CRR1, a gene encoding a putative transglycosidase, is required for proper spore wall assembly in *Saccharomyces cerevisiae*

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In *Saccharomyces cerevisiae*, sporulation is a developmental process that converts a single cell into four haploid spores. The four haploid nuclei are encapsulated within multilayered spore walls that protect them against stressful conditions. The formation of the spore-specific cell wall is a highly coordinated process that requires the participation of enzymic activities for biosynthesis, degradation, and cross-linking between components. Here the sporulation-specific gene *CRR1*, encoding a putative transglycosidase that is required for proper spore wall assembly, is described. Both the transcription of *CRR1* and the synthesis of Crr1p were induced biphasically under sporulating conditions, with a first expression peak displaying kinetics similar to those of the middle to middle-late sporulation-specific genes, and a second late peak after 24 h under these conditions. Localization studies revealed that Crr1p localized to the spore wall that surrounds each of the four ascospores within the mature asci. Mutation of this gene had no effect on the efficiency of spore formation. However, *crr1* mutant spores were sensitive to hydrolytic enzymes such as glucanase and to heat-shock treatments, underscoring the importance of this gene in the proper formation and assembly of the ascospore wall. Moreover, the deletion of *CRR1* had additive effects with respect to the sensitivity of *cda1 cda2* mutants to these treatments. Interestingly, overexpression of *CRR1* not only complemented the phenotype of the *crr1* strain but also rendered spores more resistant to the stress conditions than the wild-type. Like other mutants impaired in the formation of the spore outer layer, *crr1* mutants were permeable to Calcofluor White. Finally, detailed analysis by electron microscopy of the spore walls in the *crr1* mutants revealed a defect in the assembly of the spore wall components, suggesting a role for Crr1p in the cross-linking between the inner (glucan/mannoprotein) and the outer (chitosan/dityrosine) spore layers.

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INTRODUCTION

In *Saccharomyces cerevisiae*, meiotic development (sporulation) is induced when diploid a/α cells are starved of essential nutrients in the presence of a non-fermentable carbon source (Kupiec *et al.*, 1997). This process is controlled by the master regulatory genes *RME1*, *IME1* and *IME2* (Mitchell, 1994), and involves a coordinated

series of genetic and morphological processes, including all the events necessary for meiosis, spore wall formation and packaging of the four meiotic products into an ascus (for a review, see Kupiec *et al.*, 1997). All these events depend on the sequential activation of temporally distinct classes of sporulation-specific genes, classified as early, middle, middle-late and late (Kupiec *et al.*, 1997; Mitchell, 1994). This transcriptional programme has been now fully characterized at the genomic level by the use of DNA microarrays (Chu *et al.*, 1998; Primig *et al.*, 2000).

The sporulation programme begins when cells exit the mitotic cycle and enter the meiotic prophase. The meiotic prophase is followed by the meiosis I reductional and meiosis II equational divisions. Spore wall morphogenesis initiates with the outgrowth of the prospore wall, a double-membrane structure formed from the outer plaques of each

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Abbreviations: GFP, green fluorescent protein; SFH, short flanking homology.

A video image of the three-dimensional localization of Crr1–GFP in the spore, showing the rotation around the z axis, is available as supplementary data with the online version of this paper (at <http://mic.sgmjournals.org>).

meiosis II spindle pole body (Deng *et al.*, 1993; Knop & Strasser, 2000). As meiosis progresses, the prospore membrane extends along the outer surface of the nuclear envelope, under the control of specific septins (Fares *et al.*, 1996), and engulfment of the four haploid meiotic products, together with some portions of the mother-cell cytoplasm and organelles, occurs. Deposition of the spore wall components in the luminal space between the two layers of the prospore wall leads to the formation of mature spore walls (Guth *et al.*, 1972; Lynn & Magee, 1970; Moens & Rapport, 1971; Neiman, 1998).

Analysis of mature spore walls by electron microscopy reveals the presence of four layers (Kreger-van Rij & Veenhuis, 1970). The two inner layers, consisting mainly of glucans and mannoproteins, are similar in morphology and chemical composition to that of the vegetative cell wall (Briza *et al.*, 1988; Katohda *et al.*, 1984). The two outer layers are thought to contribute to the mechanical rigidity and the resistance to chemical and enzymic attack of the spore wall (Briza *et al.*, 1988, 1990b), and hence confer the protective nature of this structure to adverse environmental conditions. The main component of the third layer is chitosan (Briza *et al.*, 1988), although chitin is also present. Chitosan, a 1,4- β -D-glucosamine homopolymer, is produced by deacetylation of chitin chains (Kafetzopoulos *et al.*, 1993a), this reaction being catalysed by two chitin deacetylase isozymes encoded by the *CDA1* and *CDA2* genes, respectively (Christodoulidou *et al.*, 1996; Kafetzopoulos *et al.*, 1993b), whose deletion affects ascospore wall assembly (Christodoulidou *et al.*, 1999). The outermost layer of the spore wall is mainly composed of dityrosine. Dityrosine forms an insoluble macromolecule containing a high number of cross-linked tyrosine residues in its LL-, DL, and DD configurations (Briza *et al.*, 1986, 1990b). This polymer is synthesized in a two-step process catalysed by the products of the genes *DIT1* and *DIT2* (Briza *et al.*, 1994, 1996), with the formation of *N*-formyl tyrosine and *N,N'*-bis-formyl dityrosine in the first and second steps, respectively. *Dtr1p*, a sporulation-specific member of the major facilitator superfamily involved in multidrug resistance, has recently been shown to be involved in the transport of bis-formyl dityrosine from the cytoplasm of the prospore to the spore wall (Felder *et al.*, 2002).

