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Mutants of *Saccharomyces cerevisiae* with Defective Vacuolar Function

KATSUHIKO KITAMOTO,[1] KIYOSHI YOSHIZAWA,[1] YOSHINORI OHSUMI,[2] AND YASUHIRO ANRAKU[2]*

National Research Institute of Brewing, Takinogawa, Tokyo 114,[1] and Department of Biology, Faculty of Science, University of Tokyo, Hongo, Tokyo 113,[2] Japan

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Mutants of the yeast *Saccharomyces cerevisiae* that have a small vacuolar lysine pool were isolated and characterized. Mutant KL97 (*lys1 slp1-1*) and strain KL197-1A (*slp1-1*), a prototrophic derivative of KL97, did not grow well in synthetic medium supplemented with 10 mM lysine. Genetic studies indicated that the *slp1-1* mutation (for small lysine pool) is recessive and is due to a single chromosomal mutation. Mutant KL97 shows the following pleiotropic defects in vacuolar functions. (i) It has small vacuolar pools for lysine, arginine, and histidine. (ii) Its growth is sensitive to lysine, histidine, Ca^{2+}, heavy metal ions, and antibiotics. (iii) It has many small vesicles but no large central vacuole. (iv) It has a normal amount of the vacuolar membrane marker α-mannosidase but shows reduced activities of the vacuole sap markers proteinase A, proteinase B, and carboxypeptidase Y.

In the accompanying paper (7), we demonstrated that in yeast cells vacuoles are metabolically active organelles and that they regulate homeostasis of the cytosolic amino acid pool. Characterization of mutants with defects in vacuolar function is essential for determination of the physiological roles of vacuolar functions.

We previously found that 10 amino acids, i.e., arginine, lysine, histidine, phenylalanine, tryptophan, tyrosine, glutamine, asparagine, isoleucine, and leucine, are taken up actively into vacuolar membrane vesicles of yeast cells through seven H^+/amino acid antiport systems with narrow substrate specificities (17). Of these 10 amino acids, lysine is taken up by only one transport system specific for lysine and arginine. We also found that the concentration of lysine in the cytosolic pool was maintained at about 10 mM or below, even when the yeast cells were grown in lysine-rich medium, and that the excess lysine in the cytoplasm was sequestered in their vacuoles, which serve as intracellular storage compartments (7).

On the basis of these findings, we attempted to isolate mutants with a small lysine pool in their vacuoles, hoping to show that the growth of such mutants would be sensitive to lysine or the lysine analog *S*-2-aminoethyl-L-cysteine (AEC). There may be three kinds of mutants that would form a small lysine pool: class 1, defective in a specific lysine transport system of the vacuolar membrane; class 2, defective in an energy transducing system of the vacuolar membrane, such as H^+-translocating ATPase; and class 3, defective in vacuole biogenesis or its regulation. In the present study, we isolated mutants belonging to class 3 and designated the mutation *slp1*.

MATERIALS AND METHODS

The *S. cerevisiae* strains used are listed in Table 1.

YEPD and YNBD media have been described in the accompanying paper (14). Solid media were prepared by adding 2% agar (Shoei Chemicals, Tokyo, Japan) to the above media. The selection medium used for isolation of mutants was YNBD containing 8 mM lysine or YNBD containing 0.1 mM lysine and 0.5 mM AEC (Sigma Chemical Co., St. Louis, Mo.). Sporulation medium consisted of 1% potassium acetate, 0.1% yeast extract, 0.05% glucose, and 2% agar.

Mutant isolation. We assumed that a mutant with a small lysine pool caused by defective vacuolar membrane function would be sensitive to a high concentration of lysine or the lysine analog AEC, since lysine is a noncatabolizable amino acid in *S. cerevisiae* (20) and its abnormal accumulation in the cytosol, excluding the vacuoles, would probably perturb cellular metabolism. The lysine auxotrophic haploid strain A13-18 was used as the parental strain to exclude the possibility of collecting mutants defective in enzymes of the lysine biosynthetic pathway or mutants defective in lysine transport activities of the plasma membrane.

