*Proc. Natl. Acad. Sci. USA*
Vol. 91, pp. 4524–4528, May 1994
Genetics

# The sporulation-specific enzymes encoded by the *DIT1* and *DIT2* genes catalyze a two-step reaction leading to a soluble LL-dityrosine-containing precursor of the yeast spore wall

(*Saccharomyces cerevisiae*/epimerization)

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*Communicated by Leland Hartwell, January 27, 1994*

ABSTRACT **Dityrosine is a sporulation-specific component of the yeast ascospore wall that is essential for the resistance of the spores to adverse environmental conditions. Dityrosine *in vivo* exists in both the LL and DL configurations and is part of an insoluble macromolecule of unknown structure. Here we present data indicating that dityrosine of the yeast spore wall is biosynthesized by a different mechanism than dityrosine in other biological systems—e.g., the hard fertilization membrane of the sea urchin egg. We identified two soluble, low molecular weight LL-dityrosine-containing spore wall precursors in extracts of sporulating cells and one precursor containing L-tyrosine. By expression of the previously described sporulation-specific genes *DIT1* and *DIT2* in vegetative cells, it was shown that DIT1 catalyzes the reaction leading from L-tyrosine to the tyrosine-containing precursor. DIT2, which is a member of the cytochrome P450 superfamily, is responsible for the dimerization reaction leading to the dityrosine-containing precursors. Epimerization of LL- to DL-dityrosine is one of the latest steps in spore wall formation and takes place after the dityrosine-containing precursors are incorporated into the spore wall. On the basis of these findings we suggest a biosynthetic pathway for the top layer of the yeast spore wall.**

The spore wall of *Saccharomyces cerevisiae* plays a crucial role in protection of the spores from adverse environmental conditions. The two outer layers, which can be clearly distinguished from the inner layers in electron micrographs after staining with $OsO_4$ (1), seem to contribute mostly to the spores' resistance to lytic enzymes, proteases, certain organic solvents, and elevated temperature (2). The inner layers are similar in composition to the vegetative cell wall and consist mostly of glucan and mannan (3, 4). The sporulation-specific second outer layer, which is situated beneath the very thin and osmiophilic surface layer, consists of glucosamine. As shown by NMR measurements, the glucosamine molecules are linked by β1-4 glycosidic linkages, and 95% of them are polymerized into chitosan (3). The surface layer, like the second outer layer, has no equivalent in the vegetative cell wall as well. The major component of this layer is the dimerized amino acid dityrosine [2,2′-bishydroxy-5,5′-bis(α-aminopropionyl)biphenyl], which forms a highly cross-linked scaffold on the spore surface (5). A detailed analysis of the chemical structure of the spore surface is hindered by the insoluble nature of this scaffold, but a macromolecule consisting mostly of dityrosine in its LL and DL configurations can be solubilized and isolated by partial acid hydrolysis from a purified spore wall preparation (6).

Despite some progress in the elucidation of components of the spore wall, biosynthesis and assembly of the spore wall remain unclear. The spore wall arises from the prospore wall, presumably a lipid membrane, that forms around the nuclear lobes during meiosis (7). Electron microscopic investigations of sporulating cells indicate that the endoplasmic reticulum plays an important role in the deposition of spore wall components between this double membrane, and hence in the transformation of the prospore wall into the mature spore wall (8). Several sporulation-specific genes were isolated that were assumed to be involved in spore wall synthesis (9–11). However, the phenotype of the disruption mutants indicates that these genes play only a minor role during wall synthesis. No obvious aberrations of the spore wall were observed, and the only notable phenotype was a delay of the onset of the spores' resistance to diethyl ether (9, 11) or a reduction of the thermoresistance of the spores (10). Several mutants with a defect in dityrosine synthesis were isolated in our laboratory. Mutant *dit101* has a defect in a chitin synthase responsible for the synthesis of the chitosan layer (12). Spores of this mutant lack both surface layers but contain small amounts of dityrosine. Spores of the mutants *dit1* and *dit2* lack the outer spore wall layer and contain no dityrosine at all, indicating a direct role of the *DIT1* and *DIT2* gene products (DIT1 and DIT2, respectively) in dityrosine biosynthesis (2). DIT2 has a high homology with members of the cytochrome P450 superfamily and very likely is involved in the cross-linking reaction that leads from tyrosine to dityrosine. As yet, no function can be assigned to DIT1. To gain further insight into the process of the yeast spore wall synthesis and maturation we tried to identify precursors to the dityrosine-containing macromolecule of the spore wall that accumulate either during sporulation or in vegetative cells expressing the *DIT1* and/or *DIT2* genes.

