GAS2 and GAS4, a Pair of Developmentally Regulated Genes Required for Spore Wall Assembly in Saccharomyces cerevisiae

Enrico Ragni,1 Alison Coluccio,2 Eleonora Rolli,1 José Manuel Rodriguez-Peña,3 Gaia Colasante,1† Javier Arroyo,3 Aaron M. Neiman,2 and Laura Popolo1*

Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, 20133 Milano, Italy;1 Department of Biochemistry and Cell Biology, SUNY Stony Brook, Stony Brook, New York 11794-5215;2 and Departamento de Microbiología II, Universidad Complutense de Madrid, CP 28040 Madrid, Spain3

Received 9 October 2006/Accepted 15 December 2006

The GAS multigene family of Saccharomyces cerevisiae is composed of five paralogs (GAS1 to GAS5). GAS1 is the only one of these genes that has been characterized to date. It encodes a glycosylphosphatidylinositol-anchored protein functioning as a β(1,3)-glucan elongase and required for proper cell wall assembly during vegetative growth. In this study, we characterize the roles of the GAS2 and GAS4 genes. These genes are expressed exclusively during sporulation. Their mRNA levels showed a peak at 7 h from induction of sporulation and then decreased. Gas2 and Gas4 proteins were detected and reached maximum levels between 8 and 10 h from induction of sporulation, a time roughly coincident with spore wall assembly. The double null gas2 gas4 diploid mutant showed a severe reduction in the efficiency of sporulation, an increased permeability of the spores to exogenous substances, and production of inviable spores, whereas the single gas2 and gas4 null diploids were similar to the parental strain. An analysis of spore ultrastructure indicated that the loss of Gas2 and Gas4 proteins affected the proper attachment of the glucan to the chitosan layer, probably as a consequence of the lack of coherence of the glucan layer. The ectopic expression of GAS2 and GAS4 genes in a gas1 null mutant revealed that these proteins are redundant versions of Gas1p specialized to function in a compartment at a pH value close to neutral.
branes derived from the closure of the prosopore membrane and therefore is created in the absence of a preexisting structure and (ii) the layered organization of the spore wall follows a sequential program that differs from that occurring in vegetative growth. Moreover, the spore wall contains unique constituents, such as dityrosine and chitosan (8, 33). For these reasons, spore wall assembly is an interesting model of de novo formation of a supramolecular biological structure (33). Outside the plasma membrane of the spore, the two inner layers of the spore wall are made of mannoproteins and glucan that occur in an inverse orientation with respect to the vegetative cell wall (24). The external layers are formed by chitosan, a deacetylated form of chitin, and dityrosine, an insoluble arrangement of D- and L-tyrosine residues that confers the high resistance to external stresses that is typical of spores. The ascospores are interconnected by chitosan-containing structures that form the interspore bridges (9).

Melosis and sporulation involve the induction of many genes that have been divided into categories based on their temporal expression profiles (7, 38). Many genes involved in spore wall formation and maturation are classified as middle, middle-late, and late genes (7, 33, 38). Some of these genes are specific for sporulation and have no counterparts. Examples of such genes are CDA1 and CDA2, encoding two isomers of chitin-deacetylase, and DIT1 and DIT2, encoding the first enzymes in the synthesis pathway of dityrosine. Others are paralogs of genes that function in vegetative growth, such as SHC1, which replaces CHS4 in regulating Chs3p during sporulation, and CRRI, which encodes a sporulation-specific putative transglycosidase and is related to the CHRI and CRH2 genes that are expressed only during vegetative growth (16, 40). Thus, the peculiar architecture and composition of the spore wall require the action of gene products that have to be produced specifically during this developmental process.

The GAS multigene family is composed of five paralogs, from GAS1 to GAS5 (37). GAS1 is the best characterized of these genes. It encodes a GPI-anchored glycoprotein localized predominantly in the plasma membrane and recently shown to also be covalently bound to the cell wall (37, 45). It is a key protein in yeast cell wall assembly that, through its function in the amount of cell wall (1,3)-glucans that is compensated by an increase in chitin and mannoproteins, such as dityrosine and Gas4p, can replace Gas1p in vegetative growth, but only in the absence of the gene of interest (35, 36). The roles of the other GAS genes have not yet been investigated. In this work, we describe the characterization of the GAS2-GAS4 genes pair. We analyzed their expression profile, monitored the Gas2 and Gas4 protein levels during sporulation, and determined that together they are essential for proper spore wall assembly. Moreover, we found that Gas2p and Gas4p can replace Gas1p in vegetative growth, but only in media at near-neutral pH values.

### Materials and Methods

#### Yeast strains and growth conditions

The strains used were derived from the sporulation-proficient strains W303 and SK1 and are listed in Table 1. Cells were grown in batches at 30°C in synthetic dextrose (SD) minimal medium (Difco yeast nitrogen base without amino acids at 6.7 g/liter, 2% glucose), to which the required supplements were added at concentrations of 50 mg/liter for the amino acids and uracil and 100 mg/liter for adenine, or in YPD (1% yeast extract, 2% Bacto peptone, 2% glucose). For buffered medium, 10 g/liter MES [2-(N-morpholino)ethanesulfonic acid] was added and the pH was brought to 6.5 or 5.5. For solid media, 2% agar was added to YPD or SD media (YPD and SD, respectively). Growth was monitored as the increase in absorbance at 450 nm ($A_{450}$). Duplication time ($T_D$) was calculated by the equation $T_D = \ln 2/k$, where $k$, the growth rate constant, is the slope of the line obtained by linear regression on a semilogarithmic plot of the $A_{450}$ values, whereas the growth rate, $\mu$ (h$^{-1}$), was calculated as $1/T_D$.

For tetrad dissection, diploids were sporulated on solid plates of new sporulation medium (NSM; 8.2 g sodium acetate, 1.9 g KCl, 0.35 g MgSO$_4$. 7 H2O, 0.2 g NaCl, 15 g agar per liter) at 24°C. Spore germination was carried out at 30°C on YPD. Sporulation in liquid media was carried out as follows: cells were grown in YPD, and during exponential growth phase, they were collected by centrifugation, washed once with YPA (1% yeast extract, 2% Bacto peptone, and 2% potassium acetate) and inoculated into YPA at an initial optical density of 450 nm ($OD_{450}$) of 0.2. Cells were grown overnight, and the following morning, they were collected and washed with sporulation medium (SPM; 1% potassium acetate for S. cerevisiae supplemented with 2% glucose for W303 [46]), being inoculated in prewarmed SPM-1 at a concentration of about 10$^7$ cells/ml ($OD_{450}$ of about 1 to 1.5). In order to obtain a high efficiency of sporulation, the ratio of the volume of the culture to the volume of the flask was 1:10. Cultures were allowed to sporulate under vigorous shaking for 24 to 48 h at 30°C. Diploid strains harboring YEp24-derived plasmids were inoculated into liquid, semidefined sporulation medium (SA; 10 g potassium acetate, 6.7 g yeast nitrogen base without amino acids, and 1 g yeast extract in 1 liter of 0.05 pH 5 buffer [H9262]) and grown for 3 to 4 generations. Cells were sporulated in SPm-2, a 0.5% potassium acetate solution (17).