Events of spore wall assembly are controlled by regulatory proteins. *Swm1p*, a protein localized to the nucleus during the sporulation process (Ufano *et al.*, 1999), together with *Sps1p* and *Smk1p*, members of the *Smk1p*-MAP kinase signalling pathway (Neugeborn & Mitchell, 1991), are required for full expression of the mid-late and late genes during the sporulation programme. The timing of synthesis of the different layers is controlled by these regulatory proteins. The execution of the distinct sporulation events, like the formation of the glucan, chitosan and dityrosine layers, requires distinct *Smk1p* activity thresholds (Wagner *et al.*, 1999).

With knowledge of the *S. cerevisiae* genome sequence, many

new genes potentially involved in spore morphogenesis have been pinpointed. As well as the classic genetic screenings used to identify contributors to the meiotic pathway in *S. cerevisiae*, genomic-based screenings (Briza *et al.*, 2002; Enyenihi & Saunders, 2003; Rabitsch *et al.*, 2001) have been performed, leading to the identification of many genes involved in this process. However, many sporulation genes still remain to be characterized.

Here, we describe the characterization of *CRR1*. This gene belongs to a family of cell-wall-related genes, recently characterized in our laboratory, that includes *CRH1*, *CRH2* and *CRR1* (Rodríguez-Peña *et al.*, 2000, 2002). The products encoded by *CRH1* and *CRH2* are GPI cell wall proteins involved in the cross-linking between cell wall polymers, probably glucan and chitin, at different stages of the *S. cerevisiae* vegetative cell cycle (Rodríguez-Peña *et al.*, 2000). *CRR1* is poorly expressed under vegetative growth, but highly expressed under sporulation conditions (Rodríguez-Peña *et al.*, 2000). The existence of a catalytic domain conserved between *CRH1*, *CRH2* and *CRR1* suggests a role for this gene in spore wall construction. As deduced from the results presented here, *CRR1* is required for proper spore wall assembly during spore formation.

METHODS

Strains and media. The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. For routine experiments, yeast cells were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose). To induce sporulation, diploid cells were grown in YEPD for 12 h, transferred to YEPA medium (0.5% yeast extract, 0.6% yeast nitrogen base, 0.5% peptone, 1% potassium acetate, 1.02% potassium biphthalate, pH 5.5), and adjusted to an optical density of 0.1 at 600 nm. The cells were grown for at least three generations and harvested by centrifugation when the cellular concentration had reached $1\text{--}2 \times 10^7$ cells ml⁻¹, washed twice with sporulation medium (1% potassium acetate), and resuspended at 1.5×10^7 cells ml⁻¹ in the same sporulation medium supplemented with the appropriate auxotrophic requirements. After 48 h of incubation at 30 °C, ascus formation was monitored by phase-contrast microscopy. Sporulation rates (expressed as the number of asci divided by the total number of cells) of 55 and 85% were found in the FY1679 and YPA24 strains, respectively. The *Escherichia coli* strain used as the plasmid host was DH5 α [*supE44* Δ *lacU169* (ϕ 80 *lacZ* Δ M15) *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1*]. For selective growth, bacteria were grown on Luria-Bertani (LB) medium containing 100 mg ampicillin l⁻¹.

Yeast genetics and phenotypic analyses. Tetrad analyses were performed by standard micromanipulation procedures. Markers of the segregants were verified on SD (2% glucose, 0.16% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, plus the appropriate amount of amino acids) plates lacking a particular amino acid. The sensitivity or resistance of the segregants to geneticin (encoded by the *KanMX4* module) or hygromycin B (encoded by the *hphMX4* module) was monitored on YEPD plates containing 200 mg geneticin l⁻¹ (Gibco-BRL) or 300 mg hygromycin B l⁻¹ (Roche Diagnostics), respectively. The sensitivity of asci to heat-shock treatment (55 °C) or to enzymic digestion (glusulase) was determined as described by Briza *et al.* (1990a). Briefly, in the case of glusulase treatment, about 2×10^7 cells were collected from the sporulation medium after 72 h of growth and transferred to 100 μ l

Table 1. Yeast strains used in this study

Strain	Genotype	Reference
FY1679	<i>MATa/α ura3-52/ura3-52 hisΔ200/HIS3 leu2Δ1/LEU2 trp1Δ63/TRP1 GAL2/GAL2</i>	Rodriguez-Peña <i>et al.</i> (2000)
FY1679 Δ <i>crr1</i>	Y1679 but <i>crr1::LEU2/crr1::LEU2</i>	Rodriguez-Peña <i>et al.</i> (2000)
YPA24	<i>MATa/α ura3/ura3 leu2/LEU2 his3/HIS3 trp1/TRP1 ADE2/ade2 can1/can1 cyh2/CYH2</i>	Ufano <i>et al.</i> (1999)
W303-1A	<i>MATa ura3 leu2 his3 trp1 ade2 can1</i>	Ufano <i>et al.</i> (1999)
α 131-20	<i>MATα ade2 ura3 leu1 can1 cyh2</i>	Ufano <i>et al.</i> (1999)
FG01	W303-1A but <i>crr1::hphMX4</i>	This study
FG02	α 131-20 but <i>crr1::hphMX4</i>	This study
FG10	YPA24 but <i>crr1::hphMX4/crr1::hphMX4</i>	This study
FG03	W303-1A but <i>cda1-cda2::KanMX4</i>	This study
FG04	α 131-20 but <i>cda1-cda2::KanMX4</i>	This study
FG20	YPA24 but <i>cda1-cda2::KanMX4/cda1-cda2::KanMX4</i>	This study
FG05	FG03 but <i>crr1::hphMX4</i>	This study
FG06	FG04 but <i>crr1::hphMX4</i>	This study
FG30	FG20 but <i>crr1::hphMX4/crr1::hphMX4</i>	This study
FG35	W303-1A but <i>crr1::6Myc-CRR1</i>	This study
FG40	YPA24 but <i>crr1::6Myc-CRR1/CRR1</i>	This study