## MATERIALS AND METHODS

**Sporulation Conditions.** Cells of the strain SK1 (13) were grown overnight as a lawn on YPD plates [1% yeast extract (Difco)/2% peptone (Difco)/2% glucose/2% agar] at 28°C and were quantitatively transferred to sporulation plates [1% potassium acetate/0.1% yeast extract/0.025% glucose/2% agar]. Sporulation was carried out at 28°C.

**Preparation of Cell Homogenates.** Sporulating cells were suspended in water and homogenized by shaking with glass beads in a cell homogenizer (Braun, Melsungen, Germany). The homogenate was fractionated into a wall fraction, a membrane fraction, and a soluble fraction by differential centrifugation. For the identification of dityrosine-containing precursors, sporulating or galactose-induced cells were suspended in 0.1 M Tris·HCl buffer, pH 8.3/methanol (1:1, vol/vol) and homogenized as above. An equal volume of chloroform was added to the total homogenate and the sample was centrifuged for phase separation in an Eppendorf centrifuge. Prior to loading on the HPLC column, the sample was diluted with 3 vol of 0.01 M trifluoroacetic acid, and any

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precipitate that had formed was removed by centrifugation. For the detection of the tyrosine-containing precursor, cells were homogenized in 5% trichloroacetic acid. After centrifugation, the sample was directly loaded onto the HPLC column.

**HPLC Analyses.** A Waters Nova-Pak $C_{18}$ reversed-phase column (3.9 × 150 mm, 4 μm) was used for all experiments. For the detection of dityrosine, a fluorescence detector set at the excitation wavelength of 285 nm and the emission wavelength of 425 nm was used. For the detection of tyrosine, the detector was set at 274 and 303 nm. Spore wall precursors were analyzed by developing the column with a gradient of $CH_3CN$ in 0.01 M trifluoroacetic acid [0–50% (vol/vol) $CH_3CN$ in 55 min for dityrosine-containing precursors, 0–15% $CH_3CN$ in 55 min for tyrosine-containing precursors]. Prior to dityrosine and tyrosine analyses, dried samples were hydrolyzed in 50 μl of 12 M HCl at 95°C in an open Eppendorf test tube until the liquid had evaporated. The hydrolysates were dissolved in water, and dityrosine and tyrosine were analyzed under isocratic conditions using either 5% (for dityrosine) or 2.5% (for tyrosine) $CH_3CN$ in 0.01 M trifluoroacetic acid as eluants.

**Induction of *DIT1* and *DIT2* in Vegetative Cells.** Construction of plasmids with *DIT1* or *DIT2* under control of the *GAL10* promoter (14) and containing *LEU2* or *URA3*, respectively, as selective markers will be described in detail elsewhere. For the induction experiments, diploid cells of strain W3031A-H × W3031B-T (11) containing either one or both plasmids were grown in selective medium (1% glucose). After cells had reached the mid-logarithmic phase, they were centrifuged, washed with water, and resuspended in selective medium containing 1% galactose instead of glucose. To analyze spore wall precursors, cells were harvested and homogenates were prepared as described above.

**Incorporation of [$^{14}$C]Tyrosine into Spore Wall Precursors.** Approximately $10^9$ cells were harvested and resuspended in 300 μl of 0.1 M sodium acetate buffer, pH 6.3, containing 10 μl of toluene and 1 μCi of L-[U-$^{14}$C]tyrosine (NEN; 1 Ci = 37 GBq). After incubation at 30°C for 2 h, cell debris was removed by centrifugation and the supernatant was prepared for HPLC analysis by adding an equal volume of 10% trichloroacetic acid. HPLC analysis was performed as described above, and incorporation of radioactivity was measured in a scintillation counter.

