SPR* genes.

The restriction maps of wild-type loci and mutant loci for: a. *SPR1* gene; b. *SPR2* gene; and c. *SPR9* gene. The arrows represent the transcripts of the *SPR* genes.

wild-type *SPR2* gene gives a 2.7 kb HindIII fragment, a 5.8 kb fragment for the *spr2::LEU2* mutant strain (T4C), and both for the heterozygous strain (AKAS2) (Figure 4.3c).

4.2 Detection of *SPR* messages in single mutants

To make sure that there are no *SPR* transcripts made in these *spr* mutants, total RNA was prepared from the *spr* strains. It was proven previously by Primerano et al. (1990 and unpublished data) that there are no *SPR1* and *SPR2* messages in S52B (*spr1-2/spr1-2*) and T4C (*spr2/spr2*) strains, respectively. For W7a (*spr9/spr9*), the poly A(+) RNA was prepared and hybridized with the radiolabeled 0.9 kb EcoRI fragment carrying a part of the *SPR9* gene as probe. As shown in Figure 3.2b, *SPR9* transcripts can be detected in the sporulating wild-type strain, while *SPR9* transcripts are seen in neither the W7a vegetative nor sporulating cells. The behavior of cells without one or more of the *SPR* gene product was then examined.

4.3 Phenotype detection of *SPR9*

To determine the function of the gene product of *SPR9*, the behavior of the homozygous *spr9* mutant strain during vegetative growth and during and after sporulation was examined. We first examined vegetative growth of wild-type and the homozygous *spr9* mutant strains. The growth rate of W7a (*spr9/spr9*) at 23°C, 30°C, and 34°C was compared to its isogenic parent, W66-8A. There was no obvious difference in growth rate at any of the temperatures tested, as shown in Figure 4.4. This result suggests that the *SPR9* function is not needed for vegetative growth. We then asked whether or not spore formation was affected by the *spr9* mutation at 30°C. Not only were four-spore asci formed with about the same kinetics as

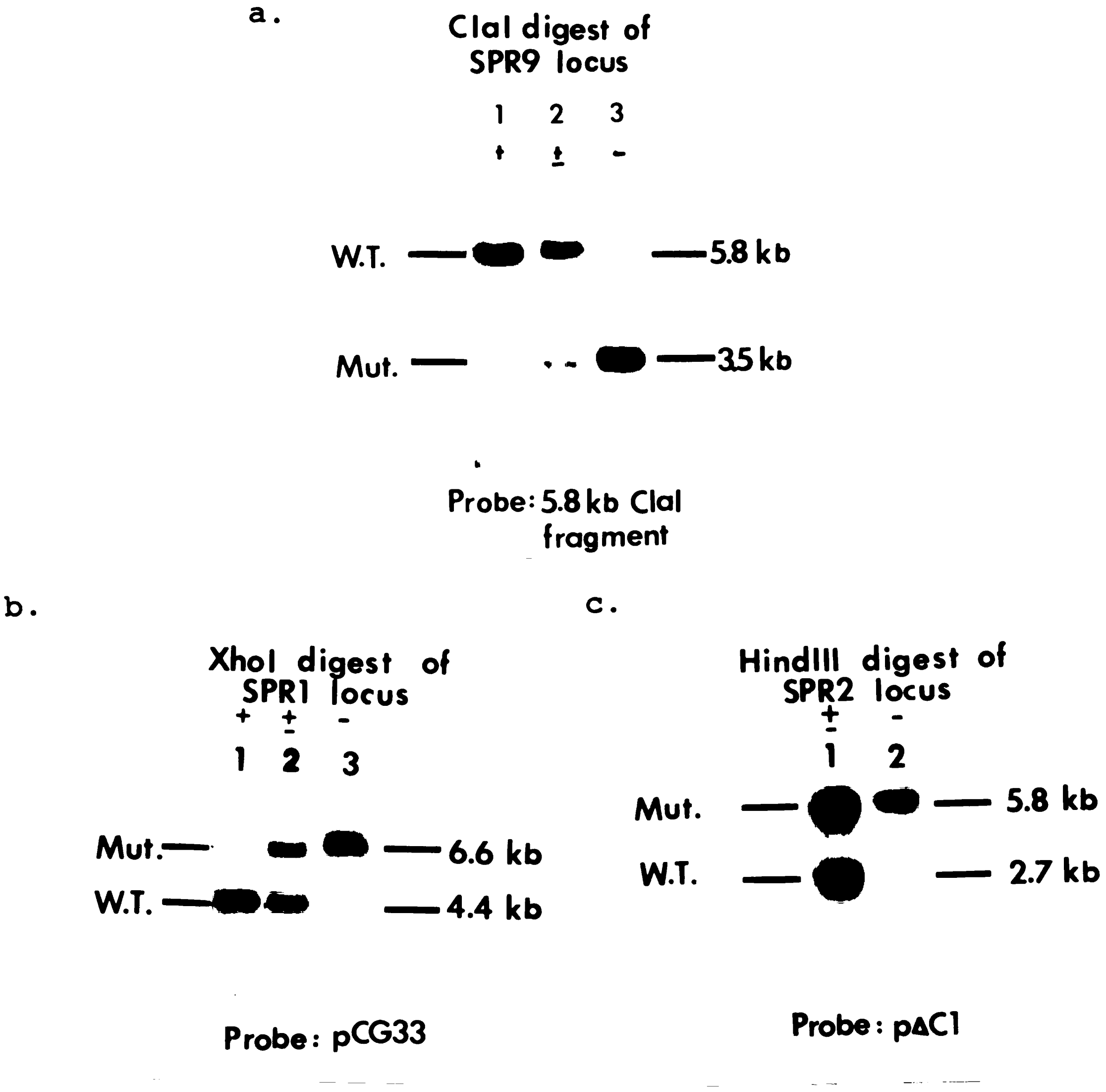


Figure 4.3: Southern blot analysis of the three *SPR* loci.

a. The genomic DNA from three strains W66-8A (lane 1), W7 (lane 2), and W7a (lane 3) was digested by ClaI enzyme for Southern blot analysis. The filter was hybridized to a radiolabeled 5.8 kb ClaI fragment for the detection of the *SPR9* locus. b. shows the detection of the *SPR1* locus in strain SCMS (lane 1), SPB1 (lane 2), and S52B (lane 3). Radiolabeled pCG33 was used as probe. The genomic DNA was digested by XhoI enzyme. c. shows the detection of the *SPR2* locus in strain SCMS (lane 1), AKAS2 (lane 2), and T4C (lane 3). The genomic DNA was digested by HindIII enzyme. Radiolabeled pΔCI was used as probe. +: wild-type band, -: mutant band.

wild-type, but also the efficiency of sporulation did not change because of the missing *SPR9* function (Figure 4.5). Both samples of sporulating cultures were also stained with DAPI at different stages for examining nuclei of the cells and examined under the phase-contrast microscope, no difference was seen among them (data not shown). These observations indicated that the *SPR9* gene may not be involved directly in the formation of spores. A similar situation has also been found in bacterial sporulation (Dion and Mandelstam, 1980; Moir, 1981). In *B. subtilis* some events occurring during sporulation are not essential for spore formation, but are essential for the subsequent germination or outgrowth steps during which the spores return to the vegetative state.

To investigate whether the *SPR9* gene is involved in events after spore maturation (such as germination) or in maturation of the spore itself (such as resistance to heat and chemicals), spores at different stages (16, 20, and 24 hours in sporulation medium) were isolated for tests. In some experiments, only spores isolated 16 hour after transfer to sporulation medium were used for examinations. Sixteen hour spores of the strains used in this study are at that stage most sensitive to heat according to our experiments.

The thermotolerance of the *spr9* mutant spores of strain W7a after 16 hours in sporulation medium was compared to that of W66-8A (wild-type strain). In this experiment, spore suspensions were incubated at various temperatures (4°C, 23°C, 30°C, and 42°C) for various times. To reduce the plating error and differences in the treatment of each sample, equal amounts of asci from wild-type and mutant strains (isogenic except at the *SPR9*::*URA3* locus) were mixed after harvesting and the mixed cultures used for all further treatment. The survivors were distinguished by scoring for the uracil requirement. At all temperatures tested there is no obvious

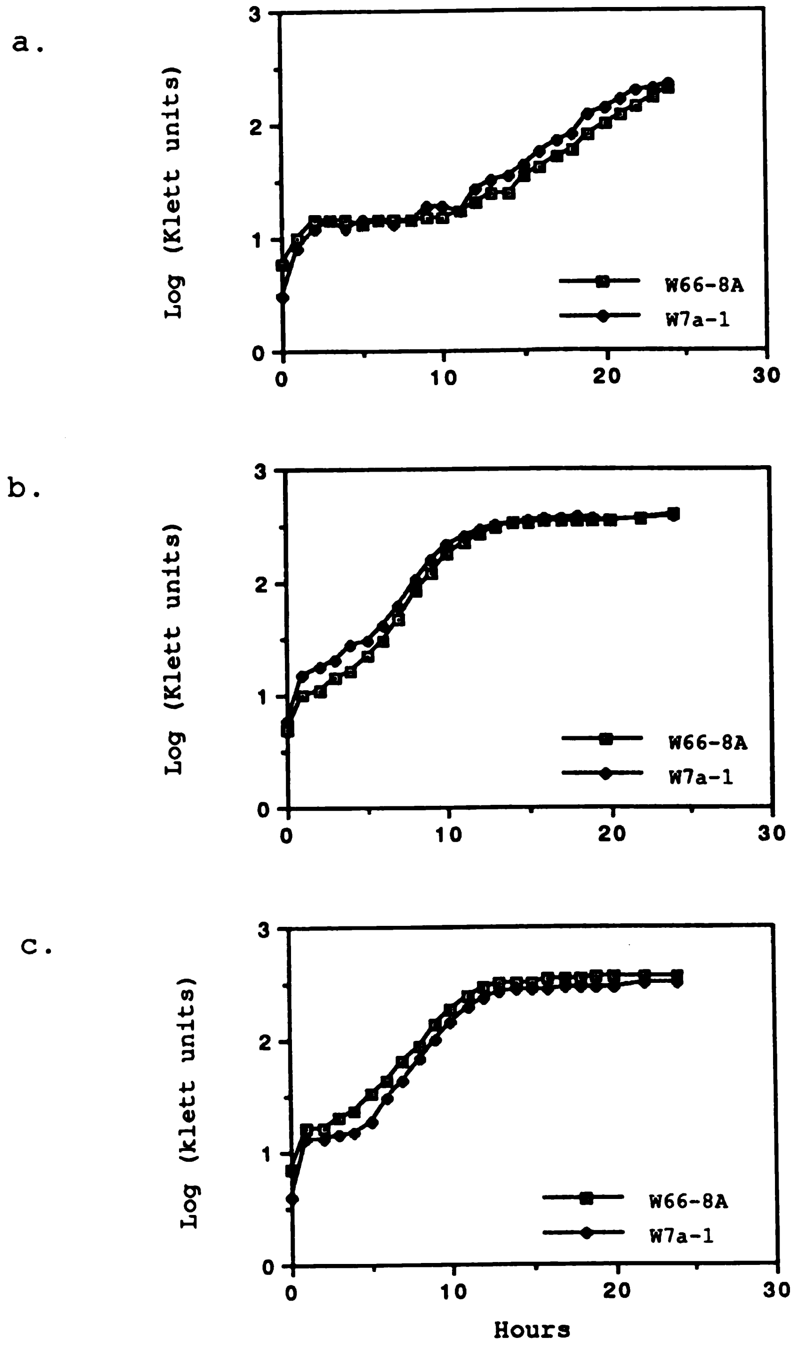


Figure 4.4: The growth curve of the wild-type and *spr9* mutant strains. Both strains were pregrown in YEPD at 30°C overnight then transferred to fresh YEPD and grown at a. 23°C, b. 30°C, c. 34°C. W88-8A: wild-type strain, W7a: *spr9* mutant strain.

