Round Shape Enlargement of the Yeast Spheroplast of *Saccharomyces cerevisiae* by HM-1 Toxin

Tadazumi KOMIYAMA,*^a Tetsuya KIMURA,^b and Yasuhiro FURUICHI^c

^a Department of Biochemistry, Niigata College of Pharmacy; 5–13–2 Kamishinei-cho, Niigata 950–2081 Japan; ^b Faculty of Bioresources, Mie University; Tsu, Mie 514–8507 Japan; and ^c GeneCare Research Institute Co., Ltd.; Kamakura 247–0063, Japan. Received November 22, 2001; accepted April 19, 2002

The effects of HM-1 killer toxin (HM-1) on yeast spheroplasts of *Saccharomyces cerevisiae* were examined under osmotically stabilized conditions. Prolonged incubation of spheroplasts in nutrient-rich media resulted in an increase in volume, accompanied by aberrant morphological changes. By contrast, spheroplasts were enlarged, maintaining a round shape, when incubated in HM-1 media. The required 50% effective dose of HM-1 was as low as 2.2×10^{-8} M, and this effect by HM-1 was specific to yeast sensitive to HM-1. Some parts of the enlarged spheroplasts were stable, but the round shape was deformed as HM-1 was removed from the medium. In both the control and HM-1-treated spheroplasts, the total protein and DNA content were increased by approximately three and four times in response to their incubations, respectively. Cytochemical analysis by 4'6-diamidino-2-phenylindol (DAPI) staining showed multiple nuclei. Consistently, actin patches of cells were evenly distributed in both the control and HM-1-treated spheroplasts. A similar enlargement of spheroplasts was observed with lipophilic antifungal compounds, aculeacin A and papulacandin B, but the effects were distinct from those of HM-1 because the spheroplasts resulted in lysis after a long incubation. The molecular mechanism(s) behind this unique observation remains to be studied, but it is clear that HM-1 is an excellent tool for studying yeast cell biology.

Key words large spheroplast; killer toxin; yeast; cell wall; beta glucan synthesis; *Saccharomyces cerevisiae*

HM-1 killer toxin (HM-1) is a strong anti-yeast protein produced by the yeast *Williopsis saturnus* var. *mrakii* IFO 0895, which was previously named *Hansenura mrakii*, and belongs to the K9-type killer toxin group.^{1–6} HM-1 consists of 88 amino acids and is stable in response to heat treatment and various pH conditions. The three-dimensional structure of this protein has been clarified using nuclear magnetic resonance.⁷ The interactions of HM-1 with sensitive yeast follows two steps: an initial binding to cell-wall polysaccharides and a secondary binding to its receptor localized in the cell membrane.^{8–11} We have previously shown that two specific arginine residues (positions 82 and 86) are required for the cytotoxic effect on yeast *Saccharomyces cerevisiae* cells.¹² HM-1 cytotoxicity is effected by disturbing the cell-wall synthesis of sensitive cells by inhibiting β -1,3-glucan synthase and the formation of pores at the budding tips of dividing cells.^{13,14} Cellular materials spouting from the pores made at the budding tip leads to cell death.⁶ Yamamoto *et al.* have reported that the cytotoxic effects of HM-1 are specific to β -1,3-glucan synthesis of sensitive cells, and that HM-1 does not inhibit other cellular events, including the synthesis of proteins, mannans, and chitin.¹³ In our previous studies, we have noted that HM-1 does not kill sensitive yeast cells if the cell walls are removed and exist as spheroplasts (unpublished data). Curiously, although spheroplasts survive against HM-1, their morphology is affected by incubation with HM-1, resulting in a perfectly round shape with an increase in the volume of spheroplasts and cellular vacuoles. In the present study, we examined the basis for this unique phenomenon caused by HM-1. Aculeacin A and papulacandin B, lipophilic antifungal antibiotics that inhibit yeast glucan synthase,^{5,15} were included as controls to compare the effects.