## RESULTS

**Dityrosine Synthesis Does Not Take Place in the Spore Wall.** Cells from different stages of sporulation were disrupted by shaking with glass beads. The homogenate was separated by centrifugation into a cell and spore wall fraction (10,000 × *g* pellet), a membrane fraction (100,000 × *g* pellet), and a fraction containing soluble components (100,000 × *g* supernatant). Aliquots of these fractions were hydrolyzed and analyzed for the presence of dityrosine. Between 10 and 20 h of sporulation, a sharp increase of dityrosine was observed in the wall fraction, corresponding to formation and maturation of spore walls. Prior to the first appearance of dityrosine in the wall fraction, we detected low amounts of dityrosine in hydrolysates of the soluble fractions. Between 12 and 20 h, when most of the dityrosine was synthesized and incorporated into the spore wall, the concentration of dityrosine in the soluble fractions remained at a constant level of about 300 pmol per $10^8$ cells; in later stages of sporulation it decreased again (Fig. 1). No dityrosine was detected in hydrolysates of the membrane fractions. The appearance of dityrosine in soluble fractions of sporulating yeast cells prior to its incorporation into spore walls suggested that dityrosine synthesis did not take place in the maturing spore wall.

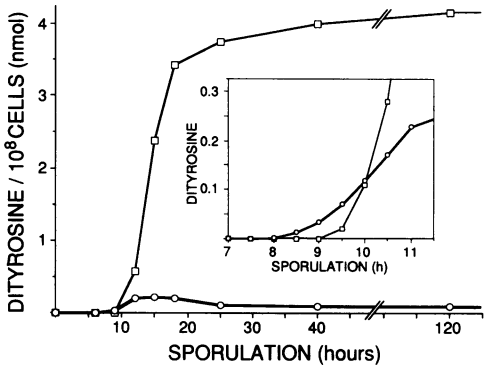


FIG. 1. Dityrosine in sporulating cells. Dityrosine was quantitated in hydrolysates of fractionated cell homogenates by HPLC using a fluorescence detector set at the excitation and emission wavelengths of dityrosine (285 and 425 nm). (*Inset*) Result of dityrosine analysis focusing on cells in the early phase of dityrosine synthesis. □, 10,000 × *g* pellet (cell and spore walls); ○, 100,000 × *g* supernatant.

These findings prompted us to investigate the presence of soluble dityrosine-containing components in sporulating yeast cells that appear transiently at the time of spore wall formation. First, the 100,000 × *g* supernatant from cells that had sporulated for 10 h was further fractionated by phase partitioning with methanol/chloroform. Most proteins precipitated and formed a thick interphase that was practically free of dityrosine. No dityrosine was detected in the chloroform phase either. Hydrolysates of the aqueous phase contained significant amounts of dityrosine. The unhydrolyzed aqueous phase was then separated by reversed-phase HPLC. Fractions were collected and analyzed for dityrosine as described in *Materials and Methods*. Both dityrosine analysis and the use of a fluorescence detector set at the excitation and emission wavelengths of dityrosine revealed the presence of two dityrosine-containing substances, eluting at 13 and 19 min. We were not able to detect free dityrosine in this extract (Fig. 2). As mentioned above, a soluble spore wall precursor can be expected to occur only transiently shortly before, or at the time of, spore wall synthesis. Therefore, we analyzed and compared extracts from cells at different stages of sporulation essentially as described above. As shown in Fig. 3, extracts from cells after 8 h of sporulation did not contain any dityrosine-containing components. The maximum concentration of the substance eluting at 19 min was found after 9 h of sporulation; at later times its concentration gradually decreased. The substance eluting at 13 min had a

