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Biochemistry

Spermidine or spermine is essential for the aerobic growth of *Saccharomyces cerevisiae*

(polyamines/S-adenosylmethionine decarboxylase/SPE2)

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ABSTRACT **A null mutation in the *SPE2* gene of *Saccharomyces cerevisiae*, encoding *S*-adenosylmethionine decarboxylase, results in cells with no detectable *S*-adenosylmethionine decarboxylase, spermidine, and spermine. This mutant has an absolute requirement for spermidine or spermine for growth; this requirement is not satisfied by putrescine. Polyamine-depleted cells show a number of microscopic abnormalities that are similar to those reported for several cell division cycle (*cdc*) and actin mutants. These include a striking increase in cell size, a marked decrease in budding, accumulation of vesicle-like bodies, absence of specific localization of chitin-like material, and abnormal distribution of actin-like material. The absolute requirement for polyamines for growth and the microscopic abnormalities are not seen if the cultures are grown under anaerobic conditions.**

Putrescine, spermidine, and spermine are widely distributed in nature, but their function is not known with any certainty (1–3). Our earlier studies indicated that *Saccharomyces cerevisiae* has an absolute requirement for putrescine for growth but only a partial requirement for spermidine or spermine. Thus, mutants of *S. cerevisiae** that are defective in the gene for ornithine decarboxylase (*SPE1*) were unable to grow in the absence of added amines; such cells cannot make putrescine and hence also lack spermidine and spermine (4, 5). On the other hand, cells with mutations in the gene (*SPE2*) for *S*-adenosylmethionine decarboxylase, an essential enzyme in the biosynthesis of spermidine and spermine, could still grow indefinitely at about 10% of the normal growth rate. Although these *spe2* mutants had no detectable spermidine or spermine (6), it seemed possible that they still produced sufficient traces of the amines to permit some growth. Therefore we prepared a null mutant by constructing a deletion-insertion mutation in the *SPE2* gene. This null mutant has an absolute requirement for either spermidine or spermine for growth. In addition, the amine-deficient cells exhibit cellular enlargement, increase in the size and number of vesicle-like bodies in the cytoplasm, delocalized chitin deposition, and abnormal distribution of actin.

The absolute requirement for spermidine or spermine for growth and the morphologic changes are only seen if the cultures are grown aerobically. Anaerobiosis eliminates the spermidine or spermine requirement.†

MATERIALS AND METHODS

Media, Growth Conditions, and Strains. YPAD medium (a rich medium) and H medium (an amine-free defined medium) (7) were used for maintenance of strains and growth studies, respectively. For selection of strains, leucine and uracil were omitted from H medium when required. H medium was sterilized by filtering the solutions through a 0.45-μm Millipore membrane or by boiling. This medium was not autoclaved since we had previously found that autoclaved media often contain impurities acquired from the steam, which permit some growth of amine-deficient mutants (4). To demonstrate an absolute requirement for spermidine or spermine for growth it was essential to use glass vessels that had been washed with 6 M HCl, rinsed with 0.1 M HCl, and dried for 4 hr at 170°C. Cultures were grown at 30°C or with shaking in air under a stream of nitrogen/5% CO_2. Growth was measured by determining the optical density at 600 nm in a Beckman DU7 spectrophotometer. [An OD_{600} of 0.2 equals $\approx 2 \times 10^6$ cells per ml (wild-type strain).]

The yeast strains are listed in Table 1. *Escherichia coli* strain DH5α (Bethesda Research Laboratories) was used for cloning and amplification of DNA. The yeast-*E. coli* shuttle vector YEp351 (10) was used as the source of the *LEU* gene. Transformation of yeast was performed by the lithium acetate method (11).

Methods for DNA manipulation were as described in refs. 12 and 13 or in the directions supplied by the commercial distributor.

Enzyme Assays. Cells grown in YPAD medium to a cell density of $1\text{–}2 \times 10^7$ cells per ml were prepared and assayed for *S*-adenosylmethionine decarboxylase activity as described (14). One unit is defined as the amount of enzyme catalyzing the release of 1 nmol of $^{14}CO_2$ from *S*-adenosyl-[*carboxyl*-^{14}C]methionine per min. Protein was assayed by the method of Bradford (15).

