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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 4 (3): 259-262, 2001
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Nystatin Sensitive and Vacuolar Protein Sorting Defective (*vps*) Mutants of *Saccharomyces cerevisiae*: Their Isolation and Characterization

M. Shah Alam Bhuiyan*, Yuji Ito, Naotaka Tanaka, Golam Sadik[1], Kiyotaka Fujita, Atsuko Nakamura, M. Helal U. Biswas[1], Hiroshi Fukui and Kaoru Takegawa.
Department of Life Sciences, Faculty of Agriculture,
Kagawa University, Kagawa 761 0795, Japan
*Present address and author for correspondence: Department of Pharmacy,
University of Rajshahi, Rajshahi-6205, Bangladesh.,
[1]Department of Pharmacy, University of Rajshahi, Rajshahi 6205, Bangladesh

Abstract: In order to obtain more insights into the molecular mechanisms of action of polyene antibiotics in yeast cells, a number of nystatin sensitive mutants of *Saccharomyces cerevisiae* were isolated by ethylmethane sulfonate mutagenesis. The mutants were characterized in terms of their sensitivity test to stresses such as high temperature or high concentration of monovalent and divalent cations. The invertase overlay assay method suggests that some of these mutants are defective for vacuolar protein sorting. By tetrad analysis four of these mutants were found to possesses single gene mutation.

Key words: *Saccharomyces cerevisiae*, mutation, nystatin sensitivity, vacuolar protein sorting

Introduction

In yeast, studies on mode of action and target of antifungal drugs, as well as the mechanism of resistance received impetus, not only because of the involvement of some yeast species in pathogenicity, but also because of uniform cell type and advantages of ease of handling associated with microbes, combined with number of novel strategies for characterization and manipulation of genes for the study of higher eucaryotic cells.

Although many antifungal drugs have been developed, nystatin and amphotericin B are among the mostly used polyene antibiotics for the treatment of fungal infections (McGinnis and Rinaldi, 1986). Several research groups have suggested that the polyene antibiotic nystatin may act by interaction with membrane sterols and cause damage to the cell membranes (Kinsky *et al*., 1966; Robin, 1971) resulting in an alteration of selective permeability (Eilam and Grossowicz, 1982; Palacios and Serrano, 1978; Lampen, 1969; Gottlieb *et al*., 1958; Lampen and Arnow, 1959; Lampen *et al*., 1959). Bolard (1986) and Lees *et al*. (1995) reported that the selectivity may be related to its greater affinity for the ergosterol of yeast membranes than cholesterol of mammalian membranes. However a study of the relationship between cellular factors for cell growth as well as their structures and level of antibiotic activity facilitated the isolation and genetical analysis of nystatin resistant mutants of *Saccharomyces cerevisiae* (Fryberg *et al*., 1974; Beezer *et al*., 1986; Levchenko *et al*., 1979; Nakanishi *et al*., 1987). But in contrast considerably less attention has been paid to nystatin sensitive mutants for understanding the mode of action of nystatin.

In the present paper, we report the isolation and characterization of nystatin sensitive mutants as a preliminary step to obtain new insights into the molecular mechanisms of action of polyene antibiotics in yeast cells.

Materials and Methods

Strains and media: The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Standard rich medium (yeast extract-peptone-dextrose, YPD) and SD minimal medium (Yeast nitrogen base, YNB) with amino acid supplements as needed were used as described (Sherman *et al*., 1979). Yeast extract fructose (YPF) medium was used for the invertase overlay assay.

Materials: Nystatin was purchased from Sigma Chemical Co. (St. Louis, Mo). All other reagents used in this study were of analytical grade and obtained from Wako Pure Chemical (Osaka, Japan).

Isolation of nystatin sensitive mutants: BHY 10 and BHY 11 wild type cells were mutagenized with ethylmethane sulfonate as described previously (Bhuiyan, 1999). Cells were grown individually in YPD medium at 27°C to an optical density at 600 nm of 1.0, centrifuged, washed, and suspended in phosphate buffer (pH 7.0). The cells were then exposed with 2% (w/v) ethylmethane sulfonate (EMS) for 1 h at 30°C for mutation and the mutagenized cells were centrifuged, washed with 5% sodium thiosulfate and suspended in YPD medium. The cells were spread on YPD plates, incubated at 30°C for 3-5 days and then the colonies were replica plated onto YPD plates containing 5 μg/ml of nystatin.

