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Vanadate-resistant yeast mutants are defective in protein glycosylation

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ABSTRACT **Spontaneous recessive orthovanadate-resistant mutants of *Saccharomyces cerevisiae* were obtained in five complementation groups, and all show defects in protein glycosylation that mimic the previously isolated *mnn* mutants. Three of the groups are allelic to the known *mnn8*, *mnn9*, and *mnn10* mutants, whereas the other two groups show other glycosylation defects. The vanadate-resistant phenotype was associated with enhanced hygromycin B sensitivity. The glycosylation phenotypes of the mutants are all reflections of defects in glycoprotein trafficking, and the easy isolation of vanadate-resistant or hygromycin B-sensitive mutants should facilitate the study of this process.**

Orthovanadate-resistant mutants of *Saccharomyces cerevisiae* have been studied extensively by Willsky and coworkers (1–3), who identified at least five complementation groups with this phenotype. Considerable effort has been expended to define the site(s) of vanadate action in the sensitive cell and, thereby, provide a clue to the possible mechanism(s) by which vanadate resistance could arise (2, 4). Although a rather high concentration of vanadate (5 mM) is required to inhibit yeast growth, it is reported that the intracellular concentration is only about 0.1 mM (3). Since vanadate enters the cell by an active process (5), one mechanism for resistance could be a change in the transport system(s) that reduces uptake or enhances excretion. Alternatively, resistant cells could develop more effective detoxification mechanisms (2) or target site(s) could in some way become desensitized. Vanadate is known to inhibit enzymes of phosphate metabolism (2, 4), thereby affecting plasma membrane ion pumps and cytoplasmic motility (5), and it is expected that vanadate could affect processing, sorting, and secretion of macromolecules.

It was recently found (R.A.H., C. Y. Chen, N. J. Simpson, and V. Chisholm, unpublished data) that the *S. cerevisiae* *mnn9* mutant, which is defective in protein glycosylation (6), is remarkably resistant to orthovanadate and sensitive to hygromycin B, compared to the parent strain X2180. We have now analyzed a number of vanadate-resistant mutants to determine whether the glycosylation defect is a general property of this class. Of the five complementation groups we identified, all show defects in protein glycosylation and three of the groups are allelic with the previously characterized *mnn8*, *mnn9*, and *mnn10* mutants (6). This result suggests that the primary sites of vanadate inhibition impinge directly on the processing of glycoproteins in the Golgi.

MATERIALS AND METHODS

Sodium orthovanadate, Na_3VO_4, was from Aldrich. Vanadate-containing plates were made by adding a freshly prepared filter-sterilized sodium orthovanadate solution to a melted sterilized agar medium, which was then poured into sterile Petri plates. Antisera that recognized the wild-type terminal $\alpha 1 \rightarrow$ 3-linked mannose determinant and the unsubstituted $\alpha 1 \rightarrow$ 6-linked mannose determinant of the *mnn2* mutant outer chain came from laboratory stocks (7). The cell surface phenotype was assessed from visible agglutination of the cells in a microtiter plate by an appropriately diluted serum. Alcian blue dye binding was assessed as described elsewhere (8).