undiluted glucosylase (type H-2; Sigma). At several times during incubation at room temperature, 50 μ l aliquots were taken from the cell suspension and plated onto YEPD plates. To determine heat tolerance, cell cultures were kept at 4 °C for 12 h and diluted to a density of 3×10^6 cells ml⁻¹ in distilled water. Then, 1 ml of the cell suspension was transferred to an Eppendorf tube, which was immersed in a water bath at 55 °C. Aliquots (100 μ l) were taken at different times during heat treatment and plated onto YEPD plates. For both phenotypic studies, the plates were incubated for 2 days at 30 °C before the number of colonies was counted. In parallel, cell survival to heat shock at 55 °C was measured at different times by spotting aliquots of cells onto YEPD plates.

Plasmid and deletant strain construction. Standard molecular biology techniques for DNA manipulations and bacterial transformations were used as described elsewhere (Sambrook *et al.*, 1989). In the construction of the haploid *CRR1* deletant strains (FG01 and FG02), the *CRR1* complete ORF was deleted, except for the start and stop codons, by using the short flanking homology (SFH) PCR technique (Wach *et al.*, 1997), which allowed the replacement of the target ORF by the *hphMX4* selection marker from plasmid pAG32 (Goldstein & McCusker, 1999). The CR1 and CR2 primers devised for this purpose are listed in Table 2. The SFH deletion cassette was obtained using the Expand High Fidelity PCR System (Roche Diagnostics). ORF replacement was identified by PCR with the help of diagnostic primers that bind either outside the target ORF (V2 and V3) or within the selection marker (HIG1 and HIG2). Finally, both *crr1* strains were mated to generate the homozygous *crr1Δ* diploid strain FG10 (see Table 1).

In order to generate strains lacking *CDA1* and *CDA2* ORFs (FG03 and FG04, respectively), we took advantage of the adjacent localization of both ORFs (*CDA1* and *CDA2*) in the yeast chromosome XII to delete them simultaneously using a unique selection marker. The deletion cassette, containing the *KanMX4* marker that confers resistance to geneticin, was obtained by means of the SFH technique using the primers CDASFW and CDASRV (see Table 2) and plasmid pFA6a-KanMX4 (Wach *et al.*, 1994) as a template. ORF replacement was identified by PCR with the help of the diagnostic primers CDASver1 and CDASver2, which bind outside the target ORFs, and K2 and K3 primers, which bind within the selection marker. Eventually, haploid strains were mated to generate the homozygous *cda1 cda2* diploid strain FG20. The same strategy was used to generate the haploid *crr1*

cda1 cda2 MATa and *MATα* strains (FG05 and FG06, respectively) and the homozygous *crr1 cda1 cda2* diploid strain FG30.

Cloning of the *CRR1* gene. The complete *CRR1* ORF plus 598 bp and 479 bp of upstream and downstream sequences, respectively, was amplified from genomic DNA (FY1679 strain) by PCR using the primers YLRup and YLRdw (see Table 2). The amplification parameters were 10 min at 97 °C and then 1 min at 94 °C, 1 min at 53 °C, 2 min at 72 °C, for 30 cycles. The PCR product of 2389 bp was cloned into the pGEM-T vector (Promega) (pJP1 plasmid). Sequence verification was carried out by sequencing both DNA strands using the walking-primer strategy on an ABI 377 automated DNA sequencer (Applied Biosystems). Plasmid pJV21C was constructed by ligating the *SacII/SacI* insert from pJP1 into the *SacII/SacI*-cleaved pRS416 vector (centromeric vector; *URA3*; Amp^r) (Sikorski & Hieter, 1989). The restriction enzymes used in this work were provided by Roche Diagnostics.

Construction of GFP and MYC fusions. In order to create a fusion protein of Crr1p with the green fluorescent protein (GFP) from *Aequorea victoria*, we took advantage of a GFP cassette flanked by two *SpeI* restriction sites generated previously (Rodriguez-Peña *et al.*, 2000, 2002). We performed site-directed mutagenesis of *CRR1* to create an artificial *SpeI* restriction site. Plasmid pJV21C containing the *CRR1* ORF was used as a template for PCR. Primers were designed to bring about the change from ACTCAA to ACTAGT (*SpeI* recognition sequence) at the 3' end of the *CRR1* ORF (24 bases from the stop codon). Briefly, two PCRs were run in parallel using the primer pairs YLR1/YLR2 and YLR3/YLR4 (see Table 2). Both the YLR1–YLR2 (496 bp) and YLR3–YLR4 (388 bp) PCR products were used as overlapping templates in a second PCR with YLR1 and YLR4 as external primers. Thus, a final product of 864 bp was generated, corresponding to a mutated internal fragment of the ORF. This PCR product was verified by sequencing and subcloned into *KpnI*-cleaved pJV21C, producing plasmid pJV21D, hence replacing the wild-type sequence with the mutant sequence. The GFP cassette was *SpeI*-digested and introduced in-frame into pJV21D to give pJV21F. Finally, the multi-copy plasmid pJV21G was constructed by ligating the *XbaI/EcoRI* insert from pJV21F into the *XbaI/EcoRI*-cleaved Yep352 vector.