MATERIALS AND METHODS

Cells and Reagents HM-1 was prepared as described previously.¹⁴ *S. cerevisiae* A451 (*MAT α* , *ura3*, *leu2*, *trp1*, *can1*, *aro7*) and *S. cerevisiae* BJ1824 (*MAT α* , *ura3*, *leu2*, *trp1*, *pep4*) cells are sensitive to HM-1. *S. cerevisiae* BJ1824 *rhk1 Δ ::URA3* (*rhk1 Δ ::URA3* in BJ1824) cells are resistant to HM-1.^{6,9,14} The YPD medium used to grow these cells consisted of 1% yeast extract and 2% each of peptone and glucose. The Zymolyase 100T and the DNA quantitation kits were products of Seikagaku Corporation (Tokyo, Japan) and Nippon Bio-Rad Laboratories (Tokyo), respectively. 4'6-Diamidino-2-phenylindol (DAPI) and rhodamine phalloidin were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Molecular Probes, Inc. (Eugene, OR, U.S.A.), respectively. Phase-contrast light and fluorescence microscopes, model BHS, were the products of the Olympus Optical Co., Ltd. (Tokyo), and Kodak TMAX 400 and Fujichrome PROVIA 400 film was used.

Preparation of Yeast Spheroplasts Spheroplasts were prepared using Zymolyase 100T, a cell-wall hydrolyzing enzyme, as described previously.¹⁶ In brief, logarithmically growing yeast cells (1.6×10^8 cells in 5-ml culture) were collected by centrifugation at 12000 rpm for 15 s at 25 °C, and the cells were washed twice with 1.0 ml of water, then suspended in 2.0 ml of cold 50 mM sodium phosphate buffer (pH 7.6) containing 10 mM EDTA, 10 mM 2-mercaptoethanol, 0.8 M sorbitol, and 0.70 mg Zymolyase 100T. The mixtures were incubated at 30 °C for 30 min, and the conversion to spheroplasts was then monitored using a light microscope. Spheroplasts were collected by centrifugation at 3000 rpm for 5 min at 25 °C, and were washed three times with 1.0 ml of 0.8 M sorbitol at 3000 rpm for 5 min at 25 °C. The spheroplasts were then suspended in 0.8 M sorbitol or YPD medium containing 0.8 M sorbitol at a concentration of 10^9 cells/ml

* To whom correspondence should be addressed. e-mail: tkomiyam@niigata-pharm.ac.jp

and were kept at 0 °C until use (Fig. 1A). Spheroplasts from *S. cerevisiae* A451 cells were used in this study unless otherwise stated.

Standard Incubation Conditions and the Monitoring of Spheroplasts Spheroplasts (10^8 cells/ml) were incubated at 30 °C with or without 2.11×10^{-7} M HM-1 in the YPD medium containing 0.8 M sorbitol as an osmotic stabilizer with occasional shaking. Cell death and morphological changes in the spheroplasts were monitored using a light and phase-contrast microscope after the cells were stained with 0.1% methylene blue dissolved in 50 mM KH_2PO_4 – Na_2HPO_4 (pH 4.6) containing 0.8 M sorbitol. Cell numbers were counted using Bürker–Türk counting chamber.

Determination of Protein and DNA Total protein and DNA were determined by the biuret method¹⁷⁾ and the Hoechst 33258 fluorescence method, respectively. Briefly, incubated spheroplasts were centrifugally washed twice with a spheroplast suspension volume of 0.6 M KCl at 2500 rpm for 5 min at 4 °C. For the total protein determination, precipitated spheroplasts were dissolved in H_2O and lysed with 0.91% sodium dodecyl sulfate (SDS), after which the biuret reagent was added. Before measurement of the absorbance at 540 nm, any visible crystalline SDS was removed by two rounds of centrifugation at 15000 rpm for 10 min at 4 °C. The bovine serum albumin standard was prepared by the same procedure. For the DNA determination, precipitated spheroplasts were suspended in 50 mM Tris–HCl (pH 8.0) containing 50 mM EDTA, and the DNA was then isolated by the method described by Philippsen *et al.*¹⁸⁾ DNA concentrations were determined according to the attached manual using calf thymus DNA as a standard.

Staining of Spheroplasts with DAPI and Rhodamine Phalloidin The staining of spheroplasts with DAPI and rhodamine phalloidin has been described by Pringle *et al.*^{19,20)}