#### Quantification of mRNA using real-time quantitative reverse transcriptase PCR (RT-PCR)

Total RNA was extracted from cells (5 × 10$^7$) collected at different time intervals after transfer to sporulation medium, using an RNeasy mini kit combined with the RNaqueous DNase on-column treatment (Qiagen GmbH, Hilden, Germany). First-strand cDNAs were synthesized from 1.5 μg of total RNA using a reverse transcription system (Promega) following the recommendations of the manufacturer, except that the incubation time of the reverse transcription reaction was extended to 45 min. As a control for genomic contamination, the same reactions were performed in the absence or presence of reverse transcriptase. Real-time PCR was performed using an ABI 7700 instrument (Applied Biosystems) in a final volume of 20 μl containing 5 μl of a 100-fold dilution of the reverse transcription reaction using 1× SYBR green PCR master mix (Applied Biosystems) together with the specific forward and reverse GAS2, GAS4, or ACT1 primers (Table 2). The primers were designed using Primer Express software 2.0 (Applied Biosystems). Each primer couple was complementary to portions that are specific for each GAS open reading frame (ORF). The real-time PCR conditions were selected according to the universal conditions (default) recommended by the manufacturer of the instrument. Each cDNA was assayed in at least duplicate PCRs for two independent experiments. Basic analysis was performed using SDS 1.9.1 software (Applied Biosystems). For further elaboration of the data, the Livak method (30) was used. Briefly, from each duplicate reaction, a ΔΔCt was calculated by subtracting the average Ct value of ACT1 from the average Ct value of the gene of interest for the same time. Then the difference between the ΔΔCt at any time and the ΔΔCt at time zero was calculated (ΔΔCt). The plotted values are 2$^\Delta\Delta$Ct.
version, and two G-to-A transitions were found at nucleotides 666, 1138, 1283, 1370, and 1648, respectively, causing the following amino acid substitutions: A219 to V, K380 to E, L428 to H, C457 to Y, and M548 to I. In the 3′-flanking region, a C-to-A transversion was present. In the GAS4 5′-flanking region, the following point mutations were found: T to C, G to A, T to C, and A to T, at nucleotides 302, 297, 262, and 257, respectively, and the insertion of an A between nucleotides 28 and 29. In the GAS4 open reading frame, an A-to-T and a T-to-C base substitution was found at nucleotides 66 and 1023, respectively, but these changes were silent. Yeast plasmids pYER-2 and pYER-4 were obtained by cloning the NheI/SalI fragment from pER-2 and pER-4 into the corresponding sites of the YEp24 vector.

**Construction of the GAS2-3xHA fusion.** The fusion gene was obtained by overlap extension PCR (18). In the first PCR step, two overlapping fragments of the designed GAS2-3xHA fusion were amplified using two sets of primers: forward primer Nhe-GAS2 and reverse primer HA3-Rev. The amplified fragment of about 2.5 kbp was cloned in TA-TOPO vector and introduced into Escherichia coli TOP10 cells. The DNA plasmids were scored for the presence of BamHI, a diagnostic site for the cloning vector and introduced into S. cerevisiae. In the first PCR step, two overlapping fragments of the designed GAS2-3xHA fusion were amplified using two sets of primers: forward primer Nhe-GAS2 and reverse primer HA3-Rev.

| Strain | Genotype | Source |
|--------|----------|--------|
| AN117-4B | MATa arg4 his3 hо::LYS2 leu2 lys2 rme1::LEU2 trp1 ura3 | A. Neiman |
| AN117-16D | MATa his3 hо::LYS2 leu2 lys2 trp1 ura3 | A. Neiman |
| AN120 | MATa/MATa arg4/ARG4 his3/his3 hо::LYS2/ly2 leu2/ly2lys2 rme1::LEU2/RME1 trp1/trp1 ura3/ura3 (cross of AN117-4B and AN117-16D) | A. Neiman |
| ER300 | AN117-4B gas2::HIS3 | This study |
| ER303 | MATa/MATa arg4/ARG4 gas2::HIS3/GAS2 his3/his3 hо::LYS2/ly2 leu2/ly2lys2 rme1::LEU2/RME1 trp1/trp1 ura3/ura3 (cross of ER300 and AN117-16D) | This study |
| ER303-6A | MATa gas2::HIS3 his3 hо::LYS2 leu2 lys2 trp1 ura3 (segregant from ER303) | This study |
| ER304 | AN117-4B gas::kanMX2 | This study |
| ER304 | MATa/MATa arg4/ARG4 gas2::kanMX2 his3/his3 hо::LYS2/ly2 leu2/ly2lys2 rme1::LEU2/RME1 trp1/trp1 ura3/ura3 (cross of ER304) | This study |
| ER308 | MATa arg4/ARG4 gas2::HIS3/GAS2 gas4::kanMX2/GAS4 his3/his3 hо::LYS2/ly2 leu2/ly2lys2 rme1::LEU2/RME1 trp1/trp1 ura3/ura3 (cross of ER300 and ER304-3B) | This study |
| ER308-9C | MATa arg4/ARG4::HIS2::LEU2 his3 hо::LYS2 leu2 lys2 rme1::LEU2 trp1 ura3 (segregant from ER308) | This study |
| ER308 | MATa arg4/ARG4::HIS3 gas4::LEU2 his3 hо::LYS2 leu2 lys2 rme1::LEU2/RME1 trp1/trp1 ura3/ura3 (cross of ER308-13A and ER308-9C) | This study |
| ER310 | AN120[2 μ·UR3](YEp24)] | This study |
| ER311 | AN120[2 μ·gas2–UR3](YEp24)] | This study |
| ER312 | ER300[2 μ·gas2–UR3](YEp24)] | This study |
| ER313 | ER309[2 μ·gas2–UR3](YEp24)] | This study |
| ER314 | ER306[2 μ·gas2–3xHA–UR3](YEp24)] | This study |
| ER315 | ER309[2 μ·gas2–3xHA–UR3](YEp24)] | This study |
| ER316 | AN120[2 μ·gas2–3xHA–UR3](YEp24)] | This study |
| ER317 | ER309[2 μ·UR3](YEp24)] | This study |