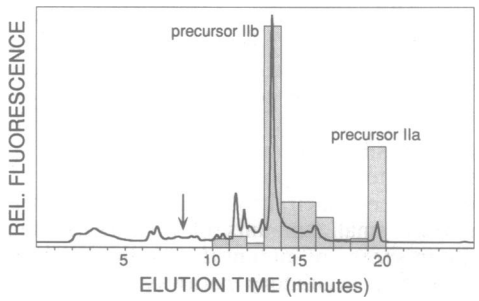


FIG. 2. HPLC analysis of a deproteinized supernatant of a homogenate from cells after 10 h of sporulation. Soluble dityrosine-containing spore wall precursors eluted at 13 and 19 min. Fractions were collected, dried, and hydrolyzed, and the amount of dityrosine was quantitated, as indicated by the shaded bars. The peak for precursor IIb is 10 pmol. Note that the sample did not contain free dityrosine, which would elute at 8 min, as indicated by the arrow. No explanation is currently available for the different fluorescence-to-dityrosine ratios observed for precursors IIa and IIb.

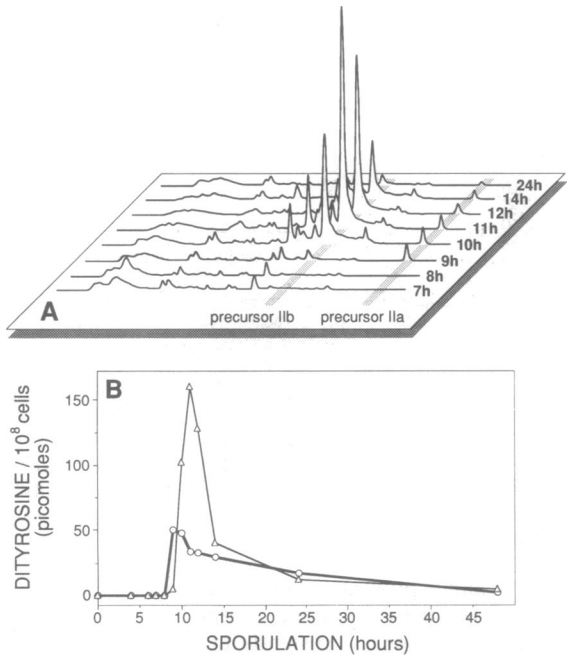


FIG. 3. Spore wall precursors IIa and IIb appear transiently during sporulation. (*A*) HPLC chromatograms of deproteinized supernatants of homogenized cells from different times of sporulation. The positions of both dityrosine-containing precursors are indicated. (*B*) Summary of the dityrosine analysis performed as in Fig. 2. ○, Dityrosine of precursor IIa; △, dityrosine of precursor IIb.

sharp concentration maximum at 11 h of sporulation. Only trace amounts of both components could be detected in mature asci. From the fact that these two components appeared in the sporulating cells before dityrosine was detected in the maturing spore walls suggested that both components were soluble precursors of the dityrosine-containing spore wall surface layer. The kinetics of their appearance also indicated that the substance eluting at 19 min is converted into the substance eluting at 13 min. We will from now on refer to them as precursor IIa and precursor IIb, respectively. To show that both substances are spore wall precursors and to exclude the possibility that they are simply soluble side-products of the spore wall maturation, we analyzed sporulating cells of the mutant *dit101*. As shown previously (12), spores of this mutant lack both outer layers as a consequence of a defect in chitin synthase III. Despite the lack of the dityrosine-containing spore wall surface layer, sporulated cultures of this mutant contained a small amount of dityrosine (≈10% of a wild-type strain). Most of the dityrosine was solubilized by extraction with 50% methanol, and HPLC analysis of this extract revealed the presence of both precursors IIa and IIb. But, in contrast to wild-type cells, both precursors were present throughout sporulation (data not shown). It is possible that the severe defect of the spore wall structure prevented incorporation of the precursors into the spore wall and hence their insolubilization. It is interesting to note that only small amounts of precursors IIa and IIb accumulate in spores of mutant *dit101*; this might be due to a yet-unidentified regulatory pathway that prevents dityrosine synthesis when the precursors cannot be incorporated into the maturing spore wall.

Preliminary analyses by size exclusion chromatography indicated that the two precursors each had a molecular weight of well below 1000. UV spectra of the precursors closely resembled those of free dityrosine.