Polyamine Assays. Polyamines were assayed by an ion-exchange method (16, 17), modified for use with HPLC by T. Oshima and N. Hamasaki (personal communication). The cell pellets obtained from 20 ml of culture were extracted with 1 ml of 5% trichloroacetic acid at room temperature. After centrifugation, the supernatant solutions were neutralized with 1 M K_2HPO_4, and 250- to 500-μl samples were chromatographed on a 4.5 × 150 mm HPLC column (Aminex A5; Bio-Rad). The elution buffer (pH 4.9) contained 1.33 M NaCl, 0.067 M sodium citrate (trisodium salt), 0.67 M KCl, 0.033 M potassium citrate (tripotassium salt), 5% isopropyl alcohol, 0.04% Brij 35, and 65 mM HCl. Amines were determined fluorometrically with *o*-phthalaldehyde (18). Putrescine, cadaverine, spermidine, and spermine were well separated from each other, eluting at 14, 22, 27, and 54 min, respectively

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*The genes for the biosynthetic pathway in yeast (1) are *SPE1*: ornithine → putrescine; *SPE2*: *S*-adenosylmethionine → decarboxylated *S*-adenosylmethionine + CO_2; *SPE3*: decarboxylated *S*-adenosylmethionine + putrescine → spermidine + methylthioadenosine; *SPE4*: decarboxylated *S*-adenosylmethionine + spermidine → spermine + methylthioadenosine.

†A preliminary report on these findings was presented at the International Polyamine Symposium in Kyoto, November 11–15, 1990, and at the annual meeting of the American Society for Biochemistry and Molecular Biology in Atlanta, April 21–25, 1991.

Table 1. Strains

Strain no.	Ploidy	Genotype	Source or ref.
2602	Haploid	*MATα ura3-52 his6 leu2*	R. B. Wickner
890410-1a	Haploid	*MAT***a** *lap1 trp3 leu2*	*
72-1D	Haploid	*MATα leu2-27 ura3 spe2-4*	8
Y340	Diploid	*MAT***a** *lap1 trp3 leu2 URA3-52 HIS6 SPE2* / *MATα LAP1 TRP3 leu2 ura3-52 his6 SPE2*	2602 × 890410-1a (this study)
Y341	Diploid	*Δspe2-5::LEU2* / *SPE2* derivative of Y340	This study
Y342	Haploid	*MATα ura3-52 leu2 Δspe2-5::LEU2*	Sporulation of Y341
Y343	Haploid	*MAT***a** *trp3 leu2 Δspe2-5::LEU2*	Sporulation of Y341
Y344	Haploid	*MAT***a** *ura3-52 his6 leu2 Δspe2-5::LEU2*	Sporulation of Y341
Y346	Diploid	*MAT***a** *trp3 URA3-52 leu2 Δspe2-5::LEU2* / *MATα TRP3 ura3-52 leu2 Δspe2-5::LEU2*	Y343 × Y342
Y347	Haploid	*MATα ura3-52 trp3 leu2 Δspe2-5::LEU2*	Sporulation of Y346

*This haploid was derived from sporulation of the diploid strain 890410 by Xie *et al.* (9).

(flow rate = 0.5 ml/min). With these conditions, 0.05 nmol of each amine could be detected.

Microscopic Analysis. Chitin was visualized by fluorescence microscopy after staining with Calcofluor white M2R (American Cyanamid, Princeton, NJ) (19). Actin was stained with rhodamine-phalloidin (Molecular Probes) (19).

Bright-field, differential interference-contrast (Nomarski) and fluorescence microscopy were used with a Zeiss Axiophot microscope (19). Cells were photographed through a 100× oil-immersion objective, using ASA 100 and ASA 1600 film.