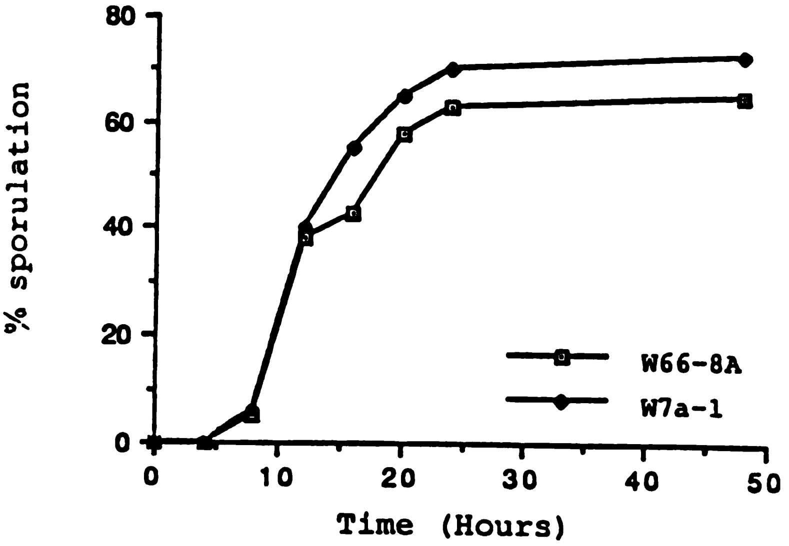


Figure 4.5: The sporulation of the wild-type and *spr9* mutant strains at 30°C. W88-8A: wild-type strain, W7a: *spr9* mutant strain.

difference in the heat tolerance of the 16 hour spores between wild-type and mutant strains (data not shown). Actually, spores from both strains can survive a prolonged time at 4°C in water (data not shown).

To test the germination ability of mutant spores, the same procedure for preparing free spores was used and these spores were spread onto YEPD plates (without pre-incubation in water) and incubated at four different temperatures. Since yeast cells can barely grow when the temperature reaches 42°C and above, germination temperatures used in this study were 23°C, 30°C, 37°C, and 40°C. For homozygous *ade2* strains, the cells will accumulate red pigments due to incapable of turning over the intermediates produced in adenine synthetic pathway. However, some of the germinated colonies turned white when the temperature was 37°C despite their *ade2* genotypes (Figure 4.6a). At 40°C, all colonies germinated were white (Figure 4.6b).

At 23°C or at 30°C, mutant spores germinated as efficiently as the wild-type spores for all four stage spores tested, however when germinated at higher temperatures (37°C and 40°C), mutant spores showed lower germination efficiency than the wild-type spores (Table 4.1 and Figure 4.7).

After 12 hours in sporulation medium at 30°C, about one third of the cells formed four-spore asci in both strains examined, and no difference in germination efficiency between the wild-type and mutant spores was observed at any temperature. In 16 h sporulating cultures, the number of four-spored asci increased to more than 40% at 30°C. When placed at 37°C, only 50% of the mutant spores germinated compared to those at 30°C, whereas the parental spores germinated as well as at 30°C. At 40°C, the germination of mutant spores dropped to 30% while the wild-type retained the same germination efficiency. Small colonies appeared on germination plates at temperatures higher than 37°C(Table 4.1) for 16 h spores. 87.5% of small colonies arose

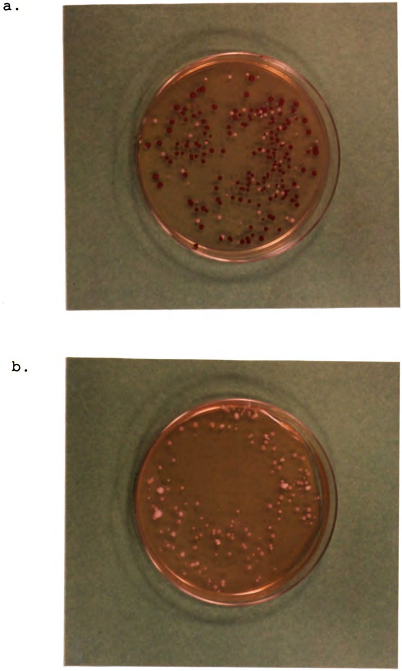


Figure 4.6: The formation of small colonies during germination at temperature ≥ 37°C.

a. The plate was incubated at 37°C for germination. b. The plate was incubated at 40°C for germination. All colonies germinated at 40°C turned white despite their *ade2* genotypes.

from mutant spores, while of the regular sized colonies, only 24% were mutants. The progeny of small colonies grew to form small colonies even at 30°C. They cannot grow in medium which contains glycerol as the sole carbon source. All these observations suggest that these small colonies are petites. The appearance of petites which may be induced by the heat during germination. The sporulation rate of 20 h cultures reached 60% at 30°C for both strains. The germination efficiency at 37°C was slightly decreased to 46% for 20 h mutant spores, while at 40°C the lowest germination of mutant spores were observed, 14%. The parental spores germinated at 37°C and 40°C with 100% and 90% efficiency, respectively. There were lower percentages of small colonies formed when a 20 h sporulating culture was germinated at either 37°C or 40°C compared to a 16 h sporulating culture at the same temperatures. For a 24 h sporulating culture, the sporulation rate was the highest at 30°C. The germination efficiency of mutant spores was also the highest among the four stages of spores examined, 68% at 37°C and 45.5% at 40°C. The 24 h wild-type spores germinated equally well at all three temperatures tested. There were very few small colonies formed for 24 h spores at either 37°C or 40°C. About equal numbers of mutant and wild-type spores gave rise to both small and large colonies at 37°C, and 47% and 37% of mutants in small and large colonies, respectively, at 40°C.

The results of heat tolerance and germination suggest that the function of the *SPR9* gene product is important to spore germination. The occurrence of the small colonies may be related to the spore stage rather than the mutation, since the percentages of small colonies decreased at both 37°C and 40°C as the spores got more mature. When germinating at temperatures higher than 37°C, both wild-type and mutant 20 h spores were most affected by heat. A much more severe deficiency of mutant spores in germination was observed at 40°C for all three stages of spores. It

Table 4.1: Germination of wild type and mutant *SPR9* spores.

	16 h spores			20 h spores			24 h spores		
Germination temperature	30°C	37°C	40°C	30°C	37°C	40°C	30°C	37°C	40°C
# of total spores	4×10^7	4×10^7	2.5×10^7	7×10^7	5×10^7	3×10^7	5.5×10^7	5×10^7	4×10^7
% of mutant spores	68%	34%	33%	62%	40%	20%	59%	52%	37%
# of mutant spores	2.7×10^7	1.4×10^7	8.3×10^7	4.3×10^7	2×10^7	6×10^6	3.3×10^7	2.6×10^7	1.5×10^7
% of mutants germinating	100%	50%	30%	100%	46%	14%	100%	68%	46%
% of W. T. spores	32%	66%	67%	38%	60%	80%	41%	48%	63%
# of W. T. spores	1.3×10^7	2.6×10^7	1.7×10^7	2.7×10^7	3×10^7	2.4×10^7	2.3×10^7	2.4×10^7	2.5×10^7
% of W. T. germinating	100%	100%	100%	100%	100%	90%	100%	100%	100%
Large : small colonies	200 : 1	4 : 1	2 : 1	200 : 1	7 : 1	3 : 1	200 : 1	9 : 1	6 : 1
% of mut. large colonies	N/A	24%	20%	N/A	40%	9%	N/A	53%	37%
% of mut. small colonies	N/A	88%	83%	N/A	78%	82%	N/A	53%	47%

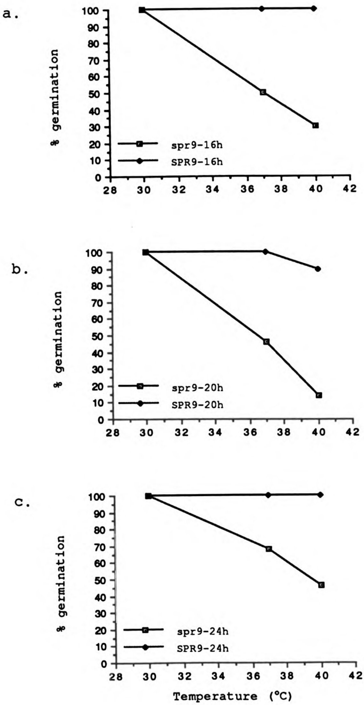


Figure 4.7: Germination efficiency of wild-type and *spr9* mutant spores.

a. 16 h spores, b. 20 h spores, c. 24 h spores is difficult at this moment to draw any further conclusions about the *SPR9* function during germination.