To obtain a C-terminal 6Myc-tagged version of Crr1p, plasmid pRS306CRR1m was constructed. The complete *CRR1* ORF, except for

Table 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5'–3')
CR1*	TTAGAGTATCATCAAGGGCTTTTGCACGAAATAAAAATATGCGTACGCTGCAGGTCGACGG
CR2*	TACGGAAGAGAGAAGCTTCGTAAGACATTATTGCTGTGACTAATCGATGAATTCGAGCTCG
V2	CGGCGTGAATATGCGAGAAAT
V3	CTGGAATCAGGAAGCTGAGAT
HIG1	GAACCTCAGTGGCAAATCCA
HIG2	TTCGCCTCGACATCATCTGC
CDASFW†	ATTAAGTGATAGATTTACTTTTACACAAGAAAAGAGATGCGTACGCTGCAGGTCGAC
CDASRV‡	TTTAATTCCTCTTATTTTCTTCAATTCCTGAAAATTAGAGCTCGAATTCATCGAT
CDASver1	AGATGTCACATTTTCCGGACC
CDASver2	GTAATTGGTTCCGTTGTTTCCT
YLRup	CTGGAATCAGGAAGCTGAGAT
YLRdw	CGGCGTGAATATCGCAGAAAT
K2	CCGTGCGGCCATCAAAATG
K3	GCCCAGATGCGAAGTTAAG
CRR1MYCFW§	GCGCGGATCCATATTACAGCTGGTGCCG
CRR1MYCRV§	GCGCGGATCCTTTTGACCGTTTCCAAGA
YLR1	TTCTAGGTACCTTCTTTTCGAATTGA
YLR2¶	ACTAGTTCTTGGAACGGTCA
YLR3¶	GACCGTTTCCAAGAAGTAGTCCAGTCATTATCTTTC
YLR4	AGTAAAGAATATAGGTACCCACAAA

*The sequence complementary to the hphMX4 marker is underlined.
†The sequence homology to the upstream sequence of the *CDA1* ORF is underlined.
‡The sequence homology to the downstream sequence of the *CDA2* ORF is underlined.
§*Bam*HI restriction sites are underlined.
||*Kpn*I restriction sites are underlined.
¶*Spe*I restriction sites are underlined.

a triplet encoding the stop codon, was amplified with the *Bam*HI-containing primers CRR1MYCFV and CRR1MYCRV (Table 2). The PCR product was digested with *Bam*HI, and the resulting fragment was introduced into plasmid pRS306m (6-Myc epitope in the integrative *URA3* vector pRS305; Sikorski *et al.*, 1989) to generate pRS306CRR1m. To integrate *CRR1-6myc* into the *CRR1* locus, pRS306CRR1m was linearized by *Asp*I digestion and transformed in a W303-1A strain. The heterozygous diploid strain (FG40) was constructed by crossing W303-1A 6Myc-Crr1 (FG35) with the wild-type strain from the opposite mating type (α 131-20).

Microscopy techniques. For phase-contrast, fluorescence and indirect immunofluorescence microscopy, cells were examined with an Eclipse TE2000U microscope (Nikon). Digital images were acquired with an Orca C4742-95-12ER charge-coupled device camera (Hamamatsu) and Aquacosmos Imaging Systems software. For Calcofluor staining, cells were incubated for 1 min in the presence of 10 µg Calcofluor White ml⁻¹.

For visualization of the GFP-Crr1 fusion protein, cells cultured in sporulation medium for 24 h were harvested by gentle centrifugation, washed twice with PBS, and finally resuspended in PBS. Samples were observed with an Eclipse TE-300 microscope (Nikon) attached to a Bio-Rad MRC1024 confocal system.

For electron microscopy, samples were prepared according to the protocol described by Miret *et al.* (1992). Briefly, about 2 × 10⁷ cells were collected after 24 h of incubation in sporulation medium and fixed with 500 µl fixative solution (sodium cacodylate buffer, pH 7.2, supplemented with 1.5 % glutaraldehyde and 2 % paraformaldehyde),

and then incubated at 4 °C for 24 h. Samples were then washed, and treated with 1 % potassium permanganate for 90 min. Fixed cells were dehydrated through a graded series of ethanol and embedded in Embed 812 resin (Electron Microscopy Science). Thin sections were stained and examined with a Zeiss EM902 electron microscope.

Quantification of mRNAs using real-time quantitative RT-PCR. Total RNA was isolated from cells (1.3 × 10⁹), collected at different time intervals after transfer to sporulation medium, by the acidic phenol method, as described previously (Ausubel *et al.*, 1993). First-strand cDNAs were synthesized from 2 µg total RNA, using the Reverse Transcription System (Promega), following the recommendations of the manufacturer. As a control for genomic contamination, the same reactions were performed in the absence of reverse transcriptase. Real-time PCR was performed using an ABI 7700 instrument (Applied Biosystems) in a final volume of 25 µl containing 5 µl of a 25-fold dilution of the reverse transcription reaction and 12.5 µl of the 2 × TaqMan Universal PCR Master Mix (Applied Biosystems), together with the specific forward and reverse primers (Sigma) and the corresponding gene-specific TaqMan probe (FAM and VIC labelled for *CRR1* and *ACT1*, respectively), designed using Primer Express Software 2.0 (Applied Biosystems). Real-time PCR conditions were selected according to the universal conditions (default conditions) recommended by the manufacturer of the instrument. Each cDNA was assayed in at least duplicate PCR reactions. Basic analysis was performed using the SDS 1.9.1 software (Applied Biosystems). For relative quantification, the abundance of each gene (*CRR1* and *ACT1*) was determined by preparing standard curves (four fivefold serial dilutions) for each gene from a common cDNA sample. For all experimental samples, the target quantity was