Preparation of a Deletion-Insertion Mutation in the Chromosomal *SPE2* Gene. We prepared a null mutation by a one-step disruption technique (20). We have previously described (8) the preparation of an M13mp18 derivative that contained the yeast *SPE2* gene (Fig. 1, line A). This DNA had been modified to contain an *Nde* I restriction site at the start site of the *SPE2* open reading frame. A *Bam*HI–*Sph* I fragment from this DNA was cloned into the *Bam*HI–*Sph* I sites of a pUC19 plasmid that had been modified previously to remove the *Nde* I site that is normally present in the vector. The latter clone was digested with the restriction enzymes *Nde* I and *Nsi* I, resulting in the excision of an 833-bp fragment beginning with the ATG initiation codon of the open reading frame of the *SPE2* gene (Fig. 1, line B). A 1970-bp *Hpa* I–*Nar* I fragment of DNA (containing the *LEU2* gene) from YEp351 (10) was inserted into this site after filling in the ends with Klenow polymerase.

This pUC19 construct was treated with the restriction enzymes *Nco* I and *Sph* I. The resultant 2829-bp fragment was used to transform a *SPE2/SPE2 leu2*$^-$*/leu2*$^-$ diploid strain (Y340, Table 1) and LEU$^+$ transformants were selected. The DNA was isolated from six of these strains and digested with *Nco* I and *Sph* I. After electrophoresis, blot hybridization showed bands consistent with the expected 2829-bp insertion.

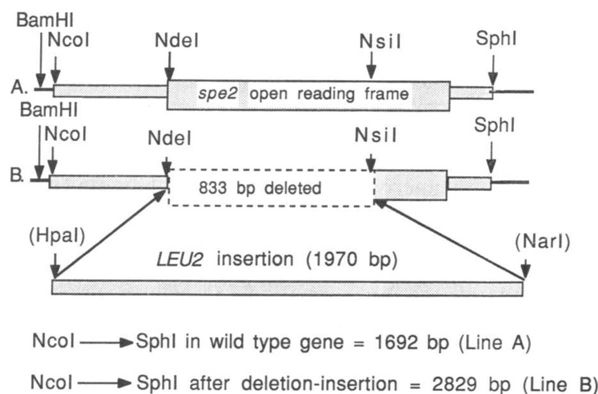

FIG. 1. Construction of the *Δspe2-5::LEU2* null mutation by deletion and insertion. The stippled bars represent yeast sequences; the solid lines represent vector sequences. bp, Base pairs.

One of these diploid strains (Y341) was sporulated, and the haploids from 16 tetrads were tested for leucine prototrophy and the absence of *S*-adenosylmethionine decarboxylase activity. In 15 of these tetrads, 2:2 segregation was observed with complete linkage between the two phenotypes. Three *Δspe2-5::LEU2* haploid strains (Y342, Y343, Y344) were selected for further studies. Southern blot hybridization of the DNA from Y342 and Y343, after digestion with *Nco* I and *Sph* I and electrophoresis, confirmed the postulated insertion-deletion mutation–i.e., a band corresponding to 2829 bp was found.

RESULTS

Absolute Requirement for Polyamines for the Growth of *Δspe2-5::LEU2* Mutants. As described above, we prepared a null mutation of the *SPE2* gene of *S. cerevisiae* by a deletion-insertion procedure. When this mutant (Y343) was grown aerobically in purified medium, the growth rate gradually decreased, presumably as a result of the depletion of the intracellular pools of spermidine and spermine remaining from the previous growth in YPAD medium. After ≈13 generations the growth rate was near zero. At this point the culture was diluted into fresh medium ("0 time" in Fig. 2).

No growth was observed in the absence of added spermidine or spermine. Addition of 10^{-6} M spermidine resulted in near-normal growth rates after a lag period (Fig. 2*A*). Substantial growth resulted from the addition of as little as 10^{-10} M spermidine (Fig. 2*B*) or 10^{-8} M spermine (Fig. 2*C*).