Invertase Overlay Assay: The invertase plate assay was done as described (Paravicini *et al*., 1992). Cells were grown at 30°C in YPD solid medium and then replica plated onto YP-fructose plates. After about 1-2 days of incubation invertase assay mixture (to overlay each plate, the mixture containing 6.0 ml of sterile water, 0.64 g of sucrose, 1.5 ml of 1 M sodium-acetate buffer, p^H 5.0, 0.3 ml of 20 mM N-ethyl maleimide, 0.15 ml of 1 mg/ml horseradish peroxidase, 0.12 ml of glucose oxidase solution, 0.9 ml of 10 mg/ml O-dianisidine, and 0.3 g of agar suspended in 0.6 ml of sterile water) was poured in thin layers onto YP- fructose plates. Then colonies were observed for the red colour change that indicated secreted invertase activity.

Tetrad analysis: The nystatin sensitive mutants were crossed with the parent strains (BHY 10 and BHY 11). Tetrad analysis

Table 1: *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source or Reference
SEY6210	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-r9*	Robinson *et al.*, 1988
SEY6211	*MATa leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101 suc2-r9*	Robinson *et al.*, 1988
BHY 10	SEY6210*; leu2-3, 112::*pBHY11(cpy-Inv *LEU2)*	Horazdovsky *et al.*, 1994
BHY 11	SEY6211*; leu2-3,112::*pBHY11(CPY-Inv *LEU2)*	Horazdovsky *et al.*, 1994

Table 2: Characterization of nystatin sensitive mutants (YIα)

Strain no.	37° C	3µg/ml clotrimazole	1.5M NaCl	1.0M KCl	0.5M $CaCl_2$	0.7M $MgCl_2$	7mM $ZnCl_2$	*vps*
Wild type	+++	+++	+++	+++	+++	+++	+++	-
1	-	+	-	++	-	-	+++	-
2	-	+	-	++	-	+	+++	-
3	+++	-	+++	+++	+++	+++	+++	-
4	-	-	-	-	-	-	+++	+
5	+++	-	++	+++	+++	+++	+++	+
6	-	+++	-	+++	-	++	-	-
7	-	++	-	++	-	++	++	-
8	++	+++	-	++	-	+++	+++	-
9	-	-	-	++	+	-	+++	+
10	+++	-	-	+++	-	-	++	-
11	+++	-	++	+++	+++	+++	+++	-
12	+	-	-	+++	+++	+++	+++	-
13	-	-	-	++	-	++	+++	+
14	-	++	-	++	-	++	+++	-
15	-	-	-	-	-	-	+	-
16	-	-	-	+++	+	++	+++	-
17	-	+	-	+++	-	++	+++	-
18	-	+	+++	+++	++	++	++	-
19	-	-	-	-	+	-	+++	-
20	-	-	-	+++	-	++	++	-
21	+	+++	-	+++	++	++	+++	-
22	+++	-	+	+++	+++	+++	+++	-
23	+++	-	+	++	++	+++	+++	-
24	-	+	-	+++	+++	+++	+++	-
25	-	-	-	+++	-	++	++	+
26	-	+++	-	++	+	-	+++	+
27	-	+	-	+++	-	++	+++	+
28	-	+	-	++	-	+	+++	-
29	+	-	-	-	-	-	++	-
30	-	-	-	+	-	-	+++	-
31	++	-	-	-	++	-	+++	-
32	++	-	++	++	-	+	-	-
33	+	+++	+	+++	++	+++	+++	-
34	-	+	++	+++	+++	+++	+++	+
35	+	+	-	+	-	++	+++	-
36	+	+	-	+++	+++	++	+++	+
37	++	-	++	+++	+++	+++	++	-
38	-	-	+++	+++	+++	+++	+	-
39	-	-	++	+++	-	+++	-	+
40	-	-	-	++	-	+	-	-
41	+++	-	-	+	-	-	+++	-
42	-	-	-	++	-	+	-	+
43	+	-	-	+++	-	++	-	+
44	-	+	-	-	-	-	-	+
45	-	+++	-	+	+	+	+++	-
46	-	-	-	-	-	-	+++	-
47	++	+++	++	+	++	-	+++	-
48	-	++	+++	+++	++	+++	+++	+
49	++	-	-	+	-	-	-	-
50	+++	+++	-	+++	+	+++	++	+
51	-	-	-	+	-	-	-	+
52	-	-	-	-	-	-	+++	-
53	-	-	-	-	-	-	-	+
54	-	-	-	-	-	-	-	-
55	-	-	-	+++	-	++	++	-
56	-	-	-	-	-	-	++	+
57	-	+	-	-	-	-	++	+
58	+	+	-	-	-	-	++	+
59	-	-	-	++	-	-	+	+
60	-	+	+	++	-	++	-	+
61	+	-	-	++	-	++	++	+
62	-	-	+++	+++	-	+++	-	+
63	-	-	-	++	-	-	-	+
64	-	-	-	-	-	-	+++	-
65	-	-	+++	+++	+	+++	+++	-
66	-	-	-	+++	-	+++	+++	-
67	++	-	-	+++	-	++	++	-