determined from the standard curve and was divided by the target quantity of the calibrator (reference sample). Thus, the calibrator ($t=0$) became the $1\times$ sample and all other quantities were expressed as an n -fold difference relative to the calibrator. In addition, amplification of *ACT1* (endogenous control) was used to standardize the amount of sample added to each reaction mixture. The following probes and forward/reverse primers were used: *ACT1*, 5'-VIC-CATGAAGGTCAAGATCATTGCTCCTCCAG-TAMRA-3', 5'-ATCACCGCTTTGGCTCCAT-3'/5'-CCAATCCAGACGGAGTA-CTTTCTT-3'; and *CRR1*, 5'-FAM-TCGATTTCACACATAGCGG-CTACACATCA-TAMRA-3', 5'-GAGGCAGAGAAAATGTTGGAA-GA-3'/5'-TTCCCGCTACTCGCTTCAA-3'.

Preparation of yeast extracts and immunoblot analysis. Cell collection (20 ml at different time intervals after transfer to sporulation medium), lysis, collection of proteins, fractionation by SDS-PAGE and transfer to nitrocellulose membranes were performed as described by Martin *et al.* (2000). Detection of actin and Myc-tagged proteins was carried out using mouse anti-actin monoclonal antibody C4 (ICN Biomedicals) and mouse anti-*c-myc* monoclonal antibody 9E10 (Covance Richmond), respectively.

RESULTS

Sporulation-specific expression of *YLR213c* (*CRR1*)

We had previously identified *CRR1* as a member of the putative family of transglycosidases which includes *CRH1*,

CRH2 and *CRR1* (Rodríguez-Peña *et al.*, 2000). In that work, we characterized the role of *CRH1* and *CRH2* in the cell wall construction process under vegetative growth conditions, and we showed that *CRR1* was mainly expressed under the meiosis developmental programme, suggesting a role for this gene in the construction of the spore wall. Here, to characterize the expression of *CRR1* under sporulation conditions more precisely, wild-type YPA24 cells were grown in pre-sporulation media, transferred to sporulation media, and incubated for 24 h at 30 °C. The expression of *CRR1* was monitored at different times by means of real-time PCR, as described in Methods. As shown in Fig. 1(a), *CRR1* was activated 6 h after transfer of the cells to the sporulation medium, with a peak of expression at about 9 h: at this time *CRR1* was induced about threefold with respect to $t=0$. The induction subsequently declined (10–14 h), disappearing after 15 h. This pattern of expression, characteristic of genes expressed midway through the sporulation process (mid to mid-late genes), almost coincides with the pattern of expression reported for this gene in the two meiotic transcriptome analyses already carried out (Chu *et al.*, 1998; Primig *et al.*, 2000). These authors monitored expression until 12 h. However, here we extended the analysis until 24 h, detecting a second peak of expression between 18 and 24 h, with a maximum sixfold induction, indicating the biphasic expression of *CRR1*.

Fig. 1. (a) Time-course of *CRR1* transcription. Levels of *CRR1* mRNA were measured by quantitative RT-PCR at the times indicated after transfer of the cells to the sporulation medium. Relative *CRR1* mRNA levels are indicated with respect to $t=0$ and normalized by *ACT1* mRNA levels measured at the same intervals. (b) Time-course of Crr1p induction during sporulation. Samples of strain YPA24 carrying Crr1p-Myc were removed from the sporulation medium at the times indicated, and extracts were made up and analysed by Western blotting using anti-Myc antibodies. The protein load was monitored using anti-actin antibodies. The relative levels of Crr1p-Myc with respect to actin protein levels are represented in the graphics. MM, molecular mass.

To examine the *CRR1* gene product, an epitope-tagged allele was constructed by fusing a sequence encoding six copies of the *c-myc* epitope to the 3' end of the coding region. The original *CRR1* allele in the YPA24 strain was replaced by this epitope-tag allele (see Methods), and the levels of Crr1p–Myc were followed through the sporulation process by Western blotting of cell extracts using anti-*c-myc* antibodies. As shown in Fig. 1(b), the protein was detected 10 h after transfer to the sporulation medium, with a first peak of expression after 12 h. The levels of the protein decreased to undetectable levels after 17 h, but then increased again after 20 h, with a second peak of maximum expression 24 h after transfer to sporulating conditions, in accordance with the transcriptional activation of *CRR1* at this time. Therefore, the levels of Crr1p during the sporulation process are in agreement with the transcriptional activation described above, suggesting that the induction of Crr1p is basically regulated at the transcriptional level.

Crr1p localizes to the spore wall envelope

To elucidate the possible role of Crr1p during sporulation, we performed localization studies using a fusion protein in which a GFP had been inserted at the C-terminus of Crr1p. Cells expressing Crr1–GFP were obtained by transformation of the FY1679 strain with plasmid pJV21G. This is a multi-copy plasmid in which the transcription of *CRR1–GFP* is controlled by the *CRR1* promoter. Forty-eight hours after transfer to sporulation conditions, in mature spores, the Crr1–GFP protein localized to the spore wall, surrounding the four haploid asci (Fig. 2a). This pattern was not observed in cells transformed with the empty vector (data not shown). Confocal analysis of the spores expressing Crr1–GFP revealed the three-dimensional

aspect of this localization (Fig. 2b). This localization pattern suggests that Crr1p may be required for spore wall synthesis during sporulation.