AEC-sensitive mutant. A cell suspension in 10 ml of 0.05 M phosphate buffer (pH 8.0) was mixed with 0.3 ml of ethyl methanesulfonate and shaken for 60 min at 30°C. The cells were then harvested and washed five times with sterile distilled water. The cell suspension was diluted serially and plated onto YEPD plates to give a density of 100 to 200 colonies per petri dish. The plates were incubated at 30°C for 2 days, and the colonies were then transferred to YNBD–0.1 mM lysine plates with or without 0.5 mM AEC by replica plating. The parental strain grew normally on a YNBD–0.1 mM lysine plate containing 0.5 mM AEC, whereas AEC-sensitive mutants formed tiny colonies. From about 5,500 colonies of mutagenized cells, 62 AEC-sensitive mutants were obtained.

Lysine-sensitive mutant. Mutagenized cells were plated onto YEPD plates as described above, and after 2 days, the colonies were replica plated onto YNBD–0.3 mM lysine and YNBD–8 mM lysine plates. Mutants that grew normally on YNBD–0.3 mM lysine plates but very slowly on YNBD–8 mM lysine plates were picked up.

For isolation of lysine-sensitive mutants, enrichment of mutants by nystatin treatment (18) was used in a few cases. For this experiment, 1 ml of the mutagenized culture was diluted with 9 ml of YNBD medium containing 8 mM lysine and was incubated overnight at 25°C. Then, 1 ml of the outgrown culture was washed twice with sterile distilled water, and the cells were suspended in 0.9 ml of lysine-free YNBD medium. The suspension was shaken for 3 h at 30°C

* Corresponding author.

TABLE 1. *S. cerevisiae* strains

Strain	Genotype	Source
X2180-1A	*MAT***a**	The Yeast Genetic Stock Center, Berkeley, Calif.
A13-18	*MAT***a** *lys1*	This laboratory
A56-1-1A	*MAT*α *ade1*	This laboratory
KL97	*MAT***a** *lys1 slp1-1*	This work
KL65	*MAT***a** *lys1 slp1-2*	This work
KL197-1A[a]	*MAT***a** *slp1-1*	This work

[a] The haploid strain KL197-1A was one of the segregants from the diploid strain constructed by crossing KL97 and A56-1-1A.

and then mixed with 0.1 ml of 100-μg/ml nystatin solution, and incubation was continued for 1.5 h. The cells were then washed twice with sterile distilled water and plated onto YNBD–0.3 mM lysine plates. After incubation at 30°C for 3 to 4 days, colonies were replica plated onto YNBD–0.3 mM lysine and YNBD–8 mM lysine plates. Mutants that were sensitive to lysine were isolated as described above. From about 1,000 mutagenized colonies with nystatin enrichment, 11 lysine-sensitive mutants were isolated, and from about 5,500 mutagenized colonies without nystatin treatment, 4 were isolated.

Enzyme assays. Cells were grown in YEPD medium for 48 h at 30°C, harvested, washed once with distilled water, and suspended in 0.1 M Tris hydrochloride buffer (pH 7.6). The cells were disintegrated with glass beads (diameter, 0.45 mm) in a homogenizer (Braun-Melsungen, Melsungen, Federal Republic of Germany). The cell lysate was centrifuged at 35,000 × *g* for 30 min, and the supernatant fluid was used for enzyme assays. Proteinase A activity was measured by the method of Lenney (8), using hemoglobin as the substrate. Proteinase B and carboxypeptidase Y were assayed by the method of Jones (5), using azocoll and *N*-benzoyl-L-tyrosine-*p*-nitroanilide (Sigma), respectively, as substrates. The activity of α-D-mannosidase was determined by the method of Opheim (16).

Differential extraction of cytosolic and vacuolar amino acid pools. The cytosolic, vacuolar, and total amino acid pools were extracted by the method described in the accompanying paper (14). The composition of amino acids in each pool was analyzed in a Hitachi 835 amino acid analyzer.

Genetic methods. The procedures described by Mortimer and Hawthorne (11) were used for sporulation and tetrad analysis.

RESULTS

Mutant selection and characterization. A total of 62 AEC-sensitive mutants and 15 lysine-sensitive mutants were selected as primary candidates for having a small lysine pool. These strains were grown in YEPD medium to the middle of the logarithmic phase, and the compositions of their total amino acid pools were examined in an amino acid analyzer. On the basis of this screening, we selected 27 mutants with small lysine pools as second candidates. These mutant cells were examined by the Cu^{2+} method (14) to determine the amino acid compositions of their cytosolic and vacuolar pools separately. These cells all had small lysine pools, but in most of them lysine was still enriched in the vacuolar pool. Consequently, the mutants which had a normal ratio of distributions of lysine in the vacuolar and cytosolic compartments were discarded.