4.4 Detection of the *SPR1* phenotype

The function of the *SPR1* gene was also investigated in this study. The *spr1* mutant strain can sporulate as efficiently as its wild-type parent (Primerano, 1990). The ability to germinate and the heat tolerance of the *SPR1* spores were examined by the same procedures used for *SPR9* strains. Two different homozygous *spr1* strains, W61A (*spr1-1::URA3/spr1-1::URA3*) and S52B (*spr1-2::LEU2/spr1-2::LEU2*), were tested for thermotolerance to show that the heat-sensitivity of *spr1* mutant spores observed in this study was not strain specific (Figure 4.9 and Figure 4.10).

To test the germination ability of the *spr1* mutant spores, strains S52B (*spr1/spr1*) and S52A (*SPR1/SPR1*) were used. For 16 h sporulating cultures, the germination efficiency of mutant spores declined as temperature increased and so did the wild-type spores. At all temperatures, the germination efficiency of mutant spores was only slightly lower than that of wild-type spores. At 37°C, 79.3% of mutant spores germinated compared to those germinated at 30°C, while 87.8% of wild-type spores germinated under the same conditions. At 40°C, the germination efficiency for mutant spores was 62.5% compared to 71.0% for wild-type spores. For a 32 h sporulating culture, no difference was observed between wild-type and mutant spores in their germination ability on YEPD plates at all temperatures tested. At 37°C and 40°C, 80% of both wild-type and mutant spores germinated compared to at 30°C (Table 4.2 and Figure 4.8).

The thermotolerance of *spr1* mutant spores was slightly lower than their isogenic wild-type spores under some conditions tested (Figure 4.9 and Figure 4.10). When

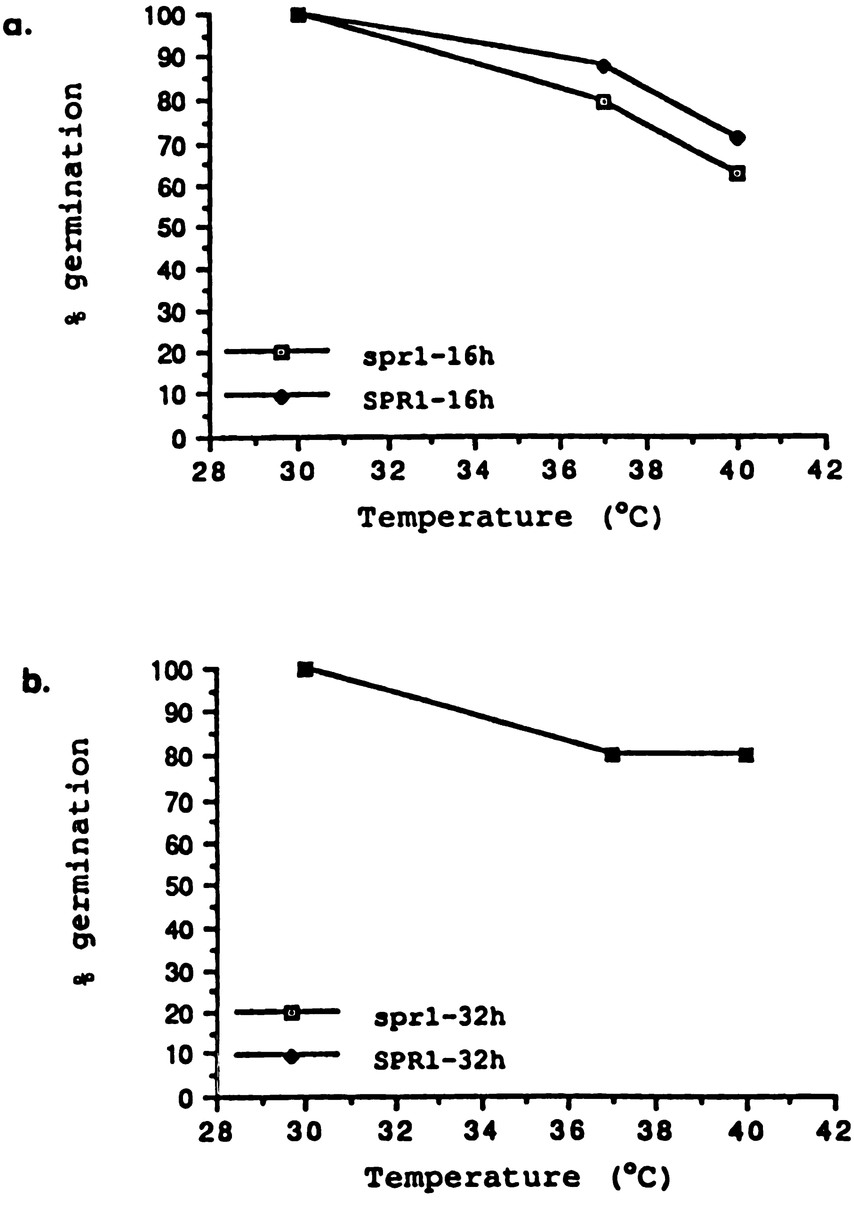


Figure 4.8: Germination efficiency of wild type and *spr1* mutant spores.

a. 16 h spores, b. 32 h spores

incubated at 4°C, 23°C or 30°C, the *spr1* mutant spores harvested at all four times survived as well as wild-type spores (data not shown). When the temperature of incubation was 42°C, the mutant spores were killed at a higher rate than the wild-type spores. This heat sensitivity of mutant spores was first apparent in 16 hour sporulating cultures. The difference in cell death between the mutant and the wild-type spores began to appear after 48 to 72 hours of incubation. After 120 h of incubation, the mutant spores showed 1% survival to the wild-type spores, while the wild-type spores had 3.3×10^{-5} survival. For 20 hour sporulating cultures, the differential killing effect of heat was less severe than to the 16 hour ones, although a 10 fold decrease in relative survival of the *spr1* mutant spores was seen at 120 h. The 24 hour mutant spores were killed by heat at the same rate as wild-type ones. This result suggests that the more mature the spores are, the less the *SPR1* function is needed.

The spores from the *SPR1/spr1* heterozygous strain (SPB1) were also tested for heat sensitivity (Figure 4.11). This strain produces 2 wild-type spores and 2 mutant spores in one ascus. Therefore, it was expected that mutant survival would be less than the wild-type survival. Surprisingly, equal numbers of wild-type and mutant spores survived at all temperatures for all four stages. This may suggest that either the *SPR1* gene product functions before the formation of the individual spore wall, or that it can diffuse through spore wall and membrane.

4.5 Phenotype detection of *SPR2*

The phenotype of the *SPR2* gene was also examined. The *spr2* mutant strain did not show any difference from wild-type strains in all tests applied. They sporulated and germinated as efficiently as wild-type (Table 4.3 and Figure 4.12), and their

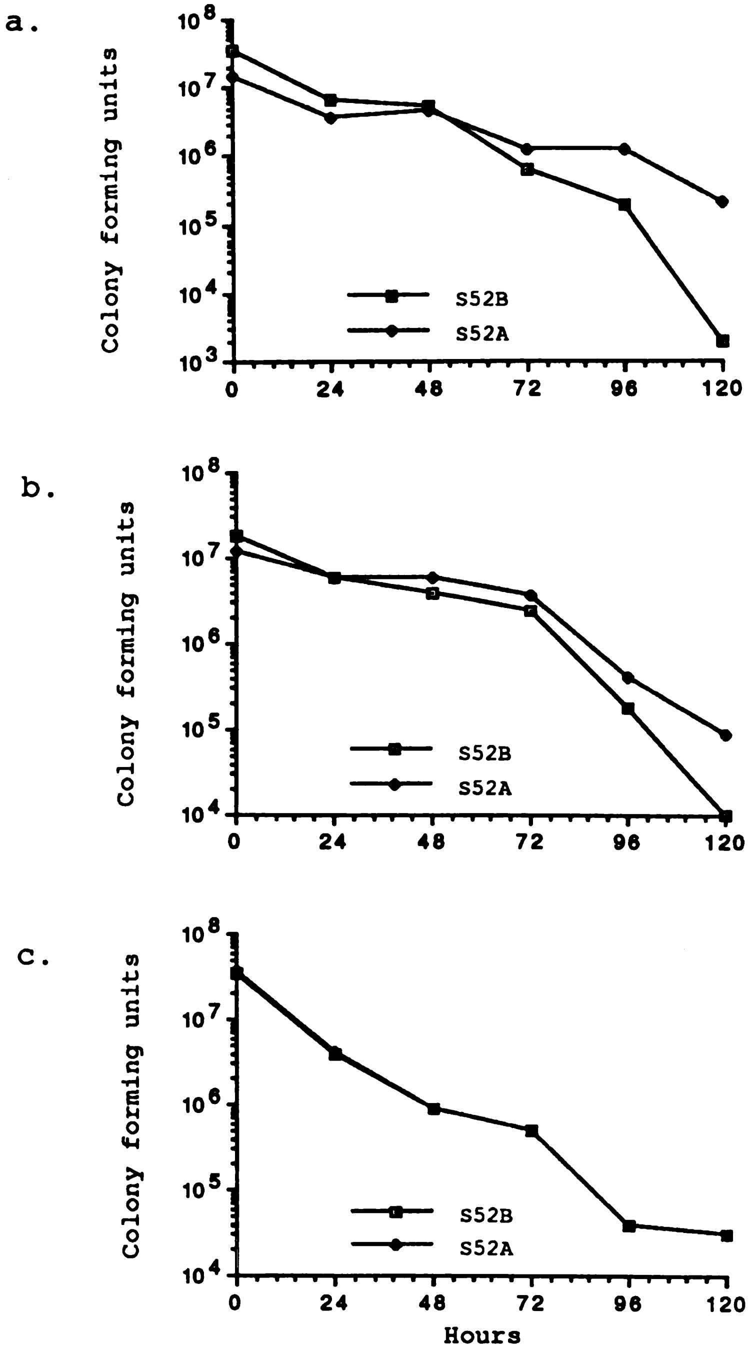


Figure 4.9: Thermotolerance of the wild-type and *spr1* mutant spores in SCMS-7 derivatives.

The wild-type spores of strain S52A and the *spr1* mutant spores of strain S52B were preincubated at 42°C for various time and then grown on YEPD plates. Spores were from a. 16 h, b. 20 h, and c. 24 h sporulating culture.