Mutation of *CRR1* leads to spores sensitive both to lytic enzymes and to stress conditions

To determine whether *CRR1* plays a role in spore morphogenesis, diploid homozygous mutants in this gene were tested for their ability to form spores. A *crr1Δ* homozygous diploid strain and its isogenic wild-type strain (FY1679) were transferred to sporulation medium, and their ability to form ascospores was monitored by phase-contrast microscopy. The efficiency of spore formation was not affected by the mutation, since mutant strains in *CRR1* sporulated as efficiently as wild-type cells, with a percentage of cells with normal ascospores of about 55 % after 72 h.

The resistance of spores to environmental stress mainly depends on the presence of an intact cell wall (Briza *et al.*, 1990a; Pammer *et al.*, 1992). On the basis of the possible participation of *CRR1* in spore wall construction, we examined the spore thermotolerance of the wild-type and mutant *crr1Δ* after exposure to 55 °C for different periods of time, as well as the sensitivity of the spores to the lytic enzyme glusulase. As shown in Figs 3(a) and 3(c), mutant spores were more sensitive to heat shock than wild-type spores. Thirty minutes of exposure to heat shock reduced viability by two orders of magnitude in the mutant strain compared to the wild-type, while no viable spores were detected in the mutant strain after 60 min of temperature treatment (Fig. 3c).

A single-copy plasmid containing the complete ORF of

Fig. 2. Localization of Crr1p in mature spores. A homozygous *crr1Δ* strain, transformed with the plasmid pJV21G expressing the Crr1p–GFP fusion protein, was sporulated. After 48 h growing under sporulating conditions, cells were analysed by confocal microscopy (a). (b) Confocal images obtained with a distance between stacks of 0.2 µm were used to reconstruct the three-dimensional localization of Crr1–GFP in the spore. A video image showing the rotation around the z axis of the three-dimensional reconstructed image is available as supplementary data with the online version of this paper at <http://mic.sgmjournals.org>. Four slides corresponding to different grades of rotation of the reconstructed three-dimensional confocal image of spores expressing Crr1–GFP are shown in (b).

Fig. 3. Phenotypic characterization of the ascospores from the *crr1/crr1* mutant strain. Wild-type cells (○), the isogenic *crr1Δ* strain (□) and the *crr1Δ* strain transformed with the plasmid pRS416-*CRR1* (◆) from FY1679 background (a, c and d) and YPA24 background (b) were incubated in sporulation medium for 24 h at 28 °C and then assayed for plating efficiencies after exposure to 55 °C (a, b and c) or to glusulase (d) at the indicated times (see Methods). Data in (a), (b) and (d) are indicated as a percentage of the initial survival rate ($t=0$). Tolerance of the spores to 55 °C was also measured in the YPA24-derived strains indicated (c) by spotting cells in YEPD after heat shock at the different times indicated. For each strain, serial 1:10 dilutions of untreated cells were spotted as a control for cell growth. WT, wild-type.

YLR213c (pRS416-*CRR1*) restored the temperature-sensitive phenotype of the mutant strain to the wild-type (Fig. 3a, c), indicating that the absence of *CRR1* was indeed responsible for the phenotype. Furthermore, a multi-copy plasmid containing the *CRR1* gene (Yep352-*CRR1*) not only restored the phenotype of the mutant strain but even rendered these spores clearly more resistant to heat shock than the wild-type ones (Fig. 3c).

The germination efficiency after different times of treatment with the lytic enzyme glusulase was also determined. As shown in Fig. 3(d), this efficiency was reduced from nearly 100 % in the wild-type to 65 % in the *crr1Δ* spores after 45 min of treatment, and from 90 % to 50 % after 2 h in the presence of the enzyme. This phenotype was complemented by the expression of *CRR1* from a multi-copy plasmid (Fig. 3d). To rule out any strain-specific effect, we

Fig. 4. Genetic interactions of *CRR1* with *CDA1* and *CDA2*. YPA24 wild-type and the isogenic *crr1* Δ (FG10), *cda1* Δ *cda2* Δ (FG20) and *crr1* Δ *cda1* Δ *cda2* Δ (FG30) strains were grown under sporulating conditions for 24 h. Then, cell survival after exposure to 55 °C at the indicated times was measured by spotting cells in YEPD (Methods). Serial 1:10 dilutions of untreated cells of each strain were spotted as a control for cell growth.

carried out the deletion of *CRR1* in the YPA24 strain (San Segundo *et al.*, 1993). In this strain, the deletion led to phenotypes identical to those observed in the original strain background, both for the heat-shock (Fig. 3b) and glucosylase treatments (data not shown).

Taken together, these results support the idea that ascospore wall maturation does not proceed properly in the *crr1/crr1* mutant. Based on these results, we looked for genetic interactions between *CRR1* and other genes involved in spore wall construction. For this purpose, we constructed triple-deleted strains in *CRR1*, *CDA1* and *CDA2*. *CDA1* and *CDA2* code for the two chitin deacetylases involved in the synthesis of chitosan, and strains mutated in both genes are defective in the synthesis and assembly of the outermost spore wall chitosan and dityrosine layers (Christodoulidou *et al.*, 1999). The sensitivity of these mutants to temperature shock (55 °C) was tested. As shown in Fig. 4, deletion of *CDA1* and *CDA2* slightly increased the sensitivity of the *crr1* mutant strain at 55 °C, again suggesting a role for Crr1p in spore wall assembly.

crr1 Δ spores have cell wall defects

To detect the chitosan layer it is possible to stain cells with Calcofluor White, which specifically stains chitin.