**Function of DIT1 and DIT2.** Spores of strains with a gene disruption of *DIT1* and/or *DIT2* lack dityrosine and the spore wall surface layer, thereby indicating that both gene products play an important role in dityrosine biosynthesis (2). To further elucidate the function of both gene products, we replaced the regions upstream of the open reading frames with *GAL10* promoter sequences (14). Vegetative cells transformed with either the GAL-DIT1 or the GAL-DIT2 plasmid or cells containing both plasmids were tested for the accumulation of products related to spore wall synthesis, as summarized in Fig. 4. No dityrosine or dityrosine-containing components were detected in cells containing the GAL-DIT1 or the GAL-DIT2 plasmids alone. On the other hand, hydrolysates of cells transformed with both the GAL-DIT1 and the GAL-DIT2 plasmid contained significant amounts of dityrosine. As shown in Fig. 4*A*, dityrosine formation and induction of the *DIT* genes followed the same time course. Extracts of induced cells containing the GAL-DIT1 and the GAL-DIT2 plasmids were separated by reversed-phase HPLC in the same way as described above for sporulating cells. Dityrosine analysis revealed that precursors IIa and IIb accumulated during induction (Fig. 4*B*). In contrast to sporulating cells, both precursors remained soluble throughout prolonged induction times (up to 36 h) and no insolubilization or incorporation into cell walls occurred (data not shown). It is interesting to note that the kinetics of the appearance of the two precursors closely resembles the kinetics observed during sporulation. At early stages of induction, only precursor IIa could be detected; from 9 h on, an excess of precursor IIb over precursor IIa was observed. As in sporulating cells, we were not able to detect free dityrosine in extracts of cells transformed with the GAL-DIT1 and the GAL-DIT2 plasmids.

On the basis of these results, we postulate the following working hypothesis for the synthesis of spore wall dityrosine: DIT1 reacts with free tyrosine to form a substrate for DIT2; DIT2 cross-links the product of DIT1 in a dimerization reaction to form the dityrosine-containing precursor IIa that, subsequently, is transformed into precursor IIb. As a corollary to this hypothesis, cells containing the GAL-DIT1 plasmid should accumulate a tyrosine-containing substance related to precursor IIa. To test this hypothesis, we analyzed deproteinized homogenates from cells transformed with the GAL-DIT1 plasmid for the presence of a tyrosine-containing low molecular weight substance. As shown in Fig. 4*C*, a prominent peak appeared after induction of the *DIT1* gene, which after hydrolysis produced free tyrosine. Only small amounts of this substance were detected in homogenates of cells transformed with both plasmids, and it was not detectable in cells containing only the GAL-DIT2 plasmid.

To unequivocally show that this tyrosine-containing substance is related to the dityrosine-containing precursors, we purified about 10 μg of it by semipreparative HPLC and artificially cross-linked it using the peroxidase/$H_2O_2$ system, which is commonly used to produce dityrosine from tyrosine in the test tube (15). The product of this reaction was analyzed by HPLC and, as shown in Fig. 5, one major dityrosine-containing peak that comigrated with precursor IIb was obtained. From this result, we conclude that the tyrosine-containing substance accumulating in vegetative cells expressing the GAL-DIT1 plasmid is the monomeric form of precursor IIb, and we will refer to it as precursor Ib. Our attempts to identify the tyrosine-containing substance related to precursor IIa in extracts of vegetative cells expressing DIT1, sporulating wild type, and *dit2* mutant cells have met with limited success up to now, and the presumed precursor Ia could not be sufficiently purified, mostly due to technical problems caused by the interference of large amounts of free tyrosine present in the cell extracts.

**L-Tyrosine Is the Substrate for DIT1.** Vegetative cells containing the GAL-DIT1 plasmid were induced on galactose media for 10 h, harvested, and permeabilized with toluene in the presence of L-[$^{14}$C]tyrosine. After 2 h at 30°C, cells were homogenized and the extracts were separated by HPLC.