On the basis of this third screening, we finally selected two AEC-sensitive mutants (KA318 and KA495) and two lysine-sensitive mutants (KL65 and KL97) as candidate mutants. The two lysine-sensitive mutants were derived from cells treated with nystatin. These four mutants all had a normal cytosolic glutamate pool but a small vacuolar lysine pool, amounting to 20 to 40% of that of the parental strain (Table 2). Interestingly, none of these mutants showed a specific decrease in the lysine pool in the vacuoles; instead, the contents of the major amino acids in the vacuolar pool, i.e., lysine, histidine, and arginine, were reduced simultaneously to 20 to 40%, 50 to 70%, and 10 to 30%, respectively, of those of the parental strain, and their distribution ratios between the vacuoles and cytosol were significantly reduced. This pleiotropic defect, observed in all four mutants, suggested that the mutation(s) was not due to a defect of lysine-specific transport into the vacuoles.

TABLE 2. Cytosolic and vacuolar amino acid pools in wild-type (A13-18) and mutant strains

Strain	Amt of amino acid (mmol/mg of protein)[a]							
	Glutamate		Lysine		Histidine		Arginine	
	C	V	C	V	C	V	C	V
A13-18	421	62	40	314	30	101	22	254
KA318	329	31	37	114	28	66	4	55
KA495	330	60	36	61	27	45	26	66
KL65	513	103	94	106	55	65	26	71
KL97	380	130	50	60	16	45	13	21

[a] Cells were grown in YEPD medium. Cytosolic and vacuolar pools were extracted differentially and analyzed in an amino acid analyzer. C, Cytosolic pool; V, vacuolar pool.

Growth properties of the mutants. The four mutants described above were grown in YNBD medium containing 5 mM lysine, harvested in the middle of logarithmic phase, and washed three times with sterile distilled water. The cells were then transferred to lysine-free YNBD medium and incubated for a further 22 h. The parental strain, A13-18 (*lys1*) grew threefold by utilizing endogenous lysine stored in the vacuoles, whereas the mutants stopped growing after 4 h and their cell numbers increased only 1.1- to 1.5-fold during incubation for 22 h (Fig. 1). These results indicated that the vacuolar lysine pool was metabolically utilizable and that growth during lysine starvation, i.e., the growth yield, was determined by the initial pool size of lysine.

The growth properties of mutants in YNBD plates supplemented with 10 mM lysine, 10 mM histidine, 10 mM arginine, 0.5 mM AEC, or 0.01 mM canavanine were examined. AEC-sensitive mutants KA318 and KA495 were not sensitive to lysine, and lysine-sensitive mutants KL65 and KL97 were not sensitive to AEC. All four mutants showed normal growth in the presence of 10 mM arginine, but three mutants, KA495, KL65, and KL97, were sensitive to canavanine. Mutant KL97 showed very poor growth in the presence of 10 mM lysine or histidine. This distinct growth phenotype was used for further genetic analysis of the mutation. On the basis of the results described below, we designated the mutation *slp1* (for small lysine pool).

Morphological observations. Phase-contrast microscopy showed that cells of strains KL97 (*slp1 lys1*) and KL197-1A (*slp1*) had no central vacuole (data not shown). Mutants KA495 and KL65 had small vacuoles that were one-half to one-third the diameter of those of the wild-type strain, while mutant KA318 had normal-sized central vacuoles. Fluorescence microscopy after staining with the fluorescent dye chloroquine (9) also showed that strain KL197-1A had no central vacuole, but many small fluorescent vesicles were