Absence of *S*-Adenosylmethionine Decarboxylase in the *Δspe2-5::LEU2* Mutant. *S*-Adenosylmethionine decarboxylase activity was determined in extracts of the *Δspe2-5::LEU2* mutant (Y343) and in extracts of a wild-type organism (2602) and of the *spe2-4* mutant (72-1D) previously described from this laboratory (6). No enzyme activity was detectable in the extracts of the insertion-deletion mutant (Y343) compared with the very low activity in the strain (72-1D) containing the point mutation (0.04 pmol of CO_2 per min per mg of protein) and the much higher activity observed in the wild-type *SPE2*$^+$ strain 2602 (8.7 pmol of CO_2 per min per mg of protein).

Lack of Detectable Spermidine or Spermine in a *Δspe2-5::LEU2* Mutant. The cells were grown for 13 generations in amine-deficient medium. The cells from 20 ml of culture (OD = 1.0; 50 mg of wet weight) were harvested by centrifugation and assayed for polyamines. Wild-type cells (2602) had 1.6 nmol of putrescine, 14.5 nmol of spermidine, and 4.1 nmol of spermine. A comparable quantity of the

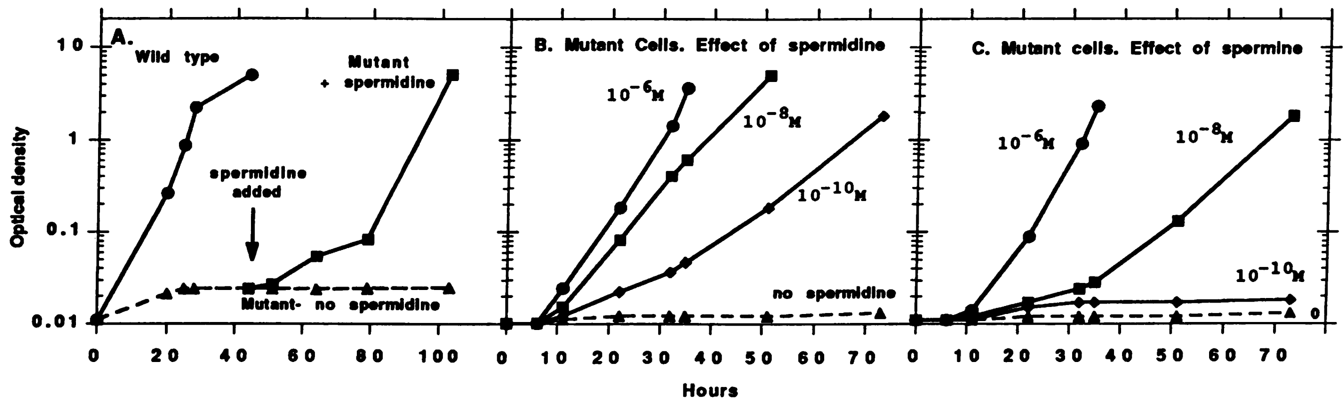

FIG. 2. Requirement of spermidine and spermine for the growth of the Δ*spe2-5*::*LEU2* mutant. Strain Y343 (Δ*spe2-5*::*LEU2*) and a wild-type (*SPE2*$^{+}$) control strain (2602) were grown in amine-free H medium for 13 generations and diluted into fresh amine-free medium at zero time. Spermidine (10^{-6} M) was added to aliquots of these cultures at 45 hr in *A*. Spermidine and spermine were added at zero time in *B* and *C*, respectively; the dotted lines represent the cultures without any added spermidine or spermine.

Δ*spe2-5*::*LEU2* mutant cells (Y343) that were depleted of amines contained 17 nmol of putrescine but no detectable spermidine or spermine (<0.05 nmol).