**W303 background**

| Strain | Genotype | Source |
|--------|----------|--------|
| W303-1A | MATa ade2–1 can1–100 his3–11,15 leu2–3,112 trp1–1 ura3–1 | P. Slominski |
| W303-1B | MATa ade2–1 can1–100 his3–11,15 leu2–3,112 trp1–1 ura3–1 | P. Slominski |
| WB2d | gas1::LEU2 (derived from W303-1B) | Vai et al. (1991) |
| G2HB | W303-1B gas2::HIS3 | This study |
| G4LB | W303-1B gas2::kanMX2 | This study |
| Y0 | Wh2[2 μ·URA3–43](YEp10)] | This study |
| Y1 | WB2d[2 μ·URA3–43–GAS1](YEp10)] | This study |
| Y2 | WB2d[2 μ·URA3–43–GAS1–GAS2 ORF(Yp23)] | This study |
| Y4 | WB2d[2 μ·URA3–43–GAS1–GAS2–ORF(Yp4)] | This study |
| W3033 | MATa/MATa ade2–1ade2–1 can1–100/can1–100 his3–11,15/his3–11,15 leu2–3,112/leu2–3,112 trp1–1/trp1–1 ura3–1/ura3–1 (cross of W303-1A and W303-1B) | This study |
| G2D2 | MATa/MATa ade2–1ade2–1 can1–100/can1–100 Gas2::GAS2 his3–11,15/his3–11,15 leu2–3,112/leu2–3,112/trp1–1/trp1–1 ura3–1/ura3–1 | This study |
| G4D2 | MATa/MATa ade2–1ade2–1 can1–100/can1–100 Gas2::LEU2/gas2::LEU2 his3–11,15/his3–11,15 leu2–3,112/leu2–3,112/trp1–1/trp1–1 ura3–1/ura3–1 | This study |
| G24D2 | MATa/MATa ade2–1ade2–1 can1–100/can1–100 Gas2::HIS3/gas2::HIS3 gas4::LEU2/gas4::LEU2 his3–11,15/his3–11,15 leu2–3,112/leu2–3,112/trp1–1/trp1–1 ura3–1/ura3–1 | This study |

* A superscript 2 indicates a diploid. 
The coding sequences for the GAS2 and GAS4 genes, previously cloned from W303-1B genomic DNA, were placed under the control of the GAS1 promoter (pGAS1) in the high-copy YEp24 vector. The plasmids were a kind gift of M. Vai (Università di Milano-Bicocca). Briefly, a 4-kbp NcoI/BamHI fragment containing the GAS1 5′-flanking region and the GAS2 ORF and its downstream sequence was excised from plasmid pG2 derived from pGEM7Zf(+) and cloned into the corresponding sites of the vector. A 3.8-kbp NcoI/BamHI fragment containing the GAS4 ORF and termination sequences cloned downstream from the GAS4 upstream region was excised from plasmid pG4 and inserted in YEp24. A 3.8-kbp NcoI/BamHI fragment containing the GAS4 ORF and termination sequences cloned downstream from the GAS4 upstream region was excised from plasmid pG4 and inserted into YEp24, YEp24-pGAS2-GAS4, and YEp24-pGAS4-GAS4 were used to transform gas2Δ strains W303-1B and AN117-4D null mutants were obtained and crossed with the strain of opposite mating type (W303-1A). The diploids were obtained from zygotes by micromanipulation of conjugating meiotic segregants carrying the desired mutations. In the SK1 genetic background, the haploid AN117-4B strain carrying the LEU2 marker are in boldface.

**TABLE 2. Oligonucleotide sequences used**

| Use and name | Sequence (5’ to 3’) |
|--------------|---------------------|
| Quantitative RT-PCR | CATTCGCGTACCTAGTTACTAGAGGTT |
| GAS2 Forward | ATGGCAGATTTGTCGACATCAA |
| GAS2 Reverse | ATGCGGATGTCGCAAGCAAAG |
| GAS4 Forward | AACACGCGGAAATTGTGCTTAT |
| GAS4 Reverse | AAGCCAAAACACAACCAAGG |
| ACT1 Forward | AGCAAGGATTCGAGGGCCCA |
| ACT1 Reverse | GCAAGATTCCAAAACAAACAA |

Cloning GAS2 and GAS4:

| Nhe-GAS2 | AGCATATTCGACTGAGTTACTAGAGGTT |
| GA4SA-Sal-dow | ATGCCTGGCTATCGTCAAAATATTAA |
| Nhe-GAS4 | AAGCCAAACACAACCAAGG |
| Sal-GAS4 | ATCGTGGCTATCGTCAAAATATTAA |

Internal tagging of GAS2:

| GAS3 Rev | GGAGACTCGTACTGTGCATA |
| GAS4 Rev | GTATTGTAAGGTGAGACT |
| Gas2orf | CCACACCATTCGTACATA |
| Gas2Valle | ACTGCCTGTACTGCTCAT |
| Gas2Test | TCGG TGTCAGTAATTCTG |

Inactivation of GAS2 and GAS4:

| Gas2Hfor | AACTCGAGGTACTTGAAACTTTTCAGT |
| Gas4Hfor | AAATGCCTGTACTGCTCAT |
| Gas4Rev | TGCATAGTCCGGGACGTCATACGGATAGC |

Construction of mutant strains: The oligonucleotides used to construct the null mutations and to test them are listed in Table 2. The short-homology PCR technique, followed by one-step gene disruption, was used for the construction of the mutant strains. Plasmid pFA6a-HIS3MX6, containing the module HIS3MX6 with the his3Δ gene from Schizosaccharomyces pombe, was used to amplify a PCR fragment used to inactivate GAS2. The 1.4-kbp PCR fragment, carrying 42 nucleotides at the ends complementary to the -32 to-10 and 1616 to-1657 segments from the start codon of GAS2, was used to transform both of the haploid strains W303-1B and AN117-4B, giving rise to strains G2H8B and ER300, respectively (Table 1). pFA6a-KanMX2, which contains the KANMX2 module, was used to amplify a 1.5-kbp PCR fragment used to inactivate GAS4 in the AN117-4B strain. The PCR fragment carried sequences complementary to the 13 to 72 and 1332 to 13391 from the GAS4 ATG at the ends, was amplified with oligonucleotides GAS4Lfor and GAS4Lrev and used to transform the W303-1B strain, creating strain G4L8B (Table 1). S. cerevisiae cells were transformed with an S.C. EasyComp transformation kit (Invitrogen). About 50 ng of genomic DNA isolated from the transformant clones was subjected to three diagnostic PCR tests to verify correct integration (the primers used are listed in Table 2). The W303-derived haploid gas2Δ and gas4Δ null mutants were obtained and crossed with the strain of opposite mating type (W303-1A). The diploids were obtained from zygotes by micromanipulation of conjugating meiotic segregants carrying the desired mutations. In the SK1 genetic background, the haploid AN117-4B strain carrying the LEU2 marker are in boldface.

**Light microscopy:** Cells were routinely observed by phase-contrast microscopy, and sporulation was scored by counting at least 200 cells after a mild sonication.

**Microscopic observation of dityrosine:** The observation of the natural fluorescence of dityrosine was performed essentially as described previously (4). Sporulating cells were collected by centrifugation and resuspended in 1 ml of S. cerevisiae 50 mM HEPES pH 7.3, 1 mM MgCl2, 1 mM DTT, 1% glycerol and 10 min incubation in the dark. Cells were washed twice with iced H2O and observed by fluorescence microscope with 465 nm and 490 nm filters.

**Assay for the presence of dityrosine:** An assay for the presence of dityrosine was performed as described previously (3, 26). Cells were streaked onto solid YPD. After a 2-day incubation at 30°C, the plates were then replica platted onto nitrocellulose filters that had been placed on YPD plates. After a 1-day incubation at 30°C, the filters were transferred to solid sporulation medium (SPM-1), colony-side up, and placed at 30°C for 3 days. To remove the ascus walls, the filters were placed in a 9-cm petri dish containing 400 μl of water, 140 μl of glusulase (from Helix pomatia; Roche), and 30 μl of 2-mercaptoethanol. After 5 h at 30°C, the filters were transferred to a dish containing 500 μl of 30% aqueous ammonia. The filters were photographed under UV light (312 nm) using a digital camera.