After incubation, spheroplasts (about 5×10^7 cells/ml) were fixed in a mixture of 5% paraformaldehyde, 20 mM MgCl_2 , 10 mM EGTA, and 0.8 M sorbitol for 3 h at room temperature. The fixed spheroplasts were collected by centrifugation at 3000 rpm, at 25 °C for 5 min, and were washed twice with a buffer consisting of Dulbecco's phosphate buffered saline without minerals but supplemented with 20 mM MgCl_2 and 10 mM EGTA (PME buffer). The spheroplasts were suspended in a small volume of PME buffer (5×10^7 cells/ml) and were kept at 4 °C until use. Four microliters of fixed spheroplasts were placed in a 4 mm diameter well of a slide glass; 10 μl of 70% ethanol was then added, and the mixture was left at 25 °C for 15 min. This procedure was repeated once. After excess moisture was removed from the slide glass, one drop of 0.2 $\mu\text{g/ml}$ DAPI solution was added to the specimens, and the mixture was kept for 15 min at 25 °C and protected from the light. For the rhodamine phalloidin staining, fixed and washed spheroplasts (2×10^5 cells) were stained by mixture with 1.2 μM rhodamine phalloidin in the dark at 25 °C for 30 min, and next at 4 °C for 13 h, and they were then centrifugally washed twice with a small volume of PME buffer at 3000 rpm at 25 °C for 5 min. The precipitated spheroplasts were finally resuspended in a few drops of 5% *n*-propyl gallate. Fluorochrome-stained cells in wells of a glass slide were observed under a fluorescence microscope using 5% *n*-propyl gallate in glycerol and immersion oil.²¹⁾

RESULTS

Enlargement of the Yeast Spheroplasts by HM-1 Figure 1A shows phase-contrast microscopy of freshly prepared spheroplasts from *S. cerevisiae* A451. Figures 1B to E show the morphological changes made by incubating spheroplasts in YPD medium, which include 0.8 M sorbitol with and

Fig. 1. Freshly Prepared Spheroplasts and Morphological Change in Spheroplasts Incubated with and without HM-1 in YPD Medium Supplemented with 0.8 M Sorbitol

The incubation conditions for the spheroplasts are described in Materials and Methods, and the spheroplasts were photographed using a phase-contrast microscope. A. Freshly prepared spheroplasts. The incubation temperature was at 30 °C for the first 8 h and 25 °C for the following 17 h. B and C. Spheroplasts incubated in the control medium. B, 5-h incubation; C, 25-h incubation. D and E. Spheroplasts incubated in the medium containing 2.11×10^{-7} M HM-1. D, 5-h incubation; E, 25-h incubation. Bar indicates 10 μm .

Fig. 2. HM-1 Dependency of the Morphological Change

The incubation conditions for the spheroplasts are described in Materials and Methods, and spheroplasts were photographed using a phase-contrast microscope. Spheroplasts, 4×10^6 cells/ml, were incubated at the following concentrations of HM-1 for 6.5 h. A, 6.57×10^{-9} M; B, 1.31×10^{-8} M; C, 2.63×10^{-8} M; D, 5.25×10^{-8} M; E, 1.05×10^{-7} M. Bar indicates $10 \mu\text{m}$.

without HM-1. In the absence of HM-1, spheroplasts enlarged, changing to various forms, aberrant and with some round forms (Figs. 1B, C), as if some of the morphological events of cell division were being reflected in the morphologies of the spheroplasts. In the presence of 2.11×10^{-7} M HM-1, however, spheroplasts enlarged enormously, maintaining their round shape (Figs. 1D, E). Compared with freshly prepared spheroplasts without subsequent culture (Fig. 1A), the degree of enlargement of spheroplasts was significant, and the diameter of some giant spheroplasts was more than $15 \mu\text{m}$, with a large vacuole. The size of enlarged spheroplasts, however, varied. Enlargement of spheroplasts was also observed by the incubation in minimum synthetic medium containing 2% glucose, 2.11×10^{-7} M HM-1 and 0.8 M sorbitol, but the size of spheroplasts did not reach that of the spheroplasts incubated in YPD medium containing 0.8 M sorbitol. In the glucose minus medium, such as from YPD or minimum synthetic medium, the size of the spheroplasts did not increase in the presence or absence of HM-1. These results suggest that the formation of large, round spheroplasts in response to HM-1 requires cell-growth conditions with osmotic stabilization.

Determination of the Concentration of HM-1 Required for Spheroplast Enlargement As large, round spheroplasts were formed only in the presence of HM-1, the minimum concentration of HM-1 required for spheroplast enlargement was determined. The morphology of spheroplasts changed dependent on HM-1 concentrations (Fig. 2), and the effective dose of 50% HM-1 was estimated as being approximately 2.2×10^{-8} M from the titration of the morphological change of spheroplasts by HM-1 (Fig. 3). This value agreed well with the IC_{50} value, 2.1×10^{-8} M, of *in vitro* killer activity in response to the sensitive yeast cells,⁶⁾ and with the IC_{50} value, 5.0×10^{-8} M, for inhibiting partially purified β -1,3-glucan synthase,¹⁴⁾ suggesting that the specific inhibition of cell-

Fig. 3. Titration of the Morphological Change of Spheroplasts by HM-1

Spheroplasts which were incubated for 12 h were counted as described in Materials and Methods. Round spheroplast percentage was calculated in reference to live cells.

wall synthesis is the basis of this spheroplast enlargement without aberrant morphology.