Vanadate-resistant mutants that arose spontaneously were selected by spreading a yeast culture on YEPD plates [1% yeast extract/2% (wt/vol) peptone/2% (wt/vol) glucose] containing 2% (wt/vol) agar and 3–10 mM sodium orthovanadate. The parent yeast strains were haploid *S. cerevisiae* X2180 (wild type) and the *mnn1* and *mnn2* mutants (8), and the plates were incubated at 30°C for several days. Colonies that grew out of the background of quiescent cells were picked and plated on YEPD. After growth at 30°C, each was again tested for vanadate-resistance and sorted into groups depending both on the level of vanadate-resistance and on the size of the colony that grew on YEPD plates from single cells, since several *mnn* mutants have the common phenotype of growing slowly (6). To assess the degree of resistance to vanadate or hygromycin B, cells were first grown as patches on YEPD/agar plates and then were replica-plated by velveteen transfer to agar plates containing various levels of the inhibitors. Plates were incubated at 30°C for 2–4 days, during which resistant cells grew as confluent patches whereas less-resistant cells failed to grow after 48 hr at 30°C but often produced papillae after 3–4 days, probably arising by spontaneous mutation. The mutants were backcrossed to wild type to determine the segregation patterns and to eliminate other defects. After assigning the isolates to complementation groups on the basis of the vanadate-resistance and invertase glycosylation patterns of hybrid diploids, each group was tested for allelism with the *mnn8*, *mnn9*, and *mnn10* mutants. We have adopted the designation *vrg* mutant, for vanadate-resistant glycosylation mutant. The *vrg5* and *vrg8* mutants were obtained from the X2180 strain, *vrg1* was from the *mnn1* strain, and *vrg2*, *vrg3*, *vrg4*, *vrg6*, and *vrg7* were from the *mnn2* strain. Although the *mnn2* strain is more sensitive to vanadate than X2180 or *mnn1*, some of the same *vrg* mutant alleles were obtained regardless of the starting strain.

Changes in protein glycosylation were assessed from the native gel electrophoresis patterns of invertase, which was detected with an activity stain on 5% gels (8). The structures of N-linked oligosaccharides released by digestion of the purified mannoprotein with endo-β-*N*-acetylglucosaminidase H were determined by ^1H NMR spectroscopy (9) and by anion-exchange HPLC (10).

RESULTS AND DISCUSSION

From 14 recessive vanadate-resistant isolates, five complementation groups were identified by assessing the growth of

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the various hybrids on vanadate-containing YEPD/agar plates. Three of the groups were allelic to *S. cerevisiae mnn8, mnn9,* and *mnn10* strains whereas two groups represented other loci (Table 1). Five isolates exhibited the *mnn9* genotype, which probably reflects the fact that this mutant is the most resistant strain we have observed and it gives the smallest colonies on YEPD plates. That five complementation groups were obtained from only 14 isolates may be attributed to the use of several vanadate levels in the selection procedure and to the secondary classification based on colony morphology. Willsky *et al.* (2) reported the selection of five complementation groups from a study of 297 vanadate-resistant isolates produced by ethyl methanesulfonate mutagenesis, whereas Kanik-Ennulat and Neff (4) identified six recessive alleles among 104 spontaneous mutants. This correspondence in numbers suggests that we have probably obtained mutants representing the same loci isolated by these investigators. Although three of the *mnn* mutants are unusually vanadate-resistant, those designated *mnn1* through *mnn6* are not, whereas *mnn2* and *mnn3* are less resistant than the wild type (Table 1).

All of the mutants we obtained showed changes in external invertase gel electrophoretic mobility, an indication that all expressed glycosylation defects (Fig. 1). The invertase gels reveal three glycosylation classes, the fastest migrating *mnn9*-like strains that have oligosaccharides with 10–14 mannoses, the *mnn8–mnn10* class that has larger oligosaccharides and migrates at intermediate rates, and the wild type that has still larger oligosaccharides and migrates as a slow diffuse band (8). The procedure does not distinguish between *mnn8* and *mnn10* invertases, which differ only slightly in size (11). Complementation between nonallelic mutants was also demonstrated by the shift in invertase migration in the heterozygous diploid, as illustrated in Fig. 2 for *vrg2* (allelic to *mnn10*) and *vrg3* (allelic to *mnn8*). Insertion of the *MNN9* gene (R.A.H., C. Y. Chen, N. J. Simpson, and V. Chisholm, unpublished data) into the *vrg6* strain also led to formation of wild-type invertase (data not shown).

The invertase mobilities of the three complementation groups with the *mnn8, mnn9,* and *mnn10* genotypes were consistent with their assignment as alleles of these three *mnn* mutants, and the structures of the N-linked oligosaccharides determined by NMR confirmed these assignments (Table 2).