Morphological Studies. After the deletion-insertion mutant (Y343) was depleted of internal polyamines by incubation in an amine-free medium, a large fraction of the cells appeared grossly abnormal microscopically (Fig. 3 *A* and *C*) compared with the mutant cells grown with spermidine (Fig. 3 *B* and *D*). This was true for the haploid Δ*spe2-5*::*LEU2* and the diploid Δ*spe2-5*::*LEU2*/Δ*spe2-5*::*LEU2* strains, but the defects were more striking in the diploid strain. The cell sizes varied from slightly larger than normal to >10 times the normal volume; the abnormally large forms were seen in 25–50% of the haploid and ≈80% of the diploid cells. These larger cells were distinctly spherical in shape, and the walls appeared thick. Buds were infrequent or completely absent in the large cells but were present in the smaller cells. The cytoplasm of the large cells contained many vesicle-like bodies (by the Nomarski technique), which were large, highly refractile, and spherical and which often filled most of the cytoplasm (Fig. 3*C*). In contrast, polyamine-supplemented cells showed one large vacuole and four or five small vesicle-like bodies in the cytoplasm (Fig. 3*D*). The appearance of the polyamine-supplemented cells was the same as that seen with the nonmutant cells (data not shown).

A number of cdc^{-} and actin^{-} strains have an abnormal microscopic picture similar to that described above (see discussion below). Since some of these strains also show defects in chitin and actin distribution, we stained the amine-deficient cells with Calcofluor (for chitin) and rhodamine-phalloidin (for actin). Staining of the amine-depleted mutant cells with Calcofluor showed diffuse staining more or less uniformly throughout the cell (Fig. 3*E*). This appearance contrasts with the rings of chitin (bud scars) seen in polyamine-supplemented mutant cells (Fig. 3*F*) or in normal cells (not shown) (21, 22). Amine-depleted and amine-supplemented Δ*spe2-5*::*LEU2* mutant cells as well as the wild-type parent were stained with rhodamine-phalloidin to visualize F-actin. Phalloidin selectively binds to the polymerized actin cytoskeleton in yeast (23); this binding may be observed as fluorescence when the phalloidin is coupled to rhodamine (19, 24). In the amine-depleted mutant cells (Fig. 3*G*) the fluorescent material was largely present in small patches located at the periphery of the cells. In spermidine-supplemented cells or in wild-type cells, there is a concentration of fluorescence toward one pole, with a pronounced concentration of fluorescence in the bud (Fig. 3*H*).

Effect of Anaerobic Growth on the Requirement for Polyamines for Growth of the Δ*spe2-5*::*LEU2* Mutant. The above experiments (Fig. 2) showed an absolute requirement for spermidine or spermine when the amine-depleted cells were grown aerobically. When these aerobic cultures were transferred to N_2/CO_2, the mutant cells began to grow after a 40-hr lag period (Fig. 4). The same results were obtained when anaerobiosis was attained by growth in stoppered cuvettes that were completely filled with the culture. The deficient cells grew in the absence of amines at ≈50% of the growth rate observed in the presence of spermidine. Furthermore, cells grown without amines under anaerobic conditions appeared normal microscopically. When anaerobic cultures were transferred back to air, the growth rate decreased again (Fig. 4).

DISCUSSION

Using a null mutation in the *SPE2* gene, we have shown that spermidine or spermine is essential for the aerobic growth of *S. cerevisiae*. Thus, the requirement for these amines is greater than that of the *SPE2* mutant that we had previously obtained by chemical mutagenesis (6).

The finding that spermidine or spermine is essential for the aerobic growth of *S. cerevisiae*, even in strains that contain a large quantity of endogenous putrescine, emphasizes the importance of spermidine and spermine for this organism and shows that these amines have a function that cannot be satisfied by putrescine.

The microscopic changes described in Fig. 3, involving abnormally large cells, markedly decreased budding, diffuse distribution of chitin, and lack of directional orientation of actin, are also seen in various cell division cycle (*cdc*) mutants as well as in mutants with defects in actin and in actin-binding proteins (25–31). In addition, the accumulation of intracellular vesicle-like material has been described for a number of secretory mutants (32) and for a myosin mutant (33). Diffuse Calcofluor staining has been commonly found in mutants defective in chitin synthesis and assembly (21, 22, 27). All of these microscopic observations have led to the suggestion that the cellular actin network may be directly involved in the polarization of intracellular secretion and in cell surface deposition in yeast (28, 34, 35) and that chitin and actin are involved in bud formation and cell division.