Stability of Round Spheroplasts Figure 4 shows a typical total cell, live cell and round cell percentage in the incubation mixture including HM-1. The percentage of total cells and round cells was maintained at about 100% through the incubations. On the other hand, the live cell percentage was decreased to 40–50% of the total cells in the initial day, and maintained similar values through the incubations. Therefore, about 50–60% of round spheroplasts are fragile and the remaining 40–50% of round spheroplasts are stable during long-term incubation. A similar stability profile was obtained for spheroplasts in the control YPD medium, which contained 0.8 M sorbitol, although there were some aberrant cells.

Effects of HM-1 on HM-1-Resistant Yeast We next examined the effects of HM-1 on HM-1-resistant cells. We found that spheroplasts prepared from HM-1-resistant *S. cerevisiae* BJ1824*rhk1Δ::URA3* were unable to maintain a round shape in the presence of HM-1, the conditions under

which spheroplasts from HM-1-sensitive *S. cerevisiae* BJ1824 undergo a round shape enlargement (Fig. 5). The spheroplasts from HM-1-resistant yeast cells, although enlarged, tended to aggregate after they formed aberrant shapes, demonstrating that the unique effects of HM-1 on the morphology of spheroplasts correlates with sensitivity to HM-1. A common property shared by HM-1-sensitive and -resistant spheroplasts is cell enlargement with an increase in volume of cellular vacuoles.

Mode of HM-1 Action HM-1 binding to sensitive cells is reversible, and the bound HM-1 is easily washed out.⁶⁾ We attempted to determine whether the formation of round spheroplasts in response to HM-1 is reversible through the removal of HM-1 from the culture media. Most round spheroplasts formed by HM-1 change their morphology to an

Fig. 4. Percentage of Total Cells, Live Cells and Round Cells in the HM-1 Containing Incubation Mixture

Incubation conditions and the cell counting method are described in Materials and Methods. The incubation temperature was 30 °C for the first 6 h and 25 °C for the subsequent incubation. Total cell numbers at first counting (6-h incubation) was estimated as 100%. Live cell % and round cell % were calculated in reference to total cells and live cells, respectively. ◇, total cell %; ○, live cell %; ●, round cell %.

aberrant form, similarly to the spheroplasts incubated in YPD medium which contained 0.8 M sorbitol in response to a 3-h incubation at 30 °C after the centrifugal washing. Furthermore, HM-1 added again at 6 h resulted in the recovery of the round form of the spheroplasts (Fig. 6A to C). Probably, aberrant cells slipped out from the partially regenerated cell wall, then became round in form by the inhibition of cell

Fig. 5. Morphological Changes in Spheroplasts Prepared from HM-1-Sensitive and -Resistant *S. cerevisiae*

Spheroplasts were photographed using a phase-contrast microscope. Spheroplasts were incubated in media containing 2.11×10^{-7} M HM-1 for 6 h. A, spheroplasts prepared from HM-1-sensitive *S. cerevisiae* BJ1824; B, spheroplasts prepared from HM-1-resistant *S. cerevisiae* BJ1824rhk1Δ::URA3. Bar indicates 10 μm.

Fig. 6. Reversibility of Morphological Change Induced by HM-1

The incubation conditions for the spheroplasts are described in Materials and Methods, and spheroplasts were photographed using a phase-contrast microscope. A to C. Spheroplasts were incubated in media containing 2.11×10^{-7} M HM-1 at 0 h, and were incubated for 3 h; they were then centrifugally washed three times at 3000 rpm at 25 °C for 5 min to remove HM-1. The same medium was incubated without HM-1 for 3 h (total 6-h incubation). HM-1 2.11×10^{-7} M was then added again, and the medium was incubated for 3 h (total 9-h incubation). A, after a 3-h incubation; B, after a 6-h incubation; C, after a 9-h incubation. D and E. After a 6-h incubation of the medium including HM-1, the medium was centrifugally washed as described for A to C, and was then incubated in the same medium without HM-1 for 3 h (9-h incubation). D, after a 6-h incubation; E, after a 9-h incubation. Bar indicates 10 μm.