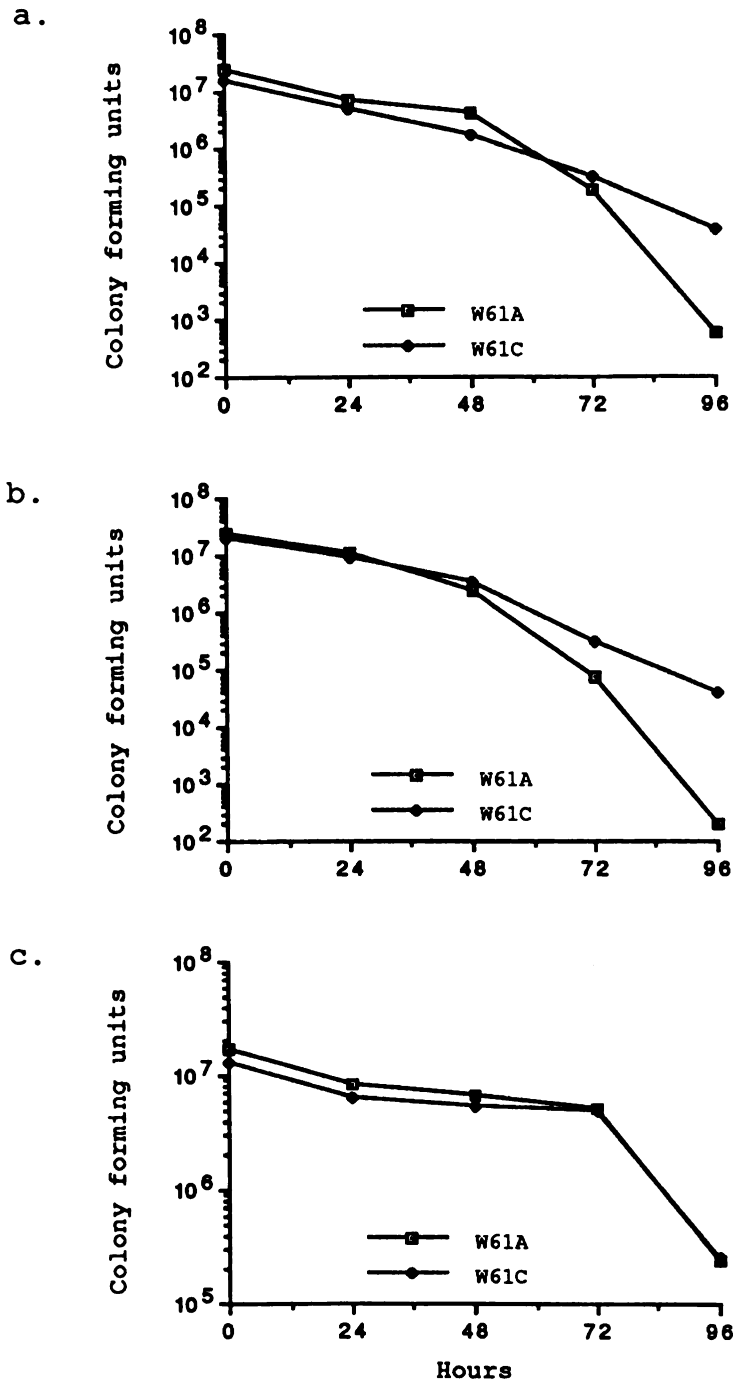


Figure 4.10: Thermotolerance of the wild-type and *spr1* mutant spores in W66-8A derivatives.

The wild-type spores of strain W61C and the *spr1* mutant spores of strain W61A were preincubated at 42°C for various time and then grown on YEPD plates. Spores were from a. 16 h, b. 20 h, and c. 24 h sporulating culture.

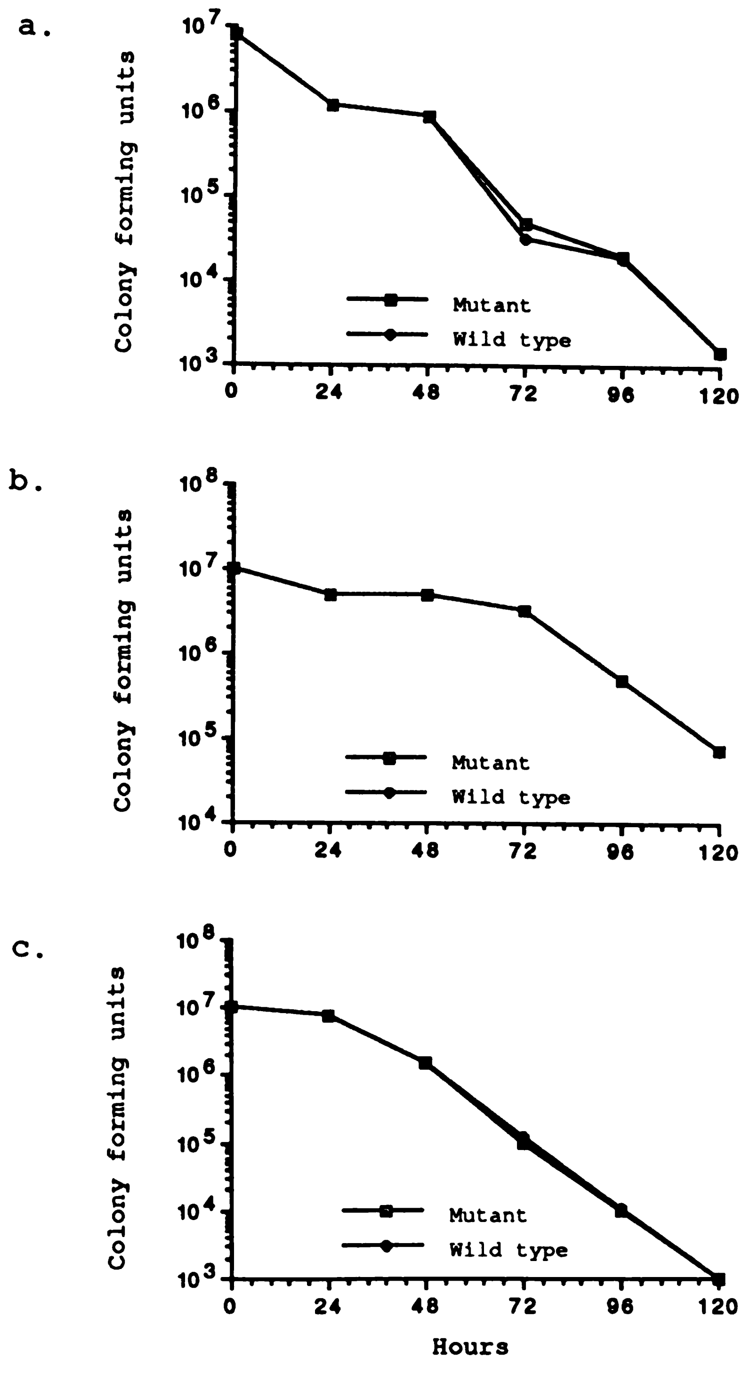


Figure 4.11: Thermotolerance of the wild-type and *spr1* mutant spores of *SPB1*. Strain SPB1 (*SPR1*/*spr1*) will produce two mutant spores and two wild-type spores in one ascus. The spore suspensions were preincubated at 42°C for various time and then grown on YEPD plates. Spores were from a. 16 h, b. 20 h, and c. 24 h sporulating culture.

resistance to heat was the same as wild-type. We have detected no phenotype for the *SPR2* gene; we do not know whether the *SPR2* gene product has an important function in the events during and after sporulation. Further studies should be done in order to identify the role of the *SPR2* product during sporulation.

Table 4.2: Germination of wild-type and *spr1* mutant spores.

	16 h spores			32 h spores		
Germination temperature	30°C	37°C	40°C	30°C	37°C	40°C
# of total spores	3.0 x 10^7	2.5 x 10^7	2.0 x 10^7	2.6 x 10^7	2.0 x 10^7	2.0 x 10^7
% of mutant spores	52%	51%	50%	50%	50%	50%
# of mutant spores	1.6 x 10^7	1.3 x 10^7	1.0 x 10^7	1.3 x 10^7	1.0 x 10^7	1.0 x 10^7
% of mutants germinating	100%	79%	63%	100%	80%	80%
% of W. T. spores	48%	49%	50%	50%	50%	50%
# of W. T. spores	1.4 x 10^7	1.2 x 10^7	1.0 x 10^7	1.3 x 10^6	1.0 x 10^7	1.0 x 10^7
% of W. T. germinating	100%	88%	71%	100%	80%	80%

Table 4.3: Germination of wild-type and *spr2* mutant spores.

	16 h spores			32 h spores		
Germination temperature	30°C	37°C	40°C	30°C	37°C	40°C
# of total spores	2.0 x 10^7	1.5 x 10^7	4.0 x 10^6	1.5 x 10^7	1.2 x 10^7	8.0 x 10^6
% of mutant spores	58%	63%	50%	54%	62.5%	63%
# of mutant spores	1.2 x 10^7	9.4 x 10^6	2.0 x 10^6	8.1 x 10^6	7.4 x 10^6	5.0 x 10^6
% of mutants germinating	100%	81%	17%	100%	91%	61%
% of W. T. spores	42%	37%	50%	46%	37.5%	37%
# of W. T. spores	8.8 x 10^6	5.6 x 10^6	2.0 x 10^6	6.9 x 10^6	4.6 x 10^6	3.0 x 10^6
% of W. T. germinating	100%	64%	23%	100%	67%	43%