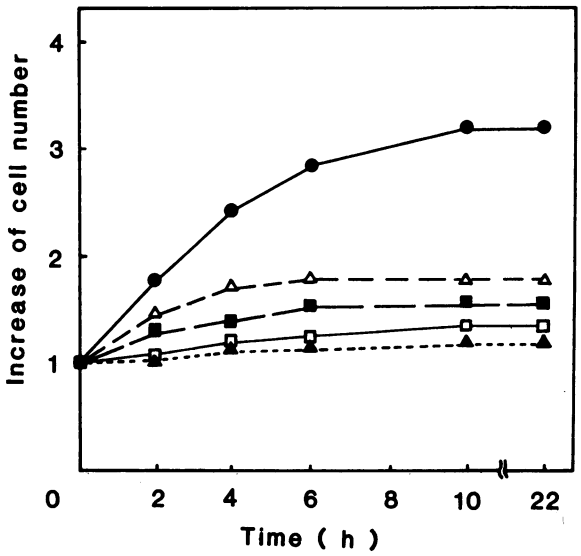

FIG. 1. Growth of mutants during lysine starvation. Cells of wild-type strain A13-18 (●) and mutant strains KA318 (■), KA495 (□), KL65 (△), and KL97 (▲) were grown in YNBD medium containing 5 mM lysine for 16 h at 30°C, harvested, and washed three times with sterile distilled water. The cells were then transferred to lysine-free YNBD medium, and their growth was monitored.

seen in the cytoplasm (Fig. 2). Figure 3 shows the ultrastructure of KL197-1A cells examined by electron microscopy by using a freeze-substitution technique (2). Cells in either the logarithmic or the stationary phase had no large central vacuole but they contained many small vesicles that were evenly distributed in the cytoplasm. The number of these vesicles seemed to increase when the cells reached stationary phase. From these observations, we concluded that the mutation(s) in KL97 resulted in a defect in the process(es) of central vacuolization, which consequently reduced the volume of vacuolar pools for basic amino acids and thereby conferred the mutant with a lysine-sensitive phenotype.

Genetic analyses. To determine whether lysine sensitivity or the defect of central vacuolization was the result of a single mutation, KL97 was crossed with A56-1-1A, a nonmutant haploid, and the resulting diploid was sporulated. The diploid was not lysine sensitive, indicating that the mutation was recessive. All 40 tetrads showed 2 Lys^r: 2 Lys^s segregation for lysine sensitivity, indicating that sensitivity was due to a single chromosomal mutation. Other phenotypes, histidine sensitivity and the defect in central vacuole formation, consistently cosegregated with the lysine-sensitive phenotype. Complementation analysis revealed that the mutation in KL97 did not complement that in KL65 but did complement those in the mutants KA318 and KA495. We therefore designated the mutations in KL97 and KL65 *slp1-1* (small lysine pool) and *slp1-2*, respectively. The diploid homozygous for the *slp1-1* mutation did not sporulate. Since diploids obtained by crossing KA318 or KA495 with A56-1-1A did not sporulate well, no further genetic analysis was done.

Growth phenotype of the *slp1-1* mutant. We examined the effects of amino acids on growth of the *slp1-1* mutant. Of the 20 amino acids tested, lysine and histidine severely inhibited the growth of KL197-1A (*slp1-1*). Both amino acids are highly compartmentalized in vacuoles and not catabolized in *S. cerevisiae* (20). Histidine was more inhibitory than lysine (Table 3). On the contrary, arginine, which is catabolizable and is a good nitrogen source for yeast cells, had no effect on growth.

Ca^{2+} (4, 13) and polyphosphate (15) are known to be localized in the vacuoles. Growth of KL97 was inhibited by the presence of 100 mM $CaCl_2$ but not of phosphate (Table 4). The mutant was more sensitive to heavy metal ions such as Hg^{2+}, Cd^{2+}, Fe^{2+}, and Cu^{2+} than the parental strain was (data not shown).