Table 2: Continued

Strain no.	37° C	3μg/ml clotrimazole	1.5M NaCl	1.0M KCl	0.5M $CaCl_2$	0.7M $MgCl_2$	7mM $ZnCl_2$	*vps*
68	-	++	-	+++	-	+++	+	-
69	-	++	-	++	-	+++	++	-
70	-	-	-	-	-	-	+++	+
71	+	+	-	+++	+++	+++	+++	-
72	+	+++	-	-	+++	-	+++	-
73	+++	-	+++	+++	-	+++	-	-
74	-	-	-	-	-	-	+	+
75	-	++	+++	+++	+	+++	+	+
76	-	+++	-	+	-	+	+++	+
77	-	-	-	-	-	-	-	+
78	-	-	-	++	+	-	+	+
79	-	-	-	++	-	+++	-	+

"-", No growth; "+", Mild growth; "++" "Moderate growth", "+++" "Strong growth"
vps= ["+", Vacuolar protein sorting defective; "-", Not vacuolar protein sorting defective]

Table 3: Characterization of nystatin sensitive mutants (YIa)

Strain no.	37° C	3μg/ml clotrimazole	1.5M NaCl	1.0M KCl	0.5M $CaCl_2$	0.7M $MgCl_2$	7mM $ZnCl_2$	*vps*
wild type	+++	+++	+++	+++	+++	+++	+++	-
1	-	-	-	+	-	+	+++	+
2	-	+++	-	+++	++	+++	+++	+
3	+++	+++	-	+++	+	++	+++	-
4	+	-	+++	+++	+++	+++	+++	-
5	-	-	+	+++	++	+++	++	+
6	++	+	+++	+++	+	++	++	+
7	-	+	-	+	-	-	++	+
8	-	+++	-	-	+	-	+	+
9	-	+++	++	+++	+	+++	+++	-
10	+++	+++	+++	+++	+++	+++	+++	-
11	-	+++	-	++	+++	+++	+	-
12	-	+++	++	+++	-	++	+	+
13	-	+++	++	++	+	-	+++	-
14	-	++	++	+++	+++	++	++	-
15	+++	++	++	+	+	++	+++	-
16	+	++	++	++	+	+	+++	-
17	+++	+	-	++	+++	+++	+++	-
18	-	+++	-	-	+	-	+	+
19	-	-	+	++	+++	++	+++	-
20	-	-	-	+++	+++	-	+++	-
21	++	-	+++	+++	+++	+++	+++	-
22	-	-	++	+++	+++	+	+++	-
23	++	-	+++	+++	++	+++	+++	-
24	-	-	++	++	++	++	++	-
25	-	-	-	++	-	-	++	+
26	-	-	-	-	++	+	+++	-
27	-	-	-	+++	+++	-	+++	+
28	-	+	+++	+++	+++	+++	+++	-
29	-	-	+++	-	+++	-	+++	-
30	++	-	-	-	++	-	++	+
31	+++	+	-	++	+	-	+++	+
32	-	-	+++	+++	++	++	+++	-
33	-	++	+++	+++	+++	+++	+++	+
34	-	-	++	+++	+++	+	++	-
35	++	+++	+	+++	+++	++	+++	-
36	++	+++	-	++	+++	-	+++	+
37	-	+++	-	++	-	++	+++	-
38	+++	-	+++	+++	+++	-	-	+
39	+	+++	-	++	-	++	+++	-
40	-	++	++	++	-	+++	+++	+
41	+++	+	-	++	++	++	+++	-
42	-	-	-	-	-	-	++	-
43	-	-	-	++	-	-	++	+
44	-	++	-	+	+	+	++	+