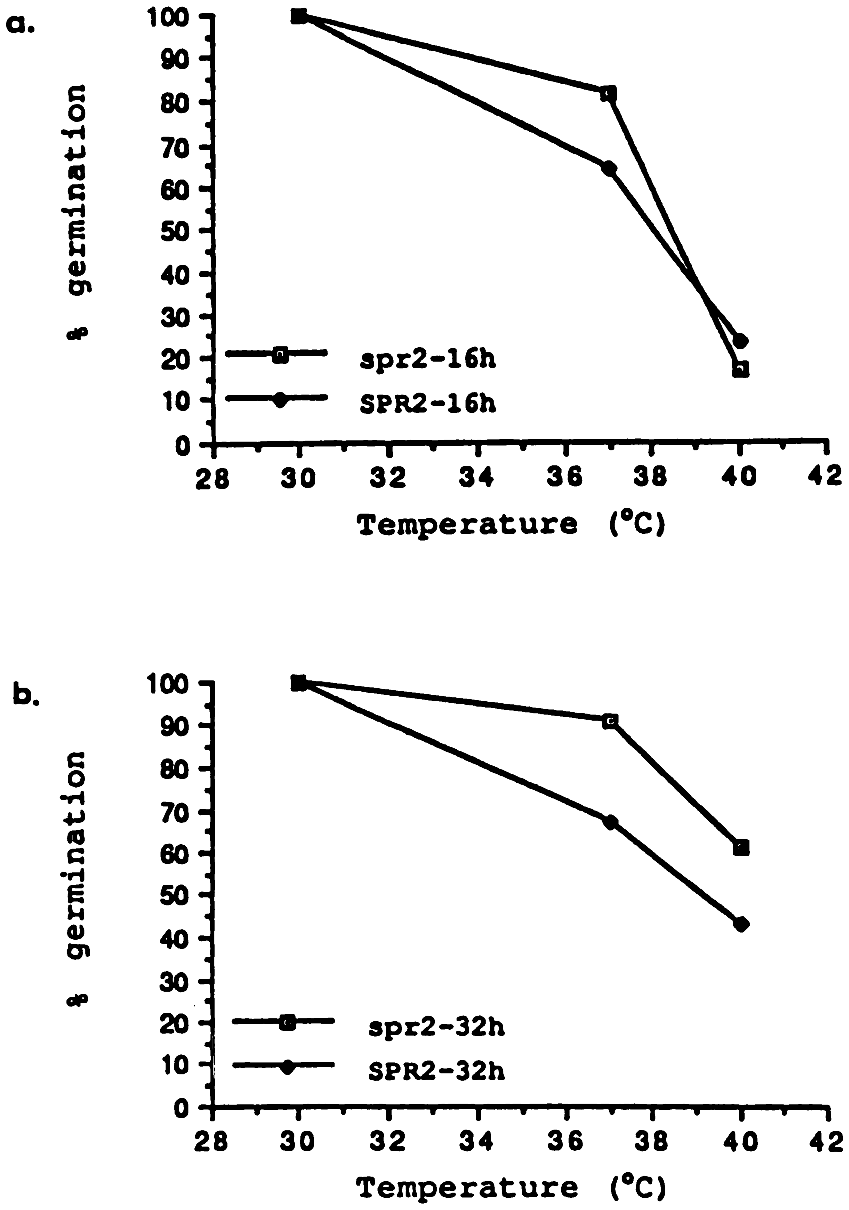


Figure 4.12: Germination efficiency of wild type and *spr2* mutant spores.

a. 16 h spores, b. 32 h spores

CHAPTER V

Construction and Analysis of Double Mutants

5.1 Construction of double mutants defective at two *SPR* loci

There were two reasons for constructing mutants defective at more than one gene; one was to determine the epistasis of these *SPR* genes during the sporulation process if they are in the same pathway; the other was to examine whether proteins of duplicated function are produced during sporulation. By mating two haploid *HO spr* single mutants, one can get a diploid that is heterozygous at both *SPR* loci. This heterozygous strain will produce spores of four different combinations of *SPR* genotypes after sporulation. They are wild-type, mutant at either *SPR* locus, and mutant at both *SPR* loci. Each spore will then diploidize and become homozygous for all loci except the mating type locus.

In this study, all the strain constructions made were between derivatives of two strains, W66-8A and SCMS7-1 (Figure 5.1). SCMS7-1 is auxotrophic for leucine, being defective at *leu2-3,112*. For the derivatives of SCMS7-1, the *LEU2* gene was inserted into the wild-type *SPR* gene and prototrophy for leucine was used to detect cells that contained *spr* mutations. W66-8A is also auxotrophic for leucine, at the *leu1* locus. In order not to confuse the *leu1* and *leu2* genotypes, we decided to isolate

LEU1 revertants of W66-8A derivatives before mating with SCMS7-1 derivatives, so that the auxotrophic phenotype for leucine observed in their offspring will be from *leu2* only.

The *LEU1* revertants of W66-8A derivatives were either UV-induced or spontaneous revertants. The spontaneous reversion rate for the *leu1* strain is 10^{-9}, calculated from this study, while the UV-induced rate is 10^{-7} (data not shown). Ten revertants from both treatments were checked for their genotypes and they all showed the same markers as the untreated parents except at the *leu1* locus (data not shown). Retention of the *SPR9* disruption (*URA3*) was also confirmed by Southern blot analysis of their genomic DNA; they all showed the correct construction at the *SPR9* locus (data not shown). Since these revertants are heterozygous at the *LEU1* locus, they were sporulated and diploidized to produce colonies that were homozygous at all loci except the mating type locus. The *LEU1* cells were then retested for their homozygosity at the *SPR9* locus by Southern blot analysis (data not shown). This strain was named W7a-1 and used for further constructions of double mutants.

Spore-to-spore mating (Figure 2.3d and e) was used to construct *spr1/9* double mutants. Spores from S52B (*spr1-2::LEU2/spr1-2::LEU2*) and spores from W7a-1 (*spr9::URA3)/spr9::URA3*) were placed side by side by micromanipulation on a piece of thin YEPD agar to facilitate their mating. The diploid hybrids formed by mating should be both *HO* and heterozygous for all the auxotrophic markers including the two *SPR* loci, since the two parent strains have completely different auxotrophic markers except that both are auxotrophic for adenine. Therefore, these diploids could grow on minimal medium supplemented with adenine while the cells from unmated and self-mated spores could not. The resulting diploid products carried one wild-type allele and one mutant allele for both the *SPR1* and *SPR9* loci as shown by

Southern blot analysis (Figure 5.2a). All five hybrids examined are heterozygous at both loci. These strains were then sporulated and the asci were dissected. All the colonies grown from single spores were tested for their genotypes on minimal medium with different combinations of supplements (Figure 5.1). Those colonies grown after dissection were further examined for their *SPR1* and *SPR9* loci by Southern blot analysis (Figure 5.3).

For the construction of the *SPR2*/9 double mutant, a similar approach was applied, except that the single mutant strains used as parents for spore to spore mating were T4C (*spr2::LEU2/spr2::LEU2*) and W7a-1. The genomic DNA of cells with *URA3* and *LEU2* genotypes were examined for their *SPR2* and *SPR9* loci (Figure 5.2b). All hybrids were heterozygous at both loci except the CT1 strain, which was defective only at *SPR2* but not at the *SPR9* locus and therefore was not used for further experiments. The reason for observing differences in relative intensity of the wild-type and mutant bands of the Southern blot analysis is not known at this moment. The genomic DNA of colonies grown after dissection was examined for their *SPR2* and *SPR9* loci by Southern blot analysis (Figure 5.3b).

5.2 Phenotypes of the double mutants

The disruption of both loci of the two double mutants, 4-8-a (*spr1/9*) and 5-4-d (*spr2/9*), was checked by Southern blot analysis (Figure 5.3). Their growth rates, like the single mutants, were not affected by mutations at two *SPR* loci (data not shown). The two double mutants can also sporulate when tested, but only about 40% of the population form four-spore asci at 30°C compared to 70% of the wild-type and the heterozygote (Figure 5.4). Figure 5.4a shows that 4-8-a sporulated 20% to 30% less than W66-8A (wild-type) and BS1 (heterozygous at both the *SPR1*

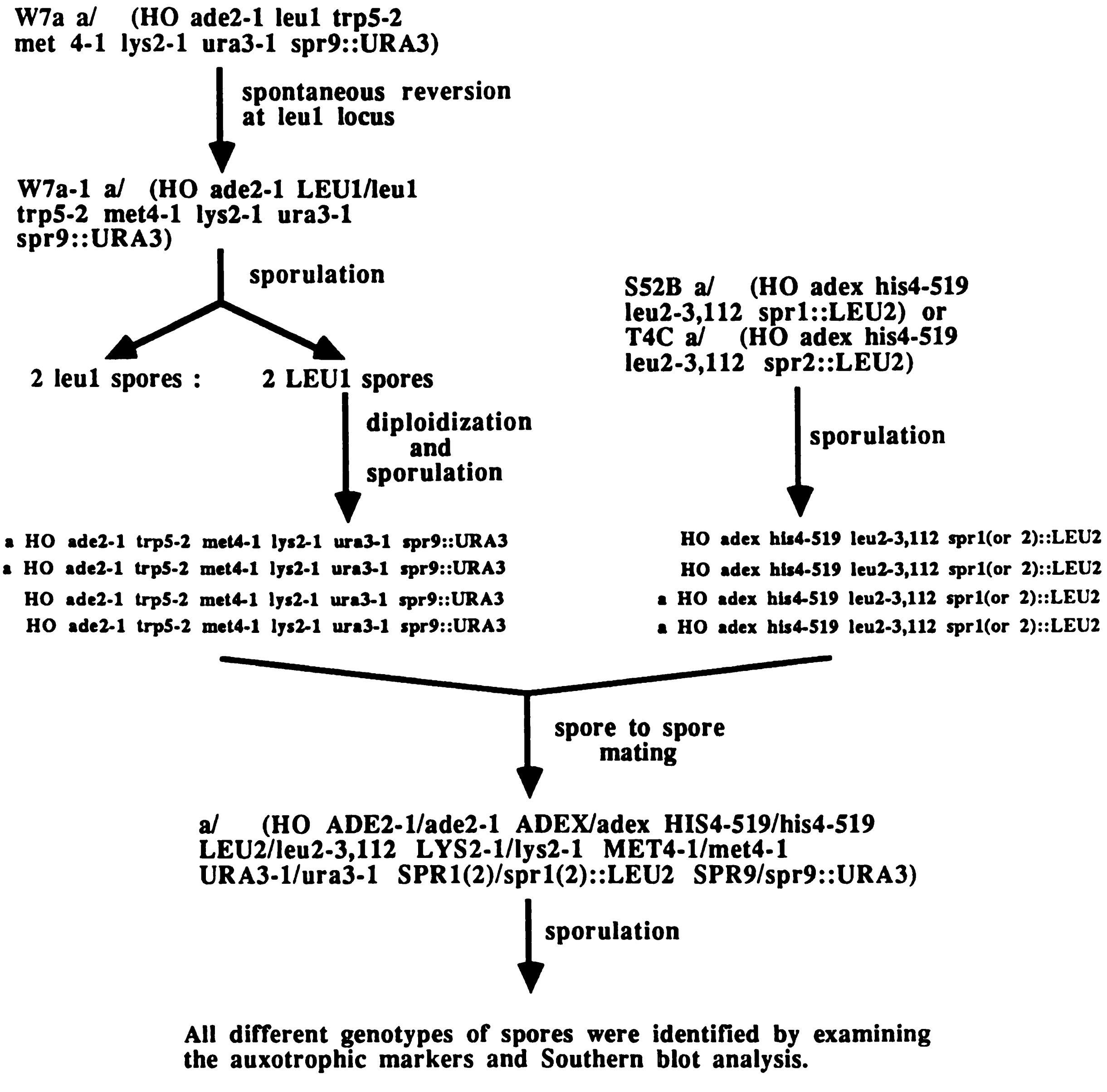


Figure 5.1: Construction of mutants defective at two *SPR* loci.