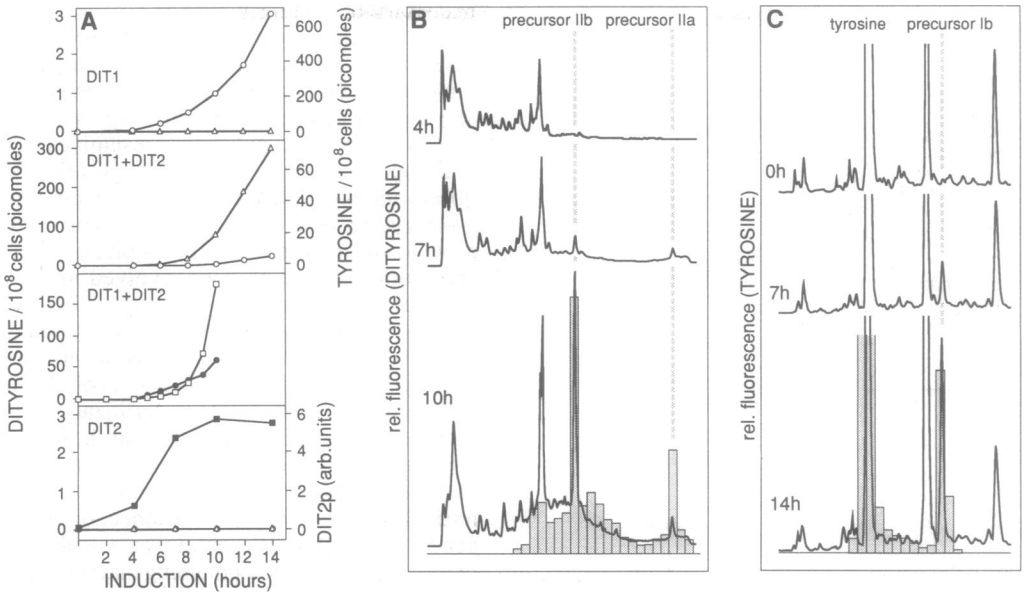


FIG. 4. Accumulation of spore wall precursors in vegetative cells after induction of *DIT1* and *DIT2*. (*A*) Summary of precursor accumulation in cells expressing *DIT1*, *DIT2*, or *DIT1* + *DIT2*. ○, Tyrosine in precursor Ib; △, dityrosine after hydrolysis of deproteinized supernatant of cell homogenates; ●, dityrosine in precursor IIa; □, dityrosine in precursor IIb; ■, expression of *DIT2*, tested with immunoblots using a polyclonal antibody against DIT2 (control). (*B*) Dityrosine-containing precursors IIa and IIb in cells expressing *DIT1* and *DIT2* at different times of induction. The fluorescence detector was set at 285 nm (excitation) and 425 nm (emission). The results of the dityrosine analysis of hydrolyzed fractions are indicated by shaded bars. The peak for precursor IIb is 15 pmol. (*C*) Tyrosine-containing precursor Ib in cells expressing *DIT1* at different times of induction. The fluorescence detector was set at 274 nm (excitation) and 303 nm (emission). The shaded bars show the results of tyrosine analysis.

About 5% of the added radioactivity was incorporated into precursor Ib. Upon hydrolysis, the incorporated radioactivity was released from the precursor and coeluted from the column with free tyrosine (data not shown). This experiment corroborates earlier results obtained by *in vivo* labeling of sporulating cells with L-[$^{14}$C]tyrosine (5) and clearly indicates that spore wall dityrosine originates from free L-tyrosine.

**Epimerization of LL-Dityrosine Occurs After Its Incorporation into the Spore Wall.** We analyzed the ratio of DL- to LL-dityrosine incorporated into the spore wall and compared it to the values obtained from the soluble precursors (Fig. 6). Dityrosine that was incorporated into precursors IIa and IIb remained in the LL conformation throughout the early and middle phases of sporulation—i.e., at the times when most of these precursors had accumulated. Only in later stages of sporulation did precursor IIb contain small amounts of DL-dityrosine. On the other hand, spore walls contained large amounts of DL-dityrosine. Interestingly, the ratio of DL- to LL-dityrosine changed dramatically during maturation of the spore walls, suggesting that epimerization of LL-dityrosine occurred after incorporation of the precursors into the spore wall. Epimerization that had occurred before or simultaneously with the incorporation of the precursor molecules would have resulted in a constant ratio of DL- to LL-dityrosine.