**DNA staining:** Cells were collected by centrifugation at 13,000 rpm for 1 min, and the pellet was washed twice with distilled water (dH2O). Then, cells were fixed with ethanol and preserved at 4°C until use. At the time of the analysis, cells were washed with dH2O and pellets were resuspended in a solution of 0.125 μg/ml of DAPI (4,6-diamidine-phenylindole). After a 10-min incubation in the dark, cells were washed twice with dH2O and examined in the fluorescence microscope. Cells with 1, 2, or 4 nuclei were counted.

**Microscopic observation of dityrosine:** The observation of the natural fluorescence of dityrosine was performed essentially as described previously (4). Sporulating cells were collected by centrifugation and resuspended in 1 ml of 5% aqueous ammonia. Cells were observed under the fluorescent microscope using UV light (DAPI filter).
Permeability and Zymolyase assays. For testing permeability to calcofluor, an aliquot corresponding to $10^7$ cells was withdrawn from the culture and mildly sonicated. Cells were pelleted, washed with 500 μl of dH$_2$O, and resuspended in 500 μl of a solution of 10 μg/ml of calcofluor white (CW; Sigma). After a 1-min incubation at room temperature, cells were centrifuged and washed three times with 800 μl of dH$_2$O. Cells were examined with an Olympus BX60 microscope connected to a DC290 Kodak digital camera. Zymolyase sensitivity was quantitated essentially as described previously (8): 100 μl of sporulated culture (approximately 0.2 at OD$_{600}$) was washed and resuspended in 1,090 μl of dH$_2$O. Ten microliters of Zymolyase 100T (ICN Biomedicals, Aurora, OH) at 10 mg/ml was added, and the cells were incubated at 37°C. At 10-min intervals, 100 μl of cells was withdrawn, diluted in dH$_2$O, and plated to determine the titer of viable cells.

Test of sensitivity to growth to calcofluor. Five microliters from a concentrated suspension of cells (total, 8 at OD$_{600}$ and 5 μl from 1:10 serial dilutions of the concentrated suspension were spotted on SDA or buffered SDA plates in the absence or presence of 2, 5, 10, 25, or 50 μg of CW per ml. Growth was checked after 2 days at 30°C.

Electron microscopy. Cells were prepared for analysis on the transmission electron microscope (TEM) using osmium tetroxide and sodium thiocarboxyl hydradize staining as described previously (8). Images were collected on an FEI BioTwin microscope at 80 kV using an AMT digital camera (Advanced Microscopy Techniques Corp., Danvers, MA). For scanning electron microscopy (SEM) studies, spheroplasts were sporulated and prepared as described previously (8), except that spores were released from the asc wall membrane by washes in 0.1% sodium dodecyl sulfate (SDS). Images were collected on a LEOM550 SEM at 2.5 kV using an in-lens detector.

Extract preparation, electrophoresis, and immunoblotting. Sporulating cells (2 x 10^6) were collected by filtration, washed, and resuspended in ice-cold dH$_2$O. After a 2-min centrifugation at 4°C, the pellets were frozen quick and stored at −20°C. After thawing, 500 μl of SB-minus buffer (0.0625 M Tris-HCl [pH 6.8], 5% SDS) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 0.02% bromophenol blue). Before loading, samples were denatured at 100°C for 3 min. Slab gels of 8% polyacrylamide gel electrophoresis (PAGE) analysis, appropriate amounts of a concentrated solution was added to the lysate in order to bring the sample to a final concentration of 10% glycerol, 5% β-mercaptoethanol, and 0.02% bromophenol blue. Before loading, samples were denatured at 100°C for 3 min. Slab gels of 8% polyacrylamide gel were used for extract separation. The electrophoresis and transfer to nitrocellulose membranes were performed using the apparatus described previously (8). The nitrocellulose membranes were blocked with 5% nonfat dry milk in TBST (0.1% Tween 20, 10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% Tween 20, and stored at 4°C overnight. Anti-Gas4p serum purified F(ab')2 fragment donkey anti-rabbit or anti-mouse immunoglobulin G was used at a dilution of 1:1,000 in Tris-buffered saline-bovine serum albumin, 0.2% Tween 20. Monoclonal mouse anti-actin antibody, clone C4 (MP Biomedicals), was used at a dilution of 1:1,000. Anti-Gas4p serum was obtained by immunizing rabbits with a soluble His-tagged form of Gas4p produced in Pichia pastoris (unpublished data). The immunization procedure was carried out by Acta International S.r.l. (Gerenzano, Varese, Italy). The optimal dilution of anti-Gas4p serum was 1:1,000 in Tris-buffered saline-bovine serum albumin, 0.2% Tween-20. Peroxidase-conjugated affinity-purified F(ab')2 fragment donkey anti-rabbit or anti-mouse immunoglobulin G was used at a dilution of 1:10,000. Bound antibodies were revealed using ECL Western blotting detection reagents (Amersham Pharmacia Biotech). Densitometric measurements of underexposed autoradiographs were performed using the Scion Image program.

RESULTS

Expression profiles of GAS2 and GAS4 genes during meiosis and sporulation. The levels of GAS2 and GAS4 mRNA were determined in a sporulation time course. An SK1 background strain was used, since it sporulates with a high degree of synchrony, completing the process in 24 h, as shown in Fig. 1, upper panel. At different time intervals from the induction of sporulation, total RNA was extracted from SK1 cells and used for a quantitative real-time RT-PCR analysis. Actin mRNA was chosen as a reference transcript, since the expression of the actin gene (ACT1) does not fluctuate significantly in sporulating SK1 cells (38). As shown in Fig. 1, central and lower panels, the expression of both GAS2 and GAS4 was limited to a short time span. A peak of expression occurred at 7 h from induction of sporulation, and by 10 h, the mRNA levels were greatly decreased. The comparison of the cycle threshold values for GAS2 ($C_T = 21.08$) and GAS4 ($C_T = 18.74$), obtained at 7 h in the same amplification experiment, indicated that GAS4 is expressed at a higher level than GAS2. Moreover,
GAS2 and GAS4 transcripts were not detectable at time zero, suggesting that these genes are not substantially transcribed in cells growing in presporulation medium (YPA).

These data are in agreement with the microarray analysis of sporulating yeast cells that showed that GAS2 and GAS4 are strongly induced during the middle phase of sporulation, leading them to be classified in cluster 5a of the middle genes (7, 38). However, the microarray analysis was limited to the first 11.5 h of the sporulation process, while our study extends the analysis to the completion of the sporulation process.