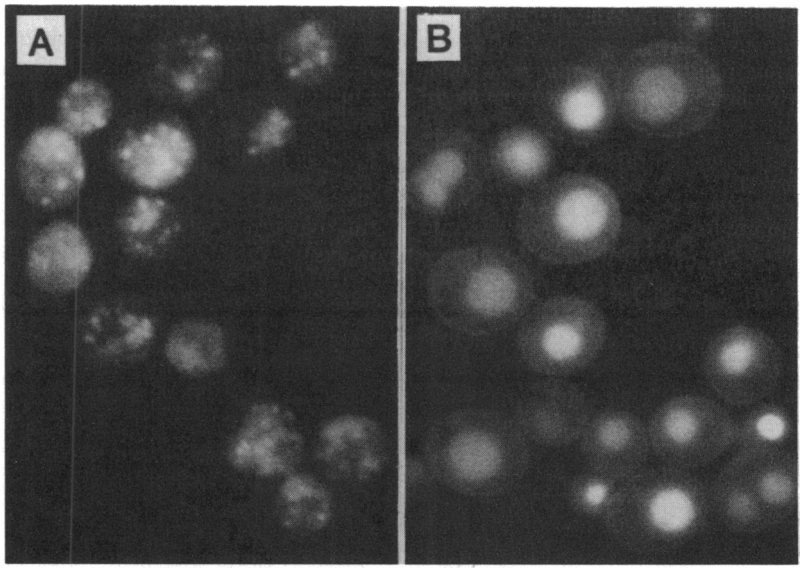

FIG. 2. Fluorescence photomicrographs of cells of *slp1-1* mutant KL197-1A (A) and wild-type strain A13-18 (B). Cells grown in YEPD medium to mid-logarithmic growth phase were suspended in 0.1 M potassium phosphate buffer (pH 7.4) at 30°C. Cell suspensions were incubated in the presence of 2% glucose and 10 mM chloroquine for 20 min. Fluorescence was observed with a Nikon Optiphoto fluorescence microscope.

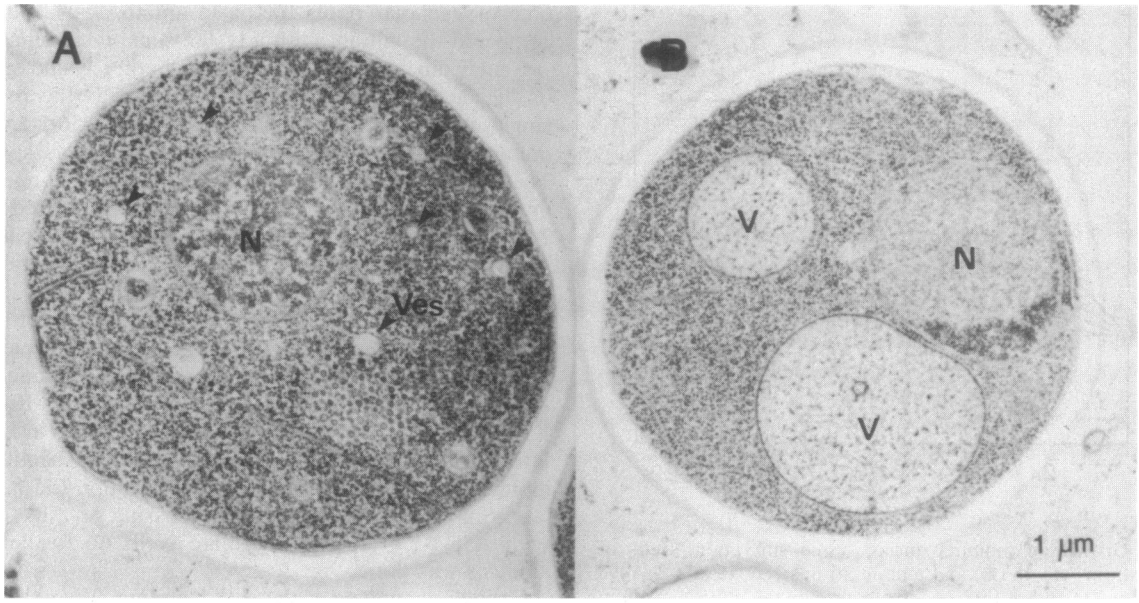

FIG. 3. Electron photomicrographs of *slp1-1* mutant KL197-1A (A) and wild-type strain A13-18 (B) cells. Cells grown in YEPD medium to mid-logarithmic phase were observed by the freeze-substitution method (2). N, Nucleus; V, vacuoles; Ves, vesicles (shown by arrowheads).

Levels of vacuolar enzymes. The vacuolar sap of *S. cerevisiae* contains proteinase A, proteinase B, and carboxypeptidase Y, whereas α-mannosidase is known to be a marker enzyme of the vacuolar membrane. We measured the activities of these marker enzymes (Table 4). The activities of the proteolytic enzymes in the *slp1-1* mutant were significantly lower than those in the parental strain. However, there was no difference in the α-mannosidase activities of the mutant and the parental strain. These results strongly suggested that the *slp1-1* mutation caused a defect in the process of vacuolization but not in the synthesis of the vacuolar membrane.