wall synthesis by HM-1. The requirement of a solid substance such as gelatin or agar for the regeneration of *S. cerevisiae* spheroplasts support this explanation.^{22,23}) A similar observation was made after an even longer 6-h pretreatment with HM-1 and subsequent washing; the round spheroplasts induced by HM-1 changed to the aberrant form in response to centrifugal washing and a 3-h incubation (Figs. 6D to E). These results indicate that the morphological change in spheroplasts between aberrant and round forms is reversible and is controlled by the addition or removal of HM-1.

Protein and DNA Synthesis As spheroplasts were enlarged by the incubations, the total protein and DNA content was determined at time 0 and at 10 h after incubation (Table 1). In both the control and HM-1-treated spheroplasts, the total protein and DNA content was increased by approximately three- and fourfold, respectively, by the 10-h incubations. This, as well as microscopic observations, indicated that the enlargement of both the control and HM-1-treated spheroplasts was in accordance with the macromolecule synthesis, although the spheroplasts did not divide.

Table 1. Protein and DNA Content in Control and HM-1-Treated Spheroplasts

Sample	Incubation time	
	0 time	10 h
Protein (μg)		
Control	213	650
Control+HM-1	225	674
DNA (μg)		
Control	0.90	3.63
Control+HM-1	0.90	3.96

The procedures for the determination of total protein and DNA are described in Materials and Methods. Incubated spheroplasts of 1.25×10^8 cells and 5.0×10^8 cells were used for the total protein and DNA determinations, respectively. Duplicate samples were analyzed, and the obtained values were averaged.

DNA Staining with DAPI We examined the chromosomes in the enlarged spheroplasts by staining the DNA with DAPI. Both the control and HM-1-treated spheroplasts contained several bright fluorescence spots indicative of a nucleus after a 15-h incubation, suggesting that multiple cycles of DNA replication occurred during the incubations (Fig. 7). These results indicate that DNA replication continues in spheroplasts, perhaps independently of the morphological changes observed in both the control and HM-1-treated cells.

Actin Staining with Rhodamine Phalloidin Gabriel *et al.*²⁴) have reported that actin, which maintains cellular architecture, is distributed evenly in spheroplasts and is locally concentrated during the regeneration of spheroplasts into competent cells. We therefore examined the profile of actin distribution in control and HM-1-treated spheroplasts (Fig. 8). No locally concentrated actin patches were found in either the control or enlarged spheroplasts exposed to HM-1, but strong actin patches were observed in some control spheroplasts having aberrant morphology.

Effects of Aculeacin A and Papulacandin B As antibiotics, aculeacin A and papulacandin B are β -1,3-glucan synthase inhibitors,^{5,15}) and we tested their effects on HM-1-sensitive and -resistant *S. cerevisiae* spheroplasts. As expected, aculeacin A and papulacandin B produced round, enlarged spheroplasts prepared from both yeast types at concentrations of $28.5 \mu\text{M}$ and $33.3 \mu\text{M}$, respectively. The concentrations of these antibiotics required for producing round, enlarged spheroplasts were $1.4 \mu\text{M}$ and $6.3 \mu\text{M}$ for aculeacin A and papulacandin B, respectively, which are parallel to the 50% doses of these compounds necessary to inhibit cell growth. Although similar in forming round, enlarged spheroplasts, the actions of these compounds were distinct from those of HM-1. First, their effects were non-specific to yeast cells, and secondly, the enlargement of spheroplasts could not be controlled by removing the compound from the medium. Finally, the enlarged spheroplasts made with ac-

Fig. 7. DAPI-Stained Spheroplasts Incubated in Control and HM-1-Included Media

The incubation conditions and DAPI staining of spheroplasts are described in Materials and Methods. Spheroplasts were stained after a 15-h incubation. A and B. Spheroplasts were incubated in the control medium. A, phase-contrast light microscopy; B, fluorescence microscopy. C and D. Spheroplasts were incubated in the medium containing HM-1. C, phase-contrast light microscopy; D, fluorescence microscopy. Bar indicates $10 \mu\text{m}$.