Calcofluor stains sporulating cells before spore wall maturation. However, this staining is not observed in mature spores because the dye is unable to permeate through the mature outer spore wall unless the spore wall is damaged or missing. This is because the dityrosine layer on the spore surface prevents the dye from entering the spore wall and binding to the chitosan layer. Thus, staining of wild-type sporulating cells with Calcofluor resulted in the staining of less than 3 % of the mature spores (Fig. 5a). However, more than 40 % of the asci of the *crr1* mutant strain contained spores whose chitosan layer became accessible for the dye and therefore displayed fluorescence after Calcofluor staining, indicating an alteration of their surface layer (Fig. 5b).

To explore the spore wall defect in greater depth, the structure of the mutant spore walls in cells grown under sporulation conditions for 48 h was compared by electron microscopy with that of wild-type spore walls. Electron micrographs representative of wild-type asci are shown in Fig. 6(a). The innermost layer, appearing as an electron-transparent layer, is similar to the vegetative cell wall and contains glucans and mannoproteins (Briza *et al.*, 1988; Katohda *et al.*, 1984). The outermost layer consists of dityrosine, which forms highly cross-linked insoluble macromolecules, and appears as a thin and highly

Fig. 5. Calcofluor staining of wild-type and *crr1/crr1* diploid cells under sporulating conditions. Wild-type YPA24 cells (a) or mutant *crr1/crr1* isogenic strain (FG10) (b) were collected 24 h after transfer to sporulation conditions, stained with Calcofluor White as described in Methods, and visualized by fluorescence microscopy.

Fig. 6. Electron micrographs of wild-type and *crr1Δ* mutants. (a) Single asci of wild-type strain FY1679 at low (left) and high (right) magnifications, showing a perfectly organized spore wall: the inner electron-transparent glucan layer (denoted with the arrow labelled 'g'), followed by the chitin/chitosan layer (denoted with the arrow labelled 'ch') and the dityrosine outermost layer (denoted with the arrow labelled 'd'); both of the latter appear as electron-dense layers. Old vegetative cell walls surround these structures. (b) Low (first two panels) and higher (remaining panels) magnifications of mutant *crr1Δ* spores. The outermost chitosan and dityrosine layers are aberrantly assembled over the glucan/mannoprotein layer, appearing as ruffled layers (marked with arrow heads). In most of the spores the dark electron-dense layer (the chitosan/dityrosine layer) is completely detached from the inner layer (denoted with arrows in the pictures). In some cases, discontinuities in the chitosan layer are even found (marked with an asterisk).

osmiophilic layer (Briza *et al.*, 1990b). Underlying this layer, and closely linked to it, is the chitosan layer, which appears as a diffuse electron-dense layer (Briza *et al.*, 1988). The electron-transparent layer and the two outer layers, the chitin/chitosan and dityrosine layers, were also present in the mutant spores (Fig. 6b). This was confirmed by chemical analysis of the spore walls, which revealed that chitosan and dityrosine levels were similar in the wild-type and mutant strains (data not shown). However, the images of the mutant spores clearly indicated the existence of an aberrant spore wall assembly process. A glance at the mutant spores indicated that the deposition of the two outer layers (chitosan and dityrosine layers) was not normal (Fig. 6b). Both electron-dense layers appeared as ruffles, instead of exhibiting the smooth aspect of wild-type spores. Instead of forming well-structured layers like the wild-type strain (Fig. 6a), both chitosan and dityrosine were deposited in an irregular fashion over the glucan layer. More detailed scrutiny of the images revealed that the ruffled appearance of the outermost layers in the mutants was the consequence of a problem in the linking between the glucan and chitosan layers. In many spores, the electron-dense (chitosan) layer was detached from the glucan/mannoprotein layer, and electron-transparent zones corresponding to the space between the two detached layers appeared (Fig. 6b, bottom panels). In some of the spores, some discontinuities in the chitosan layer were even detected. These results are in perfect agreement with the Calcofluor staining of mutant spores, and suggest that the probable function of Crr1p in the biogenesis of the spore wall is the cross-linking between glucan and chitosan.

DISCUSSION

Our knowledge of spore wall structure and biogenesis has increased considerably over the past few years, especially with respect to events related to the formation of the outer two layers, the chitin/chitosan and dityrosine layers. The enzymic activities responsible for the synthesis of chitosan have been characterized with precision. Chitin biosynthesis depends on CSIII activity (Pammer *et al.*, 1992; Valdivieso *et al.*, 1991; Sanz *et al.*, 2002). The following step – the chitin deacetylation process – depends on the enzymic activities of Cda1p and Cda2p (Briza *et al.*, 1988; Christodoulidou *et al.*, 1996, 1999), and leads to the formation of the 1,4- β -D-glucosamine polymer. There is a temporal order in the appearance of spore wall components, which begins after the closure of the prospore membrane (Lynn & Magee, 1970). Thus, mannoproteins are deposited as the prospore membrane forms, and after prospore membrane closure the glucan layers form, followed by the chitosan layer and finally the dityrosine layer (Tachikawa *et al.*, 2001). The layers are deposited from innermost to outermost with respect to the spore cytoplasm. Although not yet characterized, enzymic activities must be necessary for the formation of cross-links between these spore wall polymers. As in vegetative cell wall biogenesis, little is known about the genes encoding these cross-linking activities.