The occurrence of similar morphological abnormalities in the amine-deficient mutant raises the possibility that spermidine and spermine are also involved in bud formation and cell division and may in some way be involved in the promotion of actin polymerization and in cytoskeleton and actin functions. In support of the involvement of polyamines are earlier papers showing that polyamines facilitate the

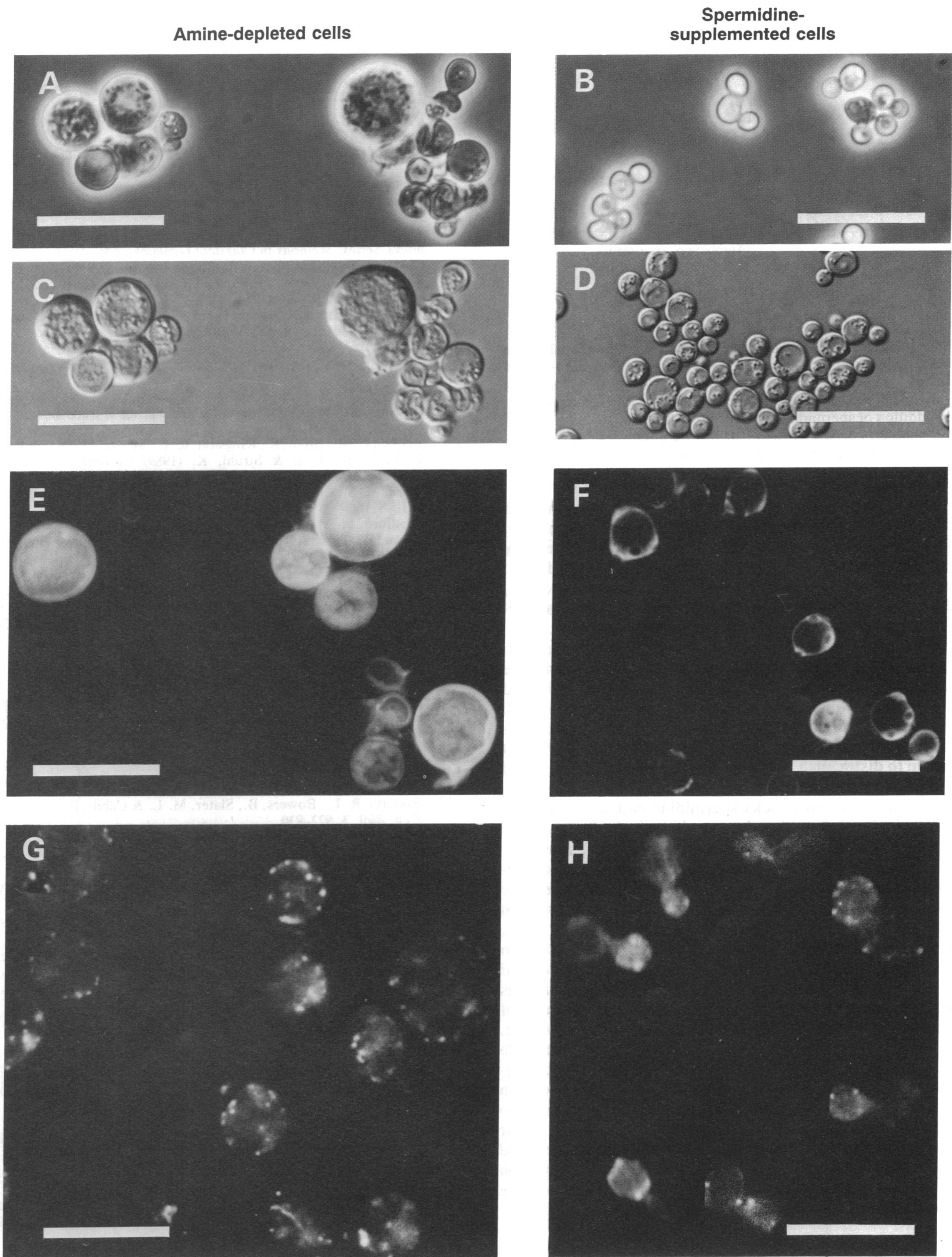

FIG. 3. Microscopic examination of Δ*spe2-5::LEU2* mutant (Y343 strain) grown with and without 10^{-6} M spermidine. (*A* and *B*) Phase-contrast microscopy. (*C* and *D*) Differential interference-contrast optics (Nomarski). (*E* and *F*) Calcofluor staining for chitin deposition. (*G* and *H*) Rhodamine-phalloidin staining for actin. The cells were grown and depleted of intracellular amines as described in the text. Staining was according to the procedures described by Pringle *et al.* (19). Polyamine-depleted cells were photographed 48 hr after their growth rate had reached zero. (Bars = 10 μm.)

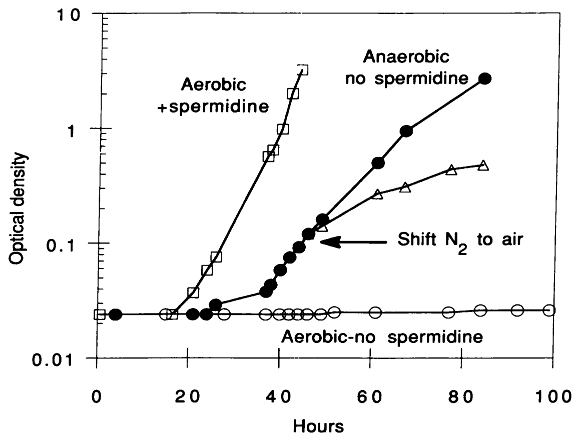

FIG. 4. Effect of aerobiosis and anaerobiosis on the growth of the Δ*spe2-5::LEU2* mutant. Strain Y343 was grown in amine-free H medium for 13 generations and diluted into fresh amine-free medium at zero time. The culture was divided at this time into three portions: (*i*) no addition of spermidine; aerobic growth (○); (*ii*) addition of 10^{-6} M spermidine; aerobic growth (□); (*iii*) no addition of spermidine; anaerobic growth (●). At 45 hr an aliquot of culture *iii* was transferred from N_2 to air (△).

polymerization of actin (36–38) and that actin fragmentation occurs in a polyamine-deficient mammalian cell mutant (39).

The polyamine-deficient cells are not dead, since they exclude trypan blue, a dye that is taken up only by dead cells. In addition, there was no decrease in the viable cell count, even when the incubation was continued in the amine-deficient medium for 4 days after growth had ceased.