**GAS2 and GAS4 protein levels during sporulation.** The levels of Gas2 and Gas4 proteins during sporulation were monitored. To detect Gas2p, a tagged version of the protein was constructed, whereas for Gas4p, a polyclonal antiserum raised against the recombinant form of Gas4p was used. Since GAS2's sequence predicts a polypeptide that has a signal peptide at the N-terminal end and the GPI attachment signal at the C-terminal end, the tag was inserted internally. To construct a fusion that did not affect the function of the protein, the Gas2 protein sequence was first analyzed with the GlobPlot 2 method that predicts the globular and disordered regions of a protein (29). The $3 \times$ HA tag was inserted in a C-terminal random coil segment of the protein between residues S509 and N510. Then, the fused gene was cloned in a multicopy plasmid.

To first verify that overexpression of the untagged GAS2 gene did not bring about any detrimental effect on sporulation, the GAS2 gene was introduced into the SK1 strain in a high-copy-number plasmid, generating the strain ER311. Overexpression of GAS2 had no relevant effect on sporulation compared to the SK1 strain harboring the empty vector and named ER310 (Table 3). Then, to check if the tagged version of the GAS2 gene was functional, the modified gene was introduced into the SK1 strain growing in presporulation medium (YPA).

The cells were grown in SA and were analyzed 24 h after the shift to SPM-2. After 10 h, the protein levels began to steadily decrease. The behavior of the Gas4p levels during sporulation is shown in Fig. 2B, lower panel. It was not influenced by overexpression of Gas2p, since the same profile was obtained in a time course experiment performed using the parental SK1 strain (data not shown). The specificity of the antiserum used was also checked. Gas4p was absent in sporulating gas2Δgas4Δ cells at the time of maximal expression (Fig. 2C, lane 1). Moreover, the serum recognized a 45-kDa band in haploid gas1Δ cells ectopically expressing GAS4 (Fig. 2C, lane 1).
2). This band was absent in the same cells transformed with the empty vector (Fig. 2C, lane 3).

The profile of Gas4p levels suggests that its regulation occurs not only at the transcription level, but also at the level of protein stability. Moreover, the apparent molecular mass of Gas4p appears higher than predicted for this polypeptide lacking the putative N- and C-terminal signal sequences (48,520 Da), suggesting that this protein is probably modified by glycosylation. Similarly, the predicted molecular mass of Gas2p lacking the N- and C-terminal signal sequences is 60,600 Da, and therefore, Gas2p could also be modified. To verify if Gas2 and Gas4 proteins are glycosylated, total extracts from sporulating cells overexpressing these proteins were subjected to treatment with EndoH, an enzyme that removes N-linked chains. The effects of short (4 h) and long (16 h) incubations were analyzed (Fig. 2D). Gas2p showed a shift in mobility at 4 h, giving rise to a band of about 62 kDa. Its pattern of deglycosylation did not change at 16 h of treatment. This indicates that only one short N-linked chain is present, in agreement with the prediction of a single potential N-glycosylation site in Gas2p. At 4 h of treatment, Gas4p showed the reduction of the intensity of the 54-kDa band and the appearance of two lower bands, of ~52 and 50 kDa. At 16 h, only the 50-kDa band was present (Fig. 2D). This result indicates that two N-linked chains are attached to Gas4p and that the two potential N-glycosylation sites present in the sequence are both used in vivo.

Loss of GAS2 and GAS4 reduces the efficiency of sporulation. The effects of the loss of GAS2 and GAS4 genes were examined in two sporulation-proficient strains: W303 and SK1. The phenotypes of haploid gas2Δ, gas4Δ, and gas2Δ gas4Δ strains and of the corresponding homozygous null diploids were first examined during vegetative growth. In YPD at 30°C, the null mutations did not cause any obvious changes in the growth rates or in cell morphologies. Moreover, two phenotypic indexes of cell wall damage, calcofluor white sensitivity and activation of the cell integrity pathway, were unaffected (data not shown). Upon induction of sporulation, the diploid W303Δ strain completed sporulation in 48 h. At this time the percentages of sporulated cells, determined in three different experiments, were 73% for the wild type and 59%, 52%, and about 30% for the gas2/gas2, gas4/gas4, and gas2 gas4/gas2 gas4 mutants, respectively, taking into account that in the double null mutant, detection of the spores was difficult. Remarkably, at 48 h, mature asci with 4 spores represented 50% of the total cells in the parental strain, whereas they represented only 15%, 16%, and 5%, respectively, of the total cells in the mutants, suggesting that the defect in spore maturation was present in...
the single mutants and became worse in the absence of both genes. Next, we verified the behavior of the homozygous null mutants during sporulation in the SK1 genetic background. As reported in Table 3, at 24 h the percentage of unsporulated cells was not appreciably affected in the gas2 Δ gas2 Δ mutant cells (B), gas4 Δ gas4 Δ mutant cells (C), gas2 Δ gas4 Δ gas2 Δ gas4 Δ mutant cells (D), whereas it increased in the gas2 Δ gas4 Δ gas2 Δ gas4 Δ mutant. Moreover, mature asci with 4 spores were only 0.7% of the total cells in the double mutant, compared to 65 to 70% of the total cells in the parental strain and in the single mutants (Table 3). The lack of clear definition of spore edges made the determination of ascal type in the double mutant difficult (see below). However, a marked increase in the numbers of monads and triads was observed. In conclusion, the mutations affect sporulation similarly in W303 Δ and SK1 strains, but the single gas2 or gas4 null mutations display greater changes in phenotype in W303 Δ than in SK1.

The morphologies of sporulating SK1-derived cells are shown in the phase-contrast micrographs of Fig. 3. As shown in panels B and C, the spores of the single mutants were round and arranged in a regular fashion, like the control (panel A), and were bright. In the double mutant (panel D), cells appeared less bright and refractile, the internal separation of the cytoplasm was unclear, and many granules were present. Moreover, the scoring of sporulated cells was difficult since spore edges were not clearly visible or appeared thin. A detail of this phenotypic trait is shown in Fig. 3F. Cells with one spore of normal size close to a small one were also frequent (about 15% of the total). Thus, the double mutation causes severe

![Image](image-url)
defects in spore formation and maturation, giving origin to a heterotypic phenotype. The morphologies of sporulating W303-derivative strains were very similar to those described above for SK1 strains (data not shown). Since in both strains the reduction of the efficiency of spore formation in the double mutant is more severe than in the single mutants, GAS2 and GAS4 genes play a partial, redundant role in sporulation. Due to its higher degree of synchrony in sporulation, further analysis of spore wall defects was carried out only for SK1-derived mutants.

The effects of overexpression of GAS2 and GAS4 were tested by analyzing SK1 strains carrying these genes on a multicopy plasmid (ER311 and ER316 strains). As shown in Table 3, no appreciable effects on sporulation efficiency were observed compared to that of the isogenic strain transformed with the empty vector (ER310), indicating that neither GAS2 nor GAS4 is detrimental for sporulation when overexpressed.