Many gene families related to the vegetative cell wall construction process have members that are specifically induced under sporulation conditions. This is the case for *SPS2*, *YCL048*, *GAS2* and *GAS4*, *SSG1/SPR1*, *YPL130*, *CWP1*, *TIR2*, *CRR1* and others (Chu *et al.*, 1998). However, no characterization of the role of these genes in the formation of the spore wall has been described, with the exception of *SSG1/SPR1*, which encodes a sporulation-specific exo-1,3- β -glucanase (Muthukumar *et al.*, 1993; San Segundo *et al.*, 1993). Here, we have characterized the role of Crr1p in spore wall assembly. *CRR1* encodes a protein of 422 aa with a predicted molecular size of 47.5 kDa, although the true molecular size deduced from Crr1p-Myc suggests some post-translational modification for the mature protein. Crr1p is encoded by the gene *CRR1* (*YLR213c*), and shows significant similarity to Crh1p and Crh2p; the latter two proteins play an important role in the vegetative cell wall construction process by cross-linking cell wall polymers (Rodríguez-Peña *et al.*, 2000). The three proteins belong to the 16th family of glycoside hydrolases (Henrissat & Bairoch, 1996), and include an N-terminal secretion signal for incorporation into the secretion pathway and a catalytic domain (DE-I/E-DXE) related to that of prokaryotic (1,3-1,4)- β -glucanases and plant transglycosidases. In contrast to *CRH1* and *CRH2*, *CRR1* does not have the C-terminal domain for GPI anchor attachment.

The pattern of expression of *CRR1* is characteristic of genes expressed midway through the sporulation process (mid to mid-late genes), being induced after 6–9 h under sporulating conditions in the strain background assayed here. As in many other genes with a similar expression pattern, detailed analysis of the *CRR1* promoter revealed the existence of an MSE consensus box (–149/GTCACAAAAA/–141). This site is recognized by Ndt80, a transcription factor involved in the regulation of middle-sporulation-specific genes (Hepworth *et al.*, 1998). Small variations in the time of induction have been reported for *CRR1*, depending on the strain background (this work; Chu *et al.*, 1998; Primig *et al.*, 2000), but the transcriptional kinetics are conserved. Similar temporal expression patterns have been described for those genes necessary for the formation of the spore walls (Briza *et al.*, 1990a), suggesting a role for *CRR1* in this process. Cluster analysis carried out by Primig *et al.* (2000), taking into account not only the time but also the levels of induction and the persistence of the induction, grouped *CRR1* in a different cluster from that of *DIT1*, which is still highly induced after 12 h, or *CDA1*, which is also highly induced, but earlier. *CRR1* grouped together with other genes involved in cell wall biogenesis, such as *CWP1* and *ECM37*. Interestingly, we found that *CRR1* was induced biphasically. In our hands, a second peak induction was found after 24 h under sporulating conditions. Only genes that are expressed very late in the sporulation process have been shown to be induced at these times. These genes are thought to play a role in spore maturation (Law & Segall, 1988), and therefore the second expression peak suggests

that Crr1p is also necessary at late times for spore maturation.

Consistent with a role in spore wall biogenesis, Crr1p localizes to the surface of ascospores. Although the efficiency of spore formation was not affected by the absence of *CRR1*, mutants in this gene had clear defects in spore wall assembly, as deduced from the phenotypes observed. First, cells lacking *CRR1* were more sensitive to heat shock and to enzymic digestion with glucanase than wild-type cells. Since the spore wall is the structure responsible for the resistance of mature spores to stress conditions, this observation clearly indicates that mutant cells in *CRR1* are unable to assemble the spore walls properly. Second, *crr1* cells were permeable to Calcofluor White, a dye that stains chitin and chitosan but is unable to permeate properly assembled spore walls. Third, the deletion of *CRR1* in a double mutant in *CDA1* and *CDA2*, the two genes responsible for the synthesis of the chitosan layer, led to an additive phenotype of resistance to temperature shock in the triple mutant. Taken together, all these data suggest a role for Crr1p in spore wall biogenesis.

In exactly which step of spore wall assembly is Crr1p involved? Our previous findings suggest that *CRH1* and *CRH2*, the two members of the yeast transglycosylase family expressed during vegetative growth, are involved in the cross-linking between cell wall polymers, particularly at the sites of polarized growth. Based on differences in the alkali-soluble and -insoluble fractions in strains deleted in *CRH1* and *CRH2*, and experiments to demonstrate colocalization of these proteins with chitin, it can be speculated that these proteins are involved in the cross-linking between glucan and chitin, possibly by transglycosylation reactions between these two polymers (Rodríguez-Peña *et al.*, 2000). Since the putative catalytic domain in Crr1p (DEIDFE) is conserved with respect to that present in Crh1p (DEIDIE) and Crh2 (DELDYE), it is very likely that Crr1p would have a similar function in spore wall assembly. The function of Crr1p in the biogenesis of the spore wall would be the cross-linking between glucan and chitin/chitosan layers. Electron microscopy experiments comparing the structure of the spore wall in wild-type and mutant cells deleted in *CRR1* support this hypothesis. In agreement with this, it was observed that overexpression of *CRR1* rendered the spores more resistant to temperature shock. It could be envisaged that overexpression of a putative transglycosylase would lead to the construction of a more compact spore wall. Interestingly, this phenotype has also been observed, following overexpression of the homologous gene of the family, *CRH2*, in the case of vegetative cells (Rodríguez-Peña *et al.*, 2000).

It is difficult to speculate about the nature of this cross-link and whether Crr1p is responsible for the cross-linking of chitin, chitosan, or both polymers to the glucan layer. Additional experiments will be needed to characterize the enzymic activity of Crr1p and to characterize further the precise link catalysed by this protein. However, to our

knowledge, this is the first protein to be identified that is involved in the cross-linking between spore wall polymers.

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