Large cells have also been reported by Goldemberg *et al.* (40) in a *S. cerevisiae* mutant that is deficient in ornithine decarboxylase [strain 179-3 (41)]. These cells also showed loss of normal nuclear structure and increased cell wall resistance to protease attack. Since the 179-3 mutant lacks putrescine in addition to spermidine and spermine, it was not possible to distinguish between an effect of putrescine and of spermidine or spermine. The Δ*spe2-5::LEU2* mutant used in the present paper only lacks spermidine and spermine, and thus our findings show that the morphological effects are due to the lack of spermidine or spermine rather than putrescine.

A most unexpected observation was the difference between the absolute requirement for spermidine or spermine observed aerobically and the absence of this requirement in nitrogen. In a N_2/5% CO_2 environment, the cultures grew, and the cells appeared normal. These cells did not contain any *S*-adenosylmethionine decarboxylase activity nor did they contain any spermidine or spermine. The latter findings suggest that an alternative pathway for the biosynthesis of these amines was not present. However, as indicated above, our analytical methods are not sufficiently sensitive to rule out such a possibility completely. It is possible that amine-deficient cells are more sensitive to oxygen damage than normal cells, but considerable work would be necessary to test this speculation. It is of interest to note that in our previous studies with *E. coli* we demonstrated a marked sensitivity of spermidine-deficient cells to oxygen damage in the presence of paraquat, presumably resulting from the formation of superoxide (42).

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