Fig. 8. Rhodamine Phalloidin-Stained Spheroplasts Incubated in Control and HM-1-Included Media

The incubation conditions and rhodamine phalloidin staining of spheroplasts are described in Materials and Methods. The incubation temperature was 30 °C for the first 3 h and 25 °C for the following 20 h. A and B. Spheroplasts were incubated in the control medium. A, phase-contrast light microscopy; B, fluorescence microscopy. C and D. Spheroplasts were incubated in a medium containing HM-1. C, phase-contrast light microscopy; D, fluorescence microscopy. Bar indicates 10 μ m.

uleacin A and papulacandin B appeared to be fragile, and they lysed during prolonged incubation.

DISCUSSION

Our results show that HM-1-sensitive yeast spheroplasts grow large in both control and HM-1-containing media in an osmotically stabilized nutrient-rich medium. However, the morphology of spheroplasts incubated in the presence and absence of HM-1 are different. Spheroplasts are perfectly round and some of these are sufficiently stable to survive for several days when incubated with HM-1, while they undergo morphological aberrations without HM-1. Thus, without cell walls, HM-1-sensitive yeast cells are no longer killed by HM-1, supporting our previous observations that HM-1-sensitive cells, such as those at a budding tip, die at the growth stage when a new cell wall is synthesized.^{6,13,14} Various morphological changes occur in yeast spheroplasts cultured in a nutrient-rich medium,²⁵ but we report here, we believe for the first time, that the morphology of spheroplasts can be manipulated by means of exposure to HM-1. Other killer toxins such as HYI killer toxin and K9 toxin^{4,5} have shown similar results in the formation of large, round spheroplasts, indicating a common property of this type of killer toxin (data not shown).

The cytotoxic action of HM-1 is reversible and is thought to have two stages.^{8–11} The formation of round, enlarged spheroplasts induced by HM-1 is reversible, depends on HM-1 concentrations, and occurs specifically with HM-1-sensitive yeast similar to the cytotoxic features of HM-1.⁶ These findings suggest that two different events are mediated through a common receptor localized in the spheroplast membrane. The exact nature of the receptor has not been clarified, but cellular β -1,3-glucan synthase, a large protein spanning the membrane sixteen times,^{26,27} could possibly be an HM-1 receptor. HM-1 perhaps interacts with β -1,3-glucan

synthase in the membrane of spheroplasts and continues to inhibit the synthesis of glucan, an important component of cell walls, resulting in perfectly round spheroplasts with smooth surfaces. By contrast, in the absence of HM-1, β -1,3-glucan synthesis continues in the membrane of spheroplasts as part of cell-wall regeneration, giving rise to rough surfaces that result in aberrant morphologies. The inner cellular architecture, which includes actin filaments, must be involved in these morphological changes. Actins regulate the morphology of cells through Rho1p, a small guanosine triphosphate-binding protein, that is also a regulatory subunit of β -1,3-glucan synthase.^{28–32} Accordingly, HM-1 may also indirectly inhibit the Rho1p function and actin polymerization through the interaction with β -1,3-glucan synthase, although this speculation remains to be substantiated by further experiments.

We found that HM-1 is a suitable reagent for preparing stable spheroplasts that have a smooth surface and a capability of synthesizing macromolecules. *In vitro* study with yeast *S. cerevisiae* cells has often been hampered by a hard cell wall that limits the import of small molecular weight compounds. We believe that spheroplasts made with HM-1 could eliminate some of these difficulties in *in vitro* studies with yeast, as HM-1 is apparently not incorporated into the spheroplasts and does not interfere with cellular macromolecular synthesis, except for in the cell wall. Furthermore, if necessary, HM-1 can be removed from spheroplasts by washing the cells. Thus, the method with HM-1 is useful in studies of the cell cycle and membrane transport.

The preparation of similar enlarged spheroplasts from *Escherichia coli* and *Bacillus megaterium* using penicillin, and from *S. cerevisiae* using 2-deoxy-glucose, has been previously reported, and some of the resulting spheroplasts have been used in studies of the measurement of membrane potential coupled with V-type ATPase using the patch-clamp technique.^{33–36} Lipophilic antibiotics, aculeacin A and papula-

candin B, that inhibit cell-wall β -1,3-glucan synthesis also yield large spheroplasts when added to the spheroplasts from both HM-1-sensitive and -resistant yeast cells. But the spheroplasts formed with these antibiotics contain lipophilic compounds that cohere to the membrane and are unremovable, which, combined with the increased fragility of the membrane, may cause the spheroplasts made with aculeacin A and papulacandin B to lyse during incubation much earlier than the spheroplasts made with HM-1.

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