Nuclear progression occurs normally in the gas2 and gas4 mutants. In order to determine if the loss of GAS2 and GAS4 genes primarily affected the meiotic process and the effect on spore morphogenesis was a consequence of this defect, the kinetics of chromosomal segregation in the mutants was monitored by nuclear DNA staining of cells undergoing sporulation. The results are shown in Fig. 4A to C. The kinetics of formation of cells with 1, 2, or 4 nuclei were not significantly affected by the mutations. Moreover, very similar percentages of tetranucleate cells were reached at 12 h from sporulation induction. Thus, meiotic progression does not appear to be significantly affected by the lack of GAS2 or GAS4 or both GAS2 and GAS4. Cells stained by DAPI are shown in Fig. 4D to G. Panels F and G show examples of double-mutant cells in which spores were not clearly distinguished, but four nuclei were present. We can conclude that Gas2 and Gas4 proteins are not required for meiotic progression.

Deletion of GAS2 and GAS4 leads to spore wall-defective phenotypes. Further evidence for defects in spore formation was obtained by analyzing the presence of dityrosine, a specific component of the outermost layer of the spore wall. A qualitative assay that exploits the fluorescence of dityrosine under UV light was performed as described in Materials and Methods. Patches of the parental and mutant strains were replica plated on a nitrocellulose filter and placed on solid sporulation medium. After 3 days, the filters were observed. As shown in Fig. 5A, the fluorescence observed for the gas2 gas4 null mutant was less intense than for the other strains. This indicates that dityrosine is present but less abundant, consistent with the low efficiency of sporulation of the double mutant. Cells undergoing sporulation in liquid medium were also analyzed with the microscope under conditions of basic pH that enhance the dityrosine fluorescence. Surprisingly, in the double mutant,

FIG. 5. Qualitative assay of dityrosine and microscopic analysis of intact asci. (A) Patches of cells of the indicated diploid strains were grown on a YPD plate, photographed (left panel), replica plated onto a nitrocellulose filter, and again photographed under UV light after 3 days on a sporulation plate (right panel). (B) SK1 (WT) and gas2 gas4 null mutant cells were collected at 24 h after induction of sporulation and visualized both with phase-contrast microscopy (upper panels) and under UV light (lower panels) after resuspension in 5% aqueous ammonia. Details are shown in the right panels.
microscopic examination of dityrosine revealed the presence of spores that were not detectable with phase-contrast microscopy (Fig. 5B), even though the number of spores per ascus was lower than for the wild type and their shape was irregular. At closer examination of the wild type, the asci showed a regular arrangement of fluorescence with a brighter signal at the contact points between spores (Fig. 5, details in the micrograph on the right). In the double mutant, the dityrosine was less regularly arranged and was also found diffusely throughout the ascus. Cells negative for dityrosine were also present. (Fig. 5, see details on the right). Thus, the double mutant seems to be defective in the normal accumulation and deposition of the dityrosine layer.

Intact cells undergoing sporulation were stained with CW, a dye that binds to chitin or chitosan fibrils but is also an indicator of defects in permeability (16). Cells with defects in spore wall assembly become permeable to this dye that otherwise would not penetrate into intact cells (34). As shown in Fig. 6A and B, in the parental cells bright areas corresponding to bud
scars of the mother cell are detected, whereas a faint internal staining of the spore edges corresponding to the chitosan layer is also visible. In the gas2 gas4/gas2 gas4 mutant, CW stains some spores internally, indicating that severe permeability defects or dead spores are present inside the asci (Fig. 6C and D). This staining of spores was not detected in the single mutants, which resembled the wild type (data not shown). In order to quantify this defect, we exploited a viability assay using Zymolyase. Zymolyase hydrolyzes the β(1,3)-glucan layer of the ascus wall and releases the spores but is not able to attack the glucan layer of the spore wall, since the chitosan and dityrosine layers limit its accessibility. Thus, the loss of the capability to form colonies after Zymolyase treatment is an index of increased spore wall permeability to Zymolyase. We performed a quantitative assay of the Zymolyase sensitivity of sporulated cells at 24 h from induction of sporulation. The results are shown in Fig. 6E. The CFU values at different times after exposure to Zymolyase were compared to the value at time zero for each strain. As shown, the wild-type cells and gas2 and gas4 null diploid mutants released spores that were still viable after longer times of treatment, whereas the viability of the gas2 gas4 double mutant cells steadily decreased, reaching a 10-fold drop in viability after 1 h of incubation. Moreover, the absolute CFU values at time zero were about four times lower for the double mutants than for the parental and single mutant cells. The number of CFU obtained from $10^7$ sporulating cells was about $4.5 \times 10^2$ for the single mutant and parental strains and about $1.1 \times 10^4$ for the double mutant. These results indicate that double mutant cells were less viable even in the absence of Zymolyase. In conclusion, defects in gas2 gas4 spore formation lead to a lowered viability and a higher permeability to the uptake of exogenous molecules into the spores.

**Cell wall ultrastructure.** To further characterize the spore wall defect in gas2 gas4 cells, the ultrastructure of the spore wall was examined by both transmission and scanning electron microscopy. TEM analysis suggests that all four of the layers of the spore wall are present and the outer chitosan and dityrosine layers appear to be intact (Fig. 7). However, frequent defects are seen at the interface between the β(1,3)-glucan and chitosan layers (Fig. 7). At these sites, the β(1,3)-glucan and chitosan layers are dissociated and an accumulation of disordered material, possibly unassembled carbohydrate chains, is seen. This accumulation results in a protrusion of the outer layers of the spore wall.

The appearance of the gas2 gas4 spores under SEM is consistent with the TEM results (Fig. 8). The surface texture of the spores appears similar to that of the wild type, and interspore bridges are present, suggesting that the assembly of the outer chitosan and dityrosine layers is largely normal. However, the spores often appear misshapen, with prominent bulges as well as extended areas of the surface that appear flat, rather than scalloped as in wild-type spores (Fig. 8). These bulging and flattened areas may correspond to the accumulations of material at the β-glucan–chitosan interface seen under TEM. In sum, the ultrastructural analysis of gas2 gas4 spores suggests

---

**TABLE 4. Effects of the ectopic expression of GAS2 and GAS4 on the growth of the gas1Δ mutant at different pH values**

| Strain     | Growth rate [µ (h⁻¹)]a | Unbuffered medium | Buffered medium (pH 5.5) | Buffered medium (pH 6.5) |
|------------|------------------------|-------------------|--------------------------|--------------------------|
| W303-1B    | 0.54                   | 0.55              | 0.48                     |                          |
| WB2d (gas1Δ) | 0.32                   | 0.30              | 0.16                     |                          |
| Y0         | 0.34                   | 0.35              | 0.17                     |                          |
| Y1         | 0.51                   | 0.46              | 0.43                     |                          |
| Y2         | 0.23                   | 0.26              | 0.33                     |                          |
| Y4         | 0.31                   | 0.42              | 0.40                     |                          |

a Growth was measured as the increase in OD₄₅₀. Values are the means of three independent experiments. The pH of the buffered medium during growth never changed by more than 0.1 unit.

---

**Fig. 8.** SEM analysis. The panels show SEM images of spores from wild-type (A) and gas2Δ gas4Δ mutant (B and C) cells. Arrows indicate interspore bridges, white arrowheads indicate regions where the spore wall is distended on the gas2Δ gas4Δ mutant spores, and black arrowheads indicate smooth surface regions on the gas2Δ gas4Δ mutant cells. Bars, 1 μm.
that the mutant is defective in attachment of the inner and outer spore wall layers to each other. This results in spores that have improperly organized, less-robust walls, consistent with the poor refractility of the spores under the light microscope (see Discussion).