"-", No growth; "+", Mild growth; "++" "Moderate growth", "+++" "Strong growth"
vps= ["+", Vacuolar protein sorting defective; "-", Not vacuolar protein sorting defective]

following sporulation of the heterologous diploids were examined for the nystatin sensitive and vacuolar protein sorting defective growth phenotypes. Ten or more tetrads were examined for each cross.

Results and Discussion

Screening for nystatin sensitive mutants: Screening of approximately 20,000 EMS mutagenized yeast colonies by replica plating on YPD plates containing 5 μg/ml of nystatin yielded 123 nystatin-sensitive mutants (79 mutants from BHY 10 and 44 mutants from BHY 11)

Characterization: The mutants were streaked on YPD plates and incubated at 37° C to check temperature sensitivity. To test whether the mutants were sensitive to high concentration of monovalent and divalent cations, they were streaked on YPD plates containing any of the different salts of NaCl, KCl, $CaCl_2$, $MgCl_2$, and $ZnCl_2$ and checked growth after incubation

at 30° C for 48 hours. Growth was also observed in presence of clotrimazole, to check whether the mutants were sensitive or not to any other antifungal drug. Results are shown in Table 2 and Table 3.

Identification of vacuolar protein sorting defective (*vps*) mutants: The fungal vacuole, analogous to the mammalian lysosome contain a variety of hydrolytic enzymes. Stack *et al.* (1995) reported that the delivery of Yeast vacuolar hydrolases contain their sorting signal in their amino acid sequence and alteration of the sorting signal in wild type CPY or CPY-invertase fusion proteins results in their appearance at the cell surface. The CPY-invertase hybrid proteins include 50 amino acids of the N-terminal signal sequence at the vacuolar hydrolase CPY that is responsible for correct sorting and transport to the vacuole (Bankitis *et al.*, 1986). A large number of vacuolar protein sorting defective (*vps*) mutants were isolated as a collection of mutants that missort and secrete such CPY-invertase fusion proteins to the cell surface (Rothman and Stevens, 1986; Robinson *et al.*,1988; Bankitis *et al.*, 1989; and Rothman *et al.*, 1989). To find out whether the protein sorting defectiveness is correlated with nystatin sensitivity, we are however interested to check the secretion of CPY-invertase fusion protein of nystatin sensitive mutants by invertase plate assay as described in Materials and Methods. The BHY 10 and BHY 11 strains are constructed by integrating the CPY-invertase fusion gene at the chromosomal *leu 2-3, 112* locus (Horazdovsky *et al.*, 1994). In wild type cells the targeting information contained in the CPY portion of the fusion protein leads to efficient sorting of fusion protein into the vacuole. In contrast some nystatin sensitive mutants missorted strong invertase activity derived from CPY-invertase fusion protein, which suggested that these mutants showed *vps* phenotypes.

Then we were interested to check whether the vacuolar protein sorting defectiveness and nystatin sensitivity were due to identical single recessive mutation. By tetrad analysis five mutants of BHY 10 (YIα-9, 42 , 51, 60 and 77) were found to be single recessive mutation on the chromosome as they showed a $2^+: 2^-$ segregation (data not shown). We previously reported that vacuole deficient (class C) *vps* mutants were sensitive to nystatin and YIα-51 was allelic to *vps 16* (Bhuiyan *et al.*, 1999). The vacuole deficient mutants do not accumulate nystatin in the vacuole, but accumulate through out the cytoplasm and perhaps this is the possibility for their nystatin sensitivity. However other nystatin sensitive mutants are awaited further study.

Acknowledgement

This work was supported by grants from Ministry of Education Science and Culture of Japan (MONBUSHO) that were awarded to the first author.

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