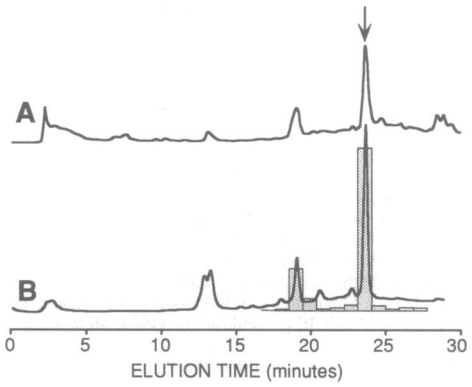


FIG. 5. Precursor Ib is the monomer of precursor IIb. (*A*) HPLC chromatogram of precursor IIb (marked by the arrow) from cells after 12 h of sporulation. (*B*) Chromatogram of the products of the reaction of precursor Ib with horseradish peroxidase and $H_2O_2$. The major peak contained dityrosine (shaded bars) and coeluted with precursor IIb from sporulating cells.

## DISCUSSION

The data presented in this report can be summarized in a biosynthetic pathway for the dityrosine-containing macromolecule, the major component of the spore wall surface (Fig. 7). In the first step, the sporulation-specific enzyme DIT1 converts L-tyrosine into the tyrosine-containing precursor Ia. In the next step, two molecules of the precursor Ia

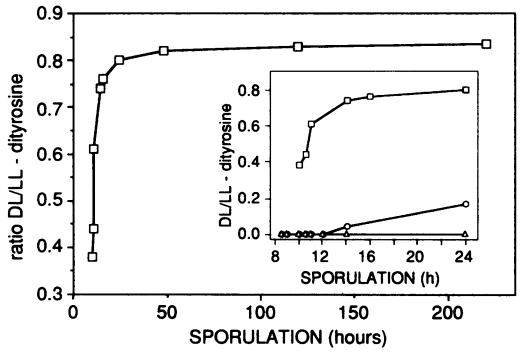


FIG. 6. Change of the ratio of DL- to LL-dityrosine in spore walls during spore maturation. Spore and cell walls at different times of sporulation were isolated and hydrolyzed, and the dityrosine diastereomers were analyzed by HPLC. (*Inset*) Comparison of the DL/LL ratio of dityrosine in spore walls (□), precursor IIa (△), and precursor IIb (○).

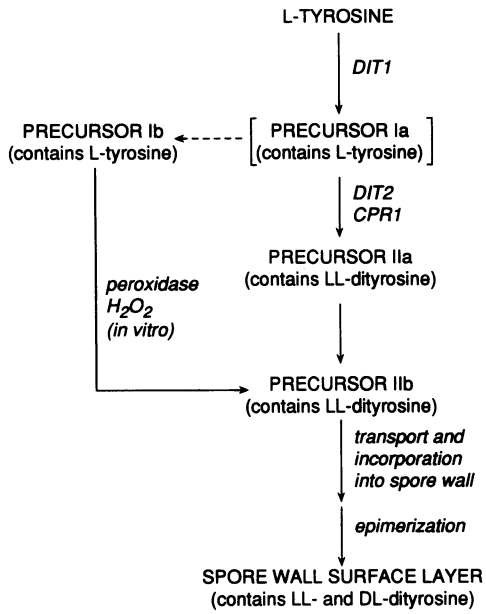


FIG. 7. The proposed pathway of dityrosine synthesis in sporulating yeast cells. The reaction leading to precursor Ib (indicated by the dashed arrow) was observed only in vegetative cells expressing *DIT1* or *DIT1* + *DIT2*. Precursor Ia is shown in brackets to indicate that we were not able to unequivocally identify it in sporulating cells. Details are given in *Discussion*.