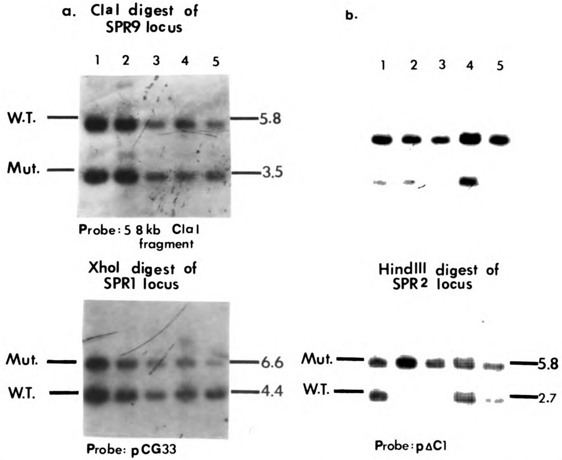


Figure 5.2: Southern blot analysis of the heterozygous strains.

The genomic DNA of cells which are prototrophic for uracil and leucine were prepared for Southern blot analysis. a. The genomic DNA of strains BS1, BS2, CS1, CS2, and DS1 was digested with ClaI enzyme for detection of the *SPR9* locus and XhoI for detection of the *SPR1* locus. b.The genomic DNA of strains BT1, BT2,CT1, CT2, and CT3 were digested with ClaI enzyme for detection of the *SPR9* locus and HindIII for detection of the *SPR2* locus. +: wild-type band, –: mutant band.

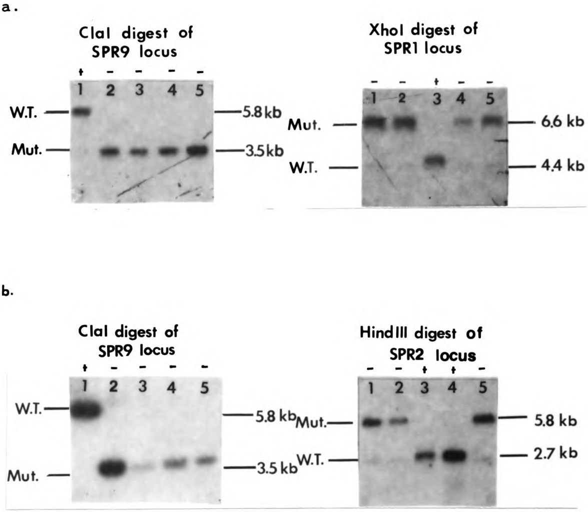


Figure 5.3: Southern blot analysis of the *spr* double mutants.

a. Southern blot analysis for the detection of *spr1/9* double mutants. The genomic DNA from 5 spore colonies was digested by ClaI enzyme for the detection of *SPR9* locus and by XhoI for *SPR1* locus. b. Southern blot analysis for the detection of *spr2/9* double mutants. The genomic DNA from 5 spore colonies was digested by ClaI enzyme for the detection of *SPR9* locus and by HindIII for *SPR1* locus. +: wild-type band, -: mutant band.

and *SPR9* loci). However, the kinetics of sporulation of 4-8-a are similar to that of the wild-type and heterozygous strains. Figure 5.4b shows the sporulation kinetics of W66-8A, CT3 (heterozygous at both the *SPR2* and *SPR9* loci), and 5-4-d. The result is similar to that of 4-8-a.

The thermotolerance of double mutant spores was also tested under the same conditions as single mutants. The result was that *spr1/9* double mutant spores were very sensitive to heat, while the *spr2/9* double mutant spores had about the same thermotolerance as *spr9* single mutant spores. Table 5.1 shows the spore viability of 4-8-a and 5-4-d after incubation at five different temperatures compared to two wild-type strains, W66-8A and SCMS-7. After incubating 3 days at 42°C, spore viability of 4-8-a dropped 10^5 and those of 5-4-d dropped 20 fold while the wild-type spores retained the same viability. At 30°C, the viability of both double mutant spores improved, a 500 fold decreases in spore viability for 4-8-a and wild-type level of viability for 5-4-d. At 4°C, the spores of wild-type and both double mutants had the same viability after 6 days of incubation. The same experiment was repeated by using micromanipulation to produce single spores, which were incubated at 42°C, and a similar result was observed (Table 5.2). As the period of incubation increased, the number of intact four-spore asci decreased for all four strains tested. However, the number of viable spores decreased much more for 4-8-a. By the third day, no viable spores were detected by plate count. Furthermore, the spores of 4-8-a were so fragile that they broke when touched with the micromanipulator needle, which was not true of the spores of the other three strains.

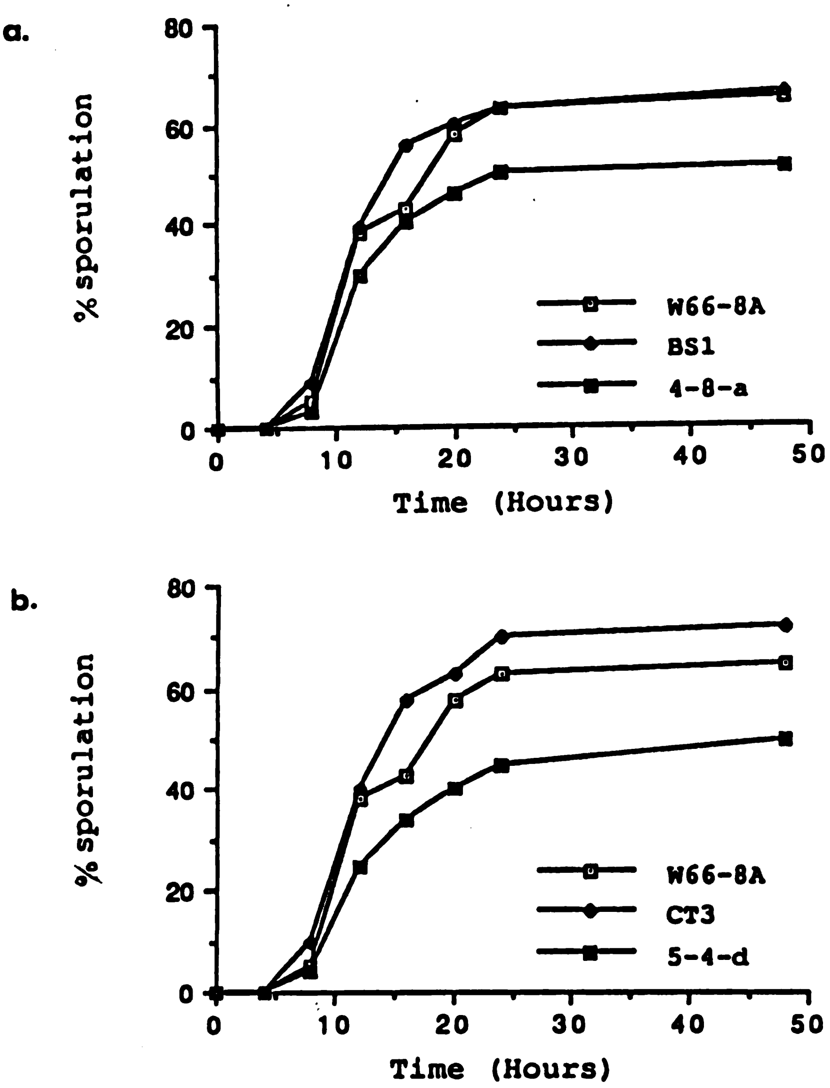


Figure 5.4: Sporulation efficiency of double mutants.

The sporulation efficiency of three strains W66-8A (wild-type), BS1 (heterozygous at both *SPR1* and *SPR9* loci), and 4-8-a (homozygous *spr1/9* at 30°C was in Figure a. Figure b shows the sporulation efficiency of strains W66-8A, CT3 (heterozygous at both *SPR2* and *SPR9* loci), and 5-4-d (homozygous *spr2/9* at 30°C.

5.3 Multiple heat effect on germination

To test if preincubation at high temperature and germination at high temperature have an additive effect on spore germination, spores of 7 strains were preincubated at 4°C, 30°C, or 42°C for 48 h and then germinated at 30°C or 40°C. The free spore suspension of each strain was divided equally into three tubes for preincubation at three different temperatures, 4°C, 30°C, and 42°C. After incubating for 48 hours, each spore suspension was spread onto two YEPD plates for germination. One plate was incubated at 30°C and the other one at 40°C. The result is shown in Table 5.4. The numbers of cells preincubated at 4°C and then germinated at 30°C were taken as the numbers of starting cells, since spores do not lose viability when incubated at 4°C. The three single mutants, S52B, T4C, and W-7-a, showed a similar heat tolerance to the wild-type strains, since the heat-sensitive phenotype of S52B mutant spores does not become evident until 72 hours. Comparing the ability of the spores to germinate at high temperature, W-7-a was sensitive to heat during germination but the other two single mutant strains were not. For the double mutant strain, 4-8-a, the absence of two *SPR* gene products was detrimental when high temperature was applied both to preincubation and germination. The number of surviving mutant cells dropped more than 100-fold when compared to wild-type spores under same conditions. For 5-4-d, the effect of the absent *SPR9* gene product was observed in the lower germination efficiency when high temperature was applied to both preincubation and germination.