Does ectopic expression of GAS2 and GAS4 complement the gas1 null mutant phenotype? The ability of GAS2 and GAS4 to complement the gas1 null mutant phenotype was examined. In order to allow the expression of these genes in vegetative growth, their promoter was replaced with the GAS1 promoter (P\textsubscript{GAS1}). The YEp24 vector and recombinant YEp24 plasmids harboring the GAS1 gene, P\textsubscript{GAS1}--GAS2 or P\textsubscript{GAS1}--GAS4, were used to transform a gas1 null mutant. The resulting transformants were named Y0 (vector), Y1 (YEp24--GAS1), Y2 (YEp24--P\textsubscript{GAS1}--GAS2), and Y4 (YEp24--P\textsubscript{GAS1}--GAS4). The kinetics of growth were monitored, and in Table 4, the values of the growth rate (μ) are shown. Interestingly, the growth rates of Y2 and Y4 were the same as those of the Y0 and gas1Δ strains, indicating that no complementation occurred in SD medium (Table 4). This was confirmed by the analysis of cell morphology (Fig. 9A). We reasoned that spore walls develop in an intracellular compartment and, thus, the pH of the environment in which Gas2p and Gas4p normally function could be close to neutrality. Cells acidify the medium during growth, and indeed, the pH of the SD medium was found to be about 3.5 at mid-exponential phase. Therefore, we tested the effect of buffering the medium to pH values of 5.5 and 6.5 on the suppression of the gas1Δ phenotype by GAS2 and GAS4 genes. As shown in Table 4, GAS4 suppressed the mutant phenotype partially at pH 5.5 and almost fully at pH 6.5. At the latter pH value, the swollen-cell morphology typical of gas1 cells, which is exacerbated by the increased pH, was totally suppressed and cells appeared similar to wild-type cells (Fig. 9A). At pH 5.5, the GAS2 gene did not complement the gas1Δ phenotype, as the Y2 strain has a growth rate lower than that of Y0, and its morphology appeared slightly worse than that of gas1. At pH 6.5, the phenotypic defects of the gas1Δ strain were partially reversed, since the Y2 cells grew slightly faster and the cells were smaller than gas1Δ mutant cells but still rounder than wild-type cells (Table 4 and Fig. 9A). The effect on gas1 mutant cells of buffering the medium to pH 6.5 was further analyzed by monitoring the suppression of hypersensitivity to CW, another phenotypic trait typical of gas1 mutant cells. In Fig. 9B, it is possible to observe that GAS2 did not suppress the hypersensitivity of gas1 cells to CW at pH 5.5. At pH 6.5, the suppression of hypersensitivity to CW by GAS2 was only partial, as shown by the plates containing 2 μg/ml of CW, and cells were still hypersensitive at higher concentrations of CW (Fig. 9B). On the other hand, GAS4 complemented CW hypersensitivity at both pH 5.5 and 6.5 (Fig. 9B). Altogether, the data indicate that both Gas2p and Gas4p are functional homologs of Gas1p, but Gas4p replaces Gas1p function better than Gas2p. Gas2p partially complements the gas1Δ phenotype only at pH 6.5.

**DISCUSSION**

The GAS gene family of *S. cerevisiae* is composed of the well-studied GAS1 gene and four paralogs (GAS2 to GAS5) that have never been investigated. This study addressed the roles of two of the paralogues, GAS2 and GAS4. Whereas GAS1 is expressed in vegetative growth and its transcription is shut down as cells enter sporulation (7, 34, 38), the GAS2 and GAS4 genes exhibit the reverse behavior (7, 38). The expression of GAS2 and GAS4 is triggered in cells undergoing sporulation and is absent during vegetative growth. In particular, we have shown in this work that GAS2 and GAS4 mRNA levels increase rapidly and reach a maximum at 7 h from the induction of sporulation and afterwards they start to decline. The time of maximal expression is coincident with the stage of sporulation in which the spore wall is formed (7). The GAS2 and GAS4 gene expression profiles are in agreement with the
The presence of MSE elements in the promoter regions of these genes. A strong match to the MSE site consensus (5'HDVKN CACAAAAAD) was found in the GAS4 promoter at positions −117 to −105 (5'GCGGCACAAAAA) from the ATG, whereas a less stringent match (5'DNCRCAAADV) was detected in the GAS2 promoter in the reverse orientation at positions −147 to −138 (5'GACACAAATT) from the start codon. The presence of a more stringent match in the GAS4 promoter could explain the higher expression level of GAS4 with respect to GAS2, since Ndt80p, the transcription factor that binds the MSE element, might recognize it with higher affinity. Ndt80p is the major regulator of the middle meiotic class of genes in which GAS2 and GAS4 were classified (7).

Microarray analysis revealed that Ndt80p itself has an expression profile very similar to those of GAS2 and GAS4 and is the main candidate for the temporal regulation of GAS2 and GAS4 expression. Indeed, the ectopic expression of Ndt80p in vegetative cells triggers the expression of GAS2 and GAS4 and the lack of Ndt80p almost completely abolishes the expression of GAS2 and GAS4 during sporulation (7). The induction of GAS2 and GAS4 gene expression might also involve a change in chromatin organization. Microarray analysis of vegetatively growing cells indicated that GAS2 and GAS4 are among the genes that are normally transcriptionally silent and become induced by histone H4 depletion (44). Thus, Ndt80p, modifications of the chromatin, and nucleosome density could all be relevant for the regulation of the expression of GAS2 and GAS4 genes.

In this study, we found that GAS2p and GAS4p levels show maxima at 8 and 10 h, respectively. In particular, the level of GAS4p starts to decline slowly after 10 h from the induction of sporulation. The boost of GAS4 mRNA production in a narrow window of the sporulation process could be crucial to attain the required level of protein. After the execution of the protein’s function, the decrease in the protein level would result from the reduction of transcription and lability of the protein. An alternative hypothesis is that a degradation mechanism specific for the Gas4 protein is triggered after GAS4p has completed its function. In both cases, the degradation of Gas4 protein, and maybe of other proteins involved in cell wall formation, could be a physiological response aimed at making amino acids available for the synthesis of late-sporulation products under conditions in which no exogenous nitrogen source is present and no net synthesis of amino acids occurs. GAS2p appears more stable, and its final localization could be the plasma membrane, based on the absence of a dibasic motif upstream of the GPI attachment site (5, 27). Further studies aimed at analyzing the localization of GAS2 and GAS4 proteins are under way.

The results of the phenotypic analysis of deletion mutants support a role for GAS2 and GAS4 gene products in spore wall assembly. A severe defect was observed only when the deletions of GAS2 and GAS4 were combined. Thus, the two genes share overlapping functions. Because the phenotype is most clearly seen when both genes are inactivated, GAS2 and GAS4 genes escaped the previous screening of a library of single diploid mutants (8).