are covalently cross-linked to form the dityrosine-containing precursor IIa. The enzyme responsible for this reaction is the cytochrome P450 DIT2. Precursor IIa is further metabolized to precursor IIb and incorporated into the maturing spore wall. Epimerization from LL- to DL-dityrosine occurs after incorporation of the soluble precursor molecules into the insoluble scaffold of the spore wall surface. It has to be emphasized that the existence of precursor Ia is hypothetical. Its presence is postulated on the basis of the following facts: (*i*) vegetative cells expressing *DIT1* accumulated a tyrosine-containing substance (precursor Ib) that was the monomer of precursor IIb and (*ii*) both in vegetative and in sporulating cells precursor IIa was transformed into precursor IIb; as a consequence, only relatively small amounts of precursor IIa (the dimer of precursor Ia) accumulated in vegetative cells expressing *DIT1* and *DIT2*. Because of the probable structural similarity between the tyrosine- and the dityrosine-containing precursors (one being simply the dimer of the other) we assume that the same reaction takes place in vegetative cells expressing *DIT1*. This would explain why these cells contain only small amounts of precursor Ia. Very likely an enzyme present in both vegetative and sporulating cells is responsible for the conversion of precursor Ia to Ib and of precursor IIa to IIb, but a spontaneous chemical reaction cannot be ruled out. A cell-free system for the conversion of L-tyrosine with purified DIT1 should enable us to unequivocally identify precursor Ia and to elucidate details about the reaction leading to precursors Ib and IIb.

From the scheme presented in Fig. 7 it follows that sporulating cells use a combination of highly specific and general household enzymes for dityrosine synthesis. Both genes *DIT1* and *DIT2* are expressed exclusively at the time of spore wall formation. DIT2 is highly specific for the reaction product of DIT1, since only cells with both enzymatic activities produce dityrosine-containing substances. This might serve a protective function, since the high specificity prevents cross-linking of the tyrosine of the cell's amino acid pool or tyrosine residues of proteins. One household enzyme, *CPR1*, was recently shown in our laboratory to be absolutely necessary for the formation of dityrosine. *CPR1* codes for a nonsporulation-specific NADPH–cytochrome P450 reductase and is needed for ergosterol synthesis (16). However, diploid cells homozygous for the disruption of this gene under sporulation conditions produce spores that lack dityrosine and whose phenotype closely resembles that of spores with a disruption of the *DIT* genes (P.B. and M.B., unpublished observations). If the conversion of precursor IIa to IIb is catalyzed by an enzyme, this has to be a household enzyme as well, since the reaction takes place in both vegetative and sporulating cells. Enzymes involved in the incorporation of the precursors into the spore wall or in the epimerization of LL-dityrosine have not yet been identified.

Dityrosine was described in several biological systems, such as the cuticles of insects (17), elastin of chicken embryos (18), collagen (19), and the hard fertilization membrane of the fertilized sea urchin egg (20). In all these systems, dityrosine is responsible for insolubilization and cross-linking of proteins. In those systems, dityrosine concentration is low; for the fertilization membrane, for instance, one dityrosine cross-link per 55,000 daltons of protein was calculated (20). In contrast to this, the dityrosine-containing macromolecule isolated from yeast spore walls contains dityrosine as the most abundant amino acid, indicating a unique structure that tempted us to postulate a nonribosomal origin of this macromolecule (6). This hypothesis is now confirmed by the discovery of soluble, low molecular weight spore wall precursors that contain either L-tyrosine or LL-dityrosine. Different from the sea urchin system, where a peroxidase cross-links tyrosine residues of ribosomally made proteins (20), the spore wall surface layer is synthesized from free tyrosine by the concerted action of several enzymes, among them DIT1 and the dityrosine-forming cytochrome P450 DIT2. Elucidation of the structure of the soluble precursors and identification of other genes involved in synthesis of the dityrosine layer should provide useful information for a better understanding of yeast spore wall biosynthesis and its regulation.

We thank Fátima Ferreira for critically reading the manuscript and many helpful discussions. We are grateful to J. C. Loper for providing the disruption strain of *CPR1*. This work was supported by the Austrian Fonds zur Förderung der Wissenschaftlichen Forschung, Project S5809-MOB (to M.B.).

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