5.4 Linkage between *SPR9* and *LYS2*

The results of CHEF analysis indicated that the *SPR9* gene lies on chromosome II where the *LYS2* gene is also located. The linkage between these two genes can be determined by recombination analysis. The first step was to construct a strain that is heterozygous at both the *SPR9* and the *LYS2* loci. The scheme used to generate the heterozygotes is the same as that for constructing double mutants (Figure 5.1). The heterozygotes from the cross of W7a-1 to S52B and the cross of W7a-1 to T4C were then sporulated and dissected for analyzing the recombination frequency between the two loci. There were 89 and 62 asci dissected from the cross involving T4C and S52B, and the percentages of four viable-spore asci were 57% and 68%, respectively. Only 92 of them were complete tetrads and were examined for the genotype of each spore. Among those 92 asci, only tetrads that were 2 URA+ : 2 URA− and 2 LYS+ : 2 LYS− were used for calculating the map distance. The reason was that there were 4 *URA3* and 2 *ura3* alleles in the heterozygotes instead of the normal 2 *URA3* and 2 *ura3* distribution. Two of the *URA3* alleles were from S52B or T4C, and the other two *URA3* were *spr9*::*URA3* alleles from W7a-1. Only the asci with the 2 URA+ : 2 URA− pattern were certain to be 2 *SPR9* : 2 *spr9*, since both the normal *URA3* and the *spr9*::*URA3* alleles were segregated into the same spore.. Some other asci were confirmed by Southern blot analysis for the 2 *SPR9* : 2 *spr9* pattern(data not shown). The numbers of parental ditype, tetratype, and nonparental ditype asci for *SPR9* and *LYS2* loci and map distance are shown in Table 5.1. Let d be the map distance in cM between two markers (Esposito and Klapholz, 1981), then

$$d = 100(\mathrm{TT} + 6\mathrm{NPD})/2(\mathrm{PD} + \mathrm{TT} + \mathrm{NPD}),$$

where PD is parental ditype ascus, TT is tetratype ascus, and NPD is nonparental ditype ascus. The map distance obtained from these data (about 67 cM) suggested that the *SPR9* locus and the *LYS2* locus are not linked. Thus, there are more than 50 cM between the *SPR9* locus and the *LYS2* locus.

The centromere linkage of the *SPR9* locus was also examined. Since the map distance between the centromere and a gene is about one half the percentage of second division segregation (SDS), there is a formula for determining the distance between a gene and its centromere:

For two heterozygous sites, each on different chromosomes, and with SDS frequencies X and Y, respectively, let F be the frequency of tetratype asci for the two markers (Esposito and Klapholz, 1981).

$$F = X + Y - 3/2(XY)$$

In this case, the other centromere linked gene used as reference is *HIS4* which is 20.8 cM from its centromere (Mortimer and Schild, 1980). Therefore, the percentage of SDS for *HIS4* is twice the number of the centimorgans between *HIS4* and its centromere, which is 0.40. The frequency of tetratype asci for *HIS4* and *SPR9* obtained from tetrad analysis of heterozygotes of both loci is 0.58. The data are shown in Table 5.4. The calculated distance between centromere II and *SPR9* is about 22.3 cM. Since the distance between centromere II and *LYS2* is 54 cM (Mortimer and Schild, 1980), these data indicate that *SPR9* should be on the opposite arm from *LYS2* (which is on the shorter arm). However, further experiments have to be done for precisely mapping the position of *SPR9* locus on chromosome II.

5.5 Linkages among *SPR1*, *SPR2*, and *ADE2*

Primerano et al. showed that both the *SPR1* and *SPR2* genes lie on chromosome XV by OFAGE analysis (1990 and unpublished data). Their linkage to the *ADE2* locus (chromosome XV) could be obtained from the same heterozygotes used for determining *SPR9* and *LYS2* linkage. The only difference was that there were two *ADE* loci involved in this cross: *ADE2* and *ADEX*. The *ade2* mutants are not only auxotrophic for adenine but accumulate a red pigment which turns the colonies red.

The *adeX* mutants are auxotrophic for adenine, and are white. The *ade2*/*adex* double mutants are also white. There would be three different types of asci produced by the heterozygotes containing the *ade2* and *adex* loci. In only two of them could the genotypes at these loci of all four spores be distinguished (Table 5.5), the parental ditype and nonparental ditype asci, and only those asci were used to determine the linkage to the *SPR1* and *SPR2* loci. In a type I ascus, the genotype at the *ADE2* locus of the two auxotrophic white ascospore colonies cannot be determined from the information obtained.

The map distances determined from the data in (Table 5.4) were 59 cM between *SPR1* and *ADE2* and 65 cM between *SPR2* and *ADE2*. Both of them are probably not linked to the *ADE2* locus. The centromere linkage of these two loci was also examined by using the *HIS4* locus as a reference to determine the frequency of second division segregation. The map distance from centromere XV to *SPR1* was 23.5 cM, and to *SPR2* 4.5 cM. The map distance between *ADE2* and the centromere was 65 cM (Mortimer and Schild, 1980). Further experiments are needed to establish the precise map relationship among *SPR1*, *SPR2*, and *ADE2* loci.

Table 5.1: Thermotolerance of double mutants.

		W66-8A	SCMS-7	4-8-a	5-4-d
SPR genotype		(1+)(2+)(9+)	(1+)(2+)(9+)	(1–)(2+)(9–)	(1+)(2–)(9–)
42°C	Day 0	6.5×10^7	3.4×10^7	1.2×10^7	1.5×10^7
	Day 3	1.5×10^7	2.3×10^7	$< 1.0 \times 10^2$	5.5×10^5
30°C	Day 0	1.5×10^8	1.5×10^7	5.0×10^6	1.3×10^7
	Day 3	1.0×10^7	1.0×10^7	1.0×10^4	8.0×10^6
4°C	Day 0	5.0×10^7	2.2×10^7	2.5×10^7	3.0×10^7
	Day 6	4.0×10^7	1.0×10^7	3.0×10^6	3.0×10^7

Table 5.2: Thermotolerance of double mutants by micromanipulation followed by incubation at 42°C.

		W66-8A	SCMS-7	4-8-a	5-4-d
Genotype		(1+)(2+)(9+)	(1+)(2+)(9+)	(1−)(2+)(9−)	(1+)(2−)(9−)
0 h	Total spores	44	49	40	36
	Total tetrads	11	10	11	9
	Viable spores	42	48	29	35
24 h	Total spores	42	15	24	52
	Total tetrads	6	4	1	13
	Viable spores	39	12	4	46
48 h	Total spores	36	40	*a*	36
	Total tetrads	9	10	—	9
	Viable spores	30	24	—	25
72 h	Total spores	41	32	—	40
	Total tetrads	10	4	—	10
	Viable spores	28	16	—	23

a. Spores broke when touched with needle. The plate count showed no viable spores after 72 h incubation at 42°C.

Table 5.3: Multiple temperature effect on germination.

	W66-8A	SCMS-7	S52B	T4C	W-7-a	4-8-a	5-4-d
SPR genotype	(1+)(2+)(9+)	(1+)(2+)(9+)	(1−)(2+)(9+)	(1+)(2−)(9+)	(1+)(2+)(9−)	(1−)(2+)(9−)	(1+)(2−)(9−)
Preincubation Germ.							
4°C (48 h) → 30°C	3.0×10^7	2.5×10^7	3.0×10^7	3.0×10^7	1.0×10^7	7.0×10^6	8.0×10^6
4°C (48 h) → 40°C	2.0×10^7	1.6×10^7	2.0×10^7	2.0×10^5	6.0×10^3	$< 1.0 \times 10^3$	1.5×10^5
30°C (48 h) → 30°C	1.0×10^7	1.2×10^8	6.0×10^6	1.5×10^7	1.5×10^7	6.0×10^5	6.0×10^6
30°C (48 h) → 40°C	7.0×10^6	1.0×10^8	4.0×10^6	7.0×10^6	$< 1.0 \times 10^5$	$< 1.0 \times 10^3$	$< 1.0 \times 10^5$
42°C (48 h) → 30°C	1.0×10^5	1.0×10^5	5.0×10^5	9.0×10^4	1.0×10^6	2.0×10^3	4.0×10^4
42°C (48 h) → 40°C	1.0×10^4	1.0×10^4	4.0×10^5	5.0×10^4	$< 1.0 \times 10^3$	$< 1.0 \times 10^3$	$< 1.0 \times 10^3$

Table 5.4: Linkage analysis of three *SPR* genes.

	Number of Asci				
	PD[a]	TT[b]	NPD[c]	Freq. of SDS[d]	Map distance
SPR9-LYS2[e]	5	23	6	—	unlinked
SPR9-HIS4	6	18	7	0.58	unlinked
SPR1-ADE2[f]	3	7	1	—	unlinked
SPR1-HIS4	4	13	5	0.59	unlinked
SPR2-ADE2	2	7	1	—	unlinked
SPR2-HIS4	1	8	9	0.44	unlinked

a. PD is parental ditype ascus.

b. TT is tetratype ascus.

c. NPD is nonparental ditype ascus.

d. SDS is second division segregation.

e. The distance between centromere II and *LYS2* is 54 cM.

f. The distance between centromere XV and *ADE2* is 65 cM.

Table 5.5: The three types of asci generated from *ADE2/ade2 ADEX/adex* strain.

	Type I (TT_a)				Type II (PD_b)				Type III (NPD_c)			
spore	growth_d	$ADE2_e$	*ADEX*	color_f	growth	*ADE2*	*ADEX*	color	growth	*ADE2*	*ADEX*	color
1	+	+	+	w	+	+	+	w	–	+	–	w
2	–	+	–	w	+	+	+	w	–	+	–	w
3	–	–	+	r	–	–	–	w	–	–	+	r
4	–	–	–	w	–	–	–	w	–	–	+	r

a. TT is tetratype ascus.

b. PD is parental ditype ascus.

c. NPD is nonparental ditype ascus.

d. The ability to grow on a MIN plate without adenine supplementation.

e. '+' = W. T. allele, '–' = mutant allele.

f. The color of colonies: 'w' = white, 'r' = red.

CHAPTER VI

Discussion

In this study, the *SPR9* locus was analyzed molecularly in terms of transcription, regulation, and nucleotide sequence. The phenotypes of three *SPR* genes, *SPR1*, *SPR2*, and *SPR9*, were also studied in terms of thermotolerance during spore formation and germination. Two double mutants, *spr1/9* and *spr2/9*, were constructed for investigation of the relationships among these three genes during sporulation. The results of this study show that the three *SPR* genes are not involved in the process of sporulation, but the gene products of *SPR1* and *SPR9* affect the spore thermotolerance and germination under high temperature. Despite the fact that the biochemical functions of these *SPR* genes and hence the reasons for the mutants showing the phenotypes are not known, some evidence suggests that some of the sporulation-specific gene products such as *SPR1* and *SPS100* are associated with the spore membrane (Primerano et al., 1990; Law and Segall, 1988). Additional sporulation-specific genes may be involved in the spore membrane assembly and functions.