TABLE 3. Effects of amino acids and inorganic ions on growth rate

Addition (mM) to YNBD	Multiplication rate[a]/h	
	X2180-1A (wild type)	KL197-1A (*slp1-1*)
None	0.22	0.12
Lysine		
1	0.21	0.07
5	0.18	0.06
10	0.19	0.05
Histidine		
1	0.18	0.12
5	0.19	0.00
10	0.14	0.00
Arginine		
1	0.22	0.12
5	0.22	0.12
10	0.19	0.13
Glutamate		
1	0.22	0.12
5	0.25	0.17
10	0.20	0.17
KCl (100)	0.17	0.13
NaCl (100)	0.19	0.10
$MgCl_2$ (100)	0.17	0.14
$CaCl_2$ (100)	0.19	0.05
KH_2PO_4 (100)	0.23	0.17

[a] Number of cell divisions.

DISCUSSION

To determine the physiological significance of the vacuolar compartment in amino acid metabolism in yeast cells, we carried out genetic studies to isolate mutants that cannot form a normal vacuolar lysine pool. Since lysine is taken up into vacuoles by only one transport system, the arginine-lysine transport system of the vacuolar membrane (17), we expected that lysine-sensitive mutants would be defective in this specific transport system or, less likely, defective in vacuolar H^+-translocating ATPase (19), which pleiotropically causes the formation of small basic amino acid pools (12, 17). However, the four mutants isolated showed defects of central vacuolization causing reduction in the volume of the vacuolar compartment.

Messenguy et al. (10) isolated a canavanine-hypersensitive mutant, named a small pool or *smap* mutant, which had a small pool of basic amino acids. However, this mutant showed a normal distribution of basic amino acids in the vacuolar and the cytosolic pools. Thus the *smap* mutation is

TABLE 4. Activities of vacuolar enzymes in wild-type and *slp1-1* mutant strains[a]

Vacuolar enzyme	Enzyme activity (10^3 U/mg of protein) in:	
	A13-18 (a *lys1*)	KL97 (a *lys1 slp1-1*)
Proteinase A	2.0	1.0
Proteinase B	0.96	0.10
Carboxypeptidase Y	1.76	0.30
α-Mannosidase	0.452	0.459

[a] Marker enzyme activities were determined by standard assays (see Materials and Methods).

phenotypically different from the *slp1-1* mutation, because the latter results not only in reduction of the total pools of basic amino acids but also in an abnormal distribution of these amino acids between the cytosol and vacuoles (Table 2).

Jones (6) isolated many *pep* mutants that are pleiotropically defective in proteinase activities located in the vacuoles. Of these, the *pep4* mutant was well characterized and the *PEP4* gene was identified as a structural gene for proteinase A (1). The *pep4* mutation does not cause hypersensitivity to amino acid analogs, but the mutant has a small amino acid pool and shows no competency for sporulation. In complementation tests, we found that *slp1-1* is not allelic to the *pep4-3* mutation. Six other *pep* mutants, *pep3*, *pep5*, *pep6*, *pep7*, *pep12*, and *pep14*, showed pleiotropic phenotypes such as suppression of *can1-11*, hypersensitivity to canavanine, a small amino acid pool, and no competency for sporulation (6). The *slp1-1* mutation may be allelic to one of these *pep* mutations. Genetic analysis to clarify this point is now in progress.

Recently, Emr and co-workers (3) isolated a set of *vpt* mutants with a defect in the sorting system for vacuolar enzymes that resulted in secretion of the vacuolar enzyme carboxypeptidase Y to the cell surface. Some of these mutants have no typical central vacuole (S. D. Emr, personal communication). However, we could not detect the activities of α-mannosidase and carboxypeptidase Y on the cell surface; therefore, the *slp1-1* mutant does not mislocalize vacuolar enzymes to the cell surface.

KL97, which apparently lacks a central vacuole, could still grow in YEPD medium, but its generation time was twice that of the parental strain. This means that central vacuoles may not be obligatory, at least for growth in nutrient-rich medium such as YEPD. However, the mutant is sensitive to lysine, histidine, canavanine, Ca^{2+}, and heavy metal ions. In addition, it is more sensitive than the parental strain to various drugs such as geneticin, kanamycin, tetracycline, and methylglyoxal (4, >10, 2, and 3 times more sensitive, respectively, than the parental cells, as judged by the diameter of inhibition zones on solid medium; data not shown). These results suggest that vacuoles function as a metabolically active storage compartment with a capacity for sequestering certain heavy metal ions and antibiotics. Furthermore, the vacuolar function may be essential in sporulation, because the diploid homozygous for *slp1-1* could not sporulate.

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