The effects of the loss of GAS2 and GAS4 proteins on spore wall morphogenesis are dramatic. Synthesis of all the layers of the spore cell wall occurs, but the accumulation of wall mater-
reflect the in vivo conditions under which these proteins normally function. In addition to the GAS family, several other secreted proteins involved in cell wall assembly have sporation-specific paralogs in S. cerevisiae (5, 12, 25). The behavior shown by Gas2 and Gas4 proteins in the ectopic expression experiments suggests a novel explanation for the existence of redundant families of enzymes involved in cell wall formation. Not only the different organization of the spore wall but the different environments in which the cell wall and spore wall are formed could have driven the evolution of sporation-specific paralogs specialized to function optimally at different pH values. In this regard, future studies on the paralogous enzymes will be crucial for understanding the roles of the different members of the gene families involved in spore wall formation.

ACKNOWLEDGMENTS

The work in the Popolo Lab was partially supported by Fondo Interno Ricerca Scientifica e Tecnologica 2004–2005, COFIN 2005, and the Cantrarin project (MRTN–CT–2004–512481) of the European Union to L.P. The work in the Neiman lab was partially supported by NIH grant GM72540 to A.M.N. and that in the Arroyo lab by project BIO2004–06376 from the Ministerio de Educacion y Ciencia to J.A. We thank M. Vai for the kind gift of plasmids, Michela Pacchi for technical assistance, and Rosa M. Perez-Diaz and J. Garcia-Cantalejo from the Unidad de Genomica UCM-PCM for their help with the quantitative RT-PCR experiments.

REFERENCES

1. An YJ, Ji H, Wu A, Lu R, Huang, and Y. Wei. 2005. A rapid and efficient method for multiple-site mutagenesis with a modified overlap extension PCR. Appl. Microbiol. Biotechnol. 68:774–778.
2. Boorsma, A., de Nobel, B. ter Riet, B. Bargmann, S. Brul, K. J. Hellingwerf, and A. Boorsma. 2006. Characterization of the transcriptional response to cell wall stress in Saccharomyces cerevisiae. Yeast 23:413–427.
3. Briza, P., M. Breitenbach, A. Ellinger, and J. Segall. 1990. Isolation of two developmentally regulated genes involved in spore wall maturation in Saccharomyces cerevisiae. Genes Dev. 4:1775–1786.
4. Briza, P., G. Winkler, H. Kalchhauser, and M. Breitenbach. 1986. Dityrosine is a prominent component of the yeast ascospore wall. A proof of its structure. J. Biol. Chem. 261:4288–4294.
5. Caro, L. H., T. Tettelin, J. H. Vossen, A. F. Ram, H. van den Ende, and F. M. Klis. 1997. In situ identification of glycosyl-phosphatidylinositol-anchored plasma-membrane and cell wall proteins of Saccharomyces cerevisiae. Yeast 13:1477–1489.
6. Carotti, C., E. Baguì, O. Palomares, T. Fontaine, G. Tedeschi, R. Rodriguez, J. P. Latge, M. Vai, and L. Popolo. 2004. Characterization of recombinant forms of the yeast Gas1 protein and identification of residues essential for glucansynthetase activity and folding. Eur. J. Biochem. 271:3635–3645.
7. Chau, S., J. DeRisi, M. Eisen, J. Moothudd, D. Botstein, P. O. Brown, and I. Herskowitz. 1998. The transcriptional program of sporulation in budding yeast. Science 282:699–705.
8. Coluccio, A., E. Bogenbruner, M. N. Conrad, M. E. Dresser, P. Briza, and A. M. Neiman. 2004. Morphogenetic pathway of spore wall assembly in Saccharomyces cerevisiae. Eukaryot. Cell 3:1464–1475.
9. Coluccio, A., and A. M. Neiman. 2004. Interspore bridges: a new feature of the Saccharomyces cerevisiae spore wall. Microbiology 150:1189–1196.
10. De Groot, P. W., A. F. Ram, and F. M. Klis. 2005. Features and functions of covalently linked proteins in fungal cell walls. Fungal Genet. Biol. 42:657–675.
11. Douglas, C. M., F. Four, J. A. Marrinan, N. Morin, J. B. Nielsen, A. M. Dahl, P. Mazur, W. Baginsky, W. Li, M. el-Sherbeini, et al. 1994. The Saccharomyces cerevisiae FKS1 (ETG1) gene encodes an integral membrane protein which is a subunit of 1,3-beta-D-glucan synthase. Proc. Natl. Acad. Sci. USA 91:12907–12911.
12. Ecker, M., R. Deutzmann, L. Lehle, V. Mrsa, and W. Tanner. 2006. Pir proteins of Saccharomyces cerevisiae are attached to beta-1,3-glucan by a new protein-carbohydrate linkage. J. Biol. Chem. 281:11523–11529.
13. Firon, A., G. Lesage, and H. Bussey. 2004. Integrative studies put cell wall synthesis on the yeast functional map. Curr. Opin. Microbiol. 7:617–623.
14. Garcia, R., C. Bermejo, C. Grau, R. Perez, J. M. Rodriguez-Pena, J. Francois, C. Nombela, and J. Arroyo. 2004. The global transcriptional response to transient cell wall damage in Saccharomyces cerevisiae and its regulation by the cell integrity signaling pathway. J. Biol. Chem. 279:15183–15193.
40. Sanz, M., J. A. Trilla, A. Duran, and C. Roncero. 2002. Control of chitin synthesis through Shc1p, a functional homologue of Chs4p specifically induced during sporulation. Mol. Microbiol. 43:1183–1195.
41. Shahinian, S., and H. Bussey. 2000. Beta-1,6-glucan synthesis in Saccharomyces cerevisiae. Mol. Microbiol. 35:477–489.
42. Trew, B. J., J. D. Friesen, and P. B. Moens. 1979. Two-dimensional protein patterns during growth and sporulation in Saccharomyces cerevisiae. J. Bacteriol. 138:60–69.
43. Vai, M., I. Orlandi, P. Cavadini, L. Alberghina, and L. Popolo. 1996. Candida albicans homologue of GGP1/GAS1 gene is functional in Saccharomyces cerevisiae and contains the determinants for glycosylphosphatidylinositol attachment. Yeast 12:361–368.
44. Wyrick, J. J., F. C. Holstege, E. G. Jennings, H. C. Causton, D. Shore, M. Grunstein, E. S. Lander, and R. A. Young. 1999. Chromosomal landscape of nucleosome-dependent gene expression and silencing in yeast. Nature 402:418–421.
45. Yin, Q. Y., P. W. de Groot, H. L. Dekker, L. de Jong, F. M. Klis, and C. G. de Koster. 2005. Comprehensive proteomic analysis of Saccharomyces cerevisiae cell walls: identification of proteins covalently attached via glycosylphosphatidylinositol remnants or mild alkali-sensitive linkages. J. Biol. Chem. 280:20894–20901.
46. Zlotnik, H., M. P. Fernandez, B. Bowers, and E. Cabib. 1984. Saccharomyces cerevisiae mannoproteins form an external cell wall layer that determines wall porosity. J. Bacteriol. 159:1018–1026.