Sporulation is an intricate process which switches yeast cells from one state to another. Most of the sporulation-specific genes studied so far (Percival-Smith and Segall, 1986; Garber and Segall, 1986; Law and Segall, 1988; Gottlin-Ninfa and Kaback, 1986; Primerano et al. unpublished data) do not block sporulation. This observation may be explained by the existence of more than one sporulation pathway in yeast cells to ensure sporulation. Alternatively, the genes involved in sporulation may be functionally redundant, or it may be that some of the gene products are not essential for sporulation and the absence of them only creates an undetectable defect in sporulation. However, few of the sporulation-specific genes have been examined for the behavior of the spores produced by homozygous mutant strains. Little is known about the process of spore development and germination, although they are obviously important parts of the developmental process. Mutations at *SPS100* (Law and Segall, 1988), *SPR1*, and *SPR9* (This work) are found to affect spore resistance to stress and germination competence. There may be more sporulation-specific genes that do not affect sporulation, but are nonetheless involved in these processes. More studies are needed to relate those gene functions to the biochemical events in spore development.

The protein predicted from the *SPR1* nucleotide sequence has a putative transit sequence, and the fusion protein of *SPR1-LacZ* was found in the membrane fraction in yeast cells (Primerano et al. 1990). Holaway et al. (1985) detected abundant transcripts of the *SPR1* gene in sporulating cells and the accumulation of the transcripts lasted at least 20 h after transfer to sporulation medium. In this study, we found that mutant spores missing the *SPR1* gene product are more sensitive to heat than are the wild-type spores. All these results suggest that the gene product of *SPR1* may be one of the components of the spore membrane. The mutant spores, however, regain heat resistance as sporulation progresses, and they finally reach the wild-type level of heat resistance after 24 h of incubation in sporulation medium. The delay in the heat resistance may suggest a delay in spore maturation. In the heterozygous strain, SPB1, both mutant and wild-type spores show the wild-type level of thermotolerance. This result indicates that the *SPR1* gene is maternally acting. The possibilities for the maternal action of the *SPR1* gene product are that *SPR1* functions before the formation of the spore wall or before the closure of ascospores, or that the *SPR1* protein can travel through the spore membrane freely. The nucleotide sequence of *SPR1* is not homologous to any sequence in Genebank, nor is the predicted protein homologous to any protein sequence in the protein bank (Primerano et al. 1990). Therefore, it is important in future to isolate the *SPR1* protein for biochemical analysis in order to reveal its function *in vivo* and its role in spore formation.

The *SPS100* gene isolated by Law and Segall (1988), which is expressed 12 h after transfer to sporulation medium, is also involved in spore maturation. The ether resistance of the *sps100* mutant spores is delayed when compared to that of wild-type spores. The nucleotide sequence of *SPS100* also predicts a transit sequence and shows many potential glycosylation sites for this protein. Their results also suggest that the *SPS100* protein is associated with the spore membrane. The ether-resistance of the *spr1* mutant spores was also examined in this study; the mutant spores exhibit the same level of resistance to ether as the wild-type spores (data not shown). Although different strains were used as recipients for constructing the *sps100* and *spr1* mutant strains, the kinetics of sporulation of the strains were similar. Both mutant spores became resistant to stress conditions at the same time (24 h after transfer to sporulation medium). These observations suggest that *SPS100* and *SPR1* function in protecting the spores from different kinds of stress. The reason for different gene products causing the same pattern of delay in spore maturation when missing is not clear.

The heat-sensitivity of *spr1* mutant spores and the ether-sensitivity of *sps100* mutant spores only exist during a few hours of development. Both mutant spores regained resistance in later stages of spore development. The reason that the gene functions of *SPR1* and *SPS100* are not absolutely required is not known. There may be proteins which appear later that can replace the functions of *SPR1* and *SPS100*. We may get the answer when the biochemical functions of these genes are known. One mutation of *B.subtilis*, *ger E36*, also shows a conditional phenotype. The endospores formed at 42°C were phase-gray, while when sporulated at lower temperature the spores became phase-bright (Moir, 1981). The basis for the conditional phenotype of the *ger E36* is not known, either.

The homozygous mutant strain *spr2/spr2* did not show any difference from the wild-type in any of the tests used in this study, including sporulation efficiency, thermotolerance, germination at high temperature, and resistance to ether (data not shown). The lack of phenotype of the *spr2* mutants may be due to the fact that only a limited number of criteria for testing mutant phenotypes during sporulation have been used, there may be other proteins that can replace the function of *SPR2*, or it may be simply that the function of the *SPR2* gene is nonessential. In *B. megaterium*, there are highly related acid-soluble polypeptides that are degraded to provide a source of amino acids during germination. These proteins may be redundant in function and the absence of any particular one would be without significant phenotype (Dignam and Setlow, 1980). Obviously, more experiments have to be done in order to draw any conclusions about the role of the *SPR2* gene during sporulation.

The *SPR9* gene is expressed after 4 h incubation in sporulation medium and the transcripts remain in abundance after at least 20 h more of incubation. The protein predicted from the nucleotide sequence of the *SPR9* locus is 16.8 kD, which is close to the size of one of the early sporulation-specific proteins identified by Kurtz and

Lindquist (1984). The predicted *SPR9* protein consists mostly of hydrophilic amino acids. No transit peptide sequence is present. No homologous sequences have been found for either the *SPR9* nucleotide sequence or the predicted protein sequence. The results of CHEF also revealed that there is no closely homologous sequence on any other chromosome of *S. cerevisiae.*

At temperatures higher than 37°C, the germination efficiency of the *spr9* mutant spores was found to be lower than the wild-type spores, for spores isolated from 16 h, 20 h, 24 h, and even 32 h sporulating cultures (data not shown for 32 h spores). Although reduced germination efficiency appeared in spores at all four times, the 20 h spores showed the most severe defect when germinated at high temperature. The decrease in germination efficiency did not result from a delay of response to germination signals, since prolonged germination time did not improve the germination efficiency of mutant spores (data not shown). It seems that either the maturation of mutant spores was not affected by the *spr9* defect, or the spore maturation was delayed even after 32 h of incubation in sporulation medium. If the first case is true, it suggests that the *SPR9* gene exerts its function after spore formation, or spore germination and formation are functionally independent. To test the second case, spores incubated longer than 32 h in sporulation medium should be used for the same kind of experiments. However, the heat-resistance of the *spr9* mutant spores was normal. This result suggests that the defective *SPR9* gene affects only the germination process. From the facts described above, we conclude that not only are the functions of *SPR1* and *SPR9* totally different, but they also act at different stages of spore formation.

The formation of small colonies was observed when the spores of strains W66-8A and derivatives were germinated at temperatures above 37°C. The appearance of the small colonies was most likely due to heat-induced induction of mitochondrial mutations. These mitochondrial mutants are called petites, they are defective in respiration and can not utilize glycerol as a carbon source. Petite *ade1* or *ade2* colonies are white and small. These small colonies not only are white, they also can not grow in YEPG medium. The result with germinating wild-type and *spr9* mutant spores suggests that the appearance of small colonies may be a consequence of the extent of maturation of spores germinated. This is supported by the observation that the more mature the spores are, the fewer small colonies are formed. Therefore, the fact that most small colonies are germinated from mutant spores is because that mutation at *spr9* locus makes spores more sensitive to heat during germination.

Multiple mutants, lacking more than one gene from a specific developmental pathway, are required for resolving the relationships among the genes in that pathway. If the genes involved are functioning in a sequential order, a damaged gene which functions at an early stage will block the expression of the genes functioning later. Thus, the multiple mutant will show the same phenotype as a single mutant blocked at the earliest gene. If genes disrupted are functioning independently, the multiple mutant will show a combination of mutant phenotypes in the same cell. If single mutants show no phenotype due to gene function duplication, the multiple mutants may show a defective phenotype. In this study, two double mutants were constructed to demonstrate the interrelationship among the *SPR1*, *SPR2*, and *SPR9* genes.

For *spr1/9* double mutant spores, the heat-sensitivity is much increased over the *spr1* single mutant spores. The number of viable spores of 16 h *spr1/9* spores dropped 10^5 after 72 h of incubation at 42°C, while *spr1* spores at the same stage only dropped 10-fold under the same conditions. The 16 h double mutant spores also showed a lower germination efficiency than the *spr9* single mutant spores at high temperature.

The above observations suggest that spores missing the two *SPR* gene products show greater sensitivity to heat both in viability and in germination efficiency than do the single mutants; i.e., the phenotype of a spore missing these two *SPR* gene functions is greater than the sum of the phenotypes of the two single mutants missing one *SPR* gene function. Therefore, we conclude that *SPR1* and *SPR9* genes do not function in sequence, nor do they have duplicated functions.

For *spr2/9* double mutants, the mutant spores showed increased thermosensitivity in addition to the predicted defect in germination. In addition, the efficiency of germination at high temperatures of double mutant spores was also lower than that of *spr9* single mutant spores. These results may suggest that the *spr2* defect, nonobservable in a single mutant, is revealed in a double mutant. From these result, we can also conclude that *SPR2* and *SPR9* genes do not function sequentially, nor have the same functions.

The physiology and the structure of endospores and spore germination of *Bacillus* have been studied in depth. Although the formation of endospores of bacteria is quite different from sporulation in *S. cerevisiae*, the knowledge obtained from bacterial spore formation can still serve as a model system for studying yeast spore formation. The spore coat of *B. subtilis* consists of twelve or more different peptides ranging in size from 8000 to 65,000 Mr, and the major component is a peptide of Mr 12,000. Four structural genes of spore coat, *cotA*, *cotB*, *cotC*, and *cotD* have been studied, which encode high abundance proteins of Mr 65,000, 59,000, 12,000, and 11,000, respectively. None of the *cot* mutations studied so far block the sporulation process. But the *cotD* mutant was found to germinate somewhat more slowly than the wild-type spores, and the *cotA* is responsible for sporulation-associated pigment production (Donovan et al., 1987). James and Mandelstam (1985) suggested that the spore coat of *B. subtilis* was acting as a protective shell, which participated in response to germination signals.

Future work should identify the biochemical roles of sporulation-specific genes during sporulation and later events. The regulation of expression of these genes is also an important and interesting question to be resolved. By comparing the sequence of the regulatory region of sporulation-specific genes, we may find some interesting sequences that control their developmentally regulated expression. Although such a sequence has not yet been found among these three genes, it may be that different sequences are used at different stages. Further sequencing should be performed on more sporulation-specific genes to identify such sequences, and these sequences can be used to isolate the factors, which react with them. By this process, the mechanism of regulating the expression of sporulation-specific genes may eventually be understood.

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