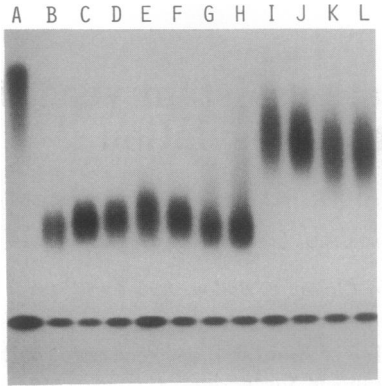

FIG. 1. Invertase native gel electrophoretic patterns. Lanes: A, X2180 wild type; B, *mnn9*; C, *vrg6*; D, *vrg7*; E, *vrg1*; F, *vrg8*; G, *vrg5*; H, *vrg4*; I, *mnn10*; J, *vrg2*; K, *mnn8*; L, *vrg3*. Nonglycosylated internal invertase migrates near the bottom of the gel whereas highly glycosylated wild-type invertase migrates near the top of the gel. The bands are visualized with an activity stain (8).

For this analysis, strains containing the *mnn1* mutation were used to simplify the interpretation by removing terminal $\alpha 1 \rightarrow$ 3-linked mannoses (8). The *mnn1 vrg6* mannoprotein yielded a small oligosaccharide by endo-β-*N*-acetylglucosaminidase H digestion with a spectrum that was identical to that of the $Man_{10}GlcNAc$ from the allelic *mnn9* strain (9). Although the *vrg1* and *mnn9* invertases were indistinguishable (Fig. 1), these two strains are nonallelic and the small oligosaccharide fraction from the *mnn1 vrg1* strain could be separated into three components with the composition $Man_{10-12}GlcNAc$. The spectra were consistent with the structures B and C in Table 2, which indicate that this mutant has a defect in outer-chain elongation that acts at a processing stage between those defined by the *mnn9* and *mnn10* mutations; that is, the outer chain is extended more than in the *mnn9* but less than in the *mnn10* strain.

Finally, the changes in protein glycosylation were also reflected in changes in cell surface carbohydrate antigenic determinants and dye binding properties (Table 3). Some strains lost most of the wild-type $\alpha 1 \rightarrow$ 3-mannosyl reactivity, whereas others showed an enhanced reactivity with this

Table 1. Growth phenotype on YEPD/agar medium containing vanadate or hygromycin

Strain	Complementation group	Orthovanadate resistance							Hygromycin B resistance			
		3	4	5	6	7	10	15	10	50	100	200
X2180 *MAT***a**	—	+	+	±	−	−	−	−	+	+	+	−
*mnn1 MAT***a**	—	+	+	+	±	−	−	−	+	+	+	−
*mnn2 MAT***a**	—	+	−	−	−	−	−	−	+	+	±	−
*mnn3 MAT***a**	—	+	−	−	−	−	−	−	+	+	+	−
*mnn4 MAT***a**	—	+	+	−	−	−	−	−	+	+	+	−
*mnn5 MAT***a**	—	+	+	−	−	−	−	−	+	+	+	−
*mnn6 MAT***a**	—	+	+	−	−	−	−	−	+	+	+	−
*mnn8 MAT***a**	I	+	+	+	+	±	−	−	+	±	−	−
vrg3 MATα	I	+	+	+	+	+	−	−	+	−*	−	−
*mnn9 MAT***a**	II	+	+	+	+	+	+†	±	±	−	−	−
vrg6 MATα	II	+	+	+	+	+	+†	−	−	−	−	−
*mnn10 MAT***a**	III	+	+	+	+	±	−	−	+	±	−	−
vrg2 MATα	III	+	+	+	+	±	−	−	+	±	−*	−
vrg1 MATα	IV	+	+	+	+	+	+†	−	+	±	−*	−
vrg7 MATα	IV	+	+	+	+	+	±‡	−	+	±	−*	−
*vrg4 MAT***a**	V	+	+	+	+	+	−	−	+	−*	−	−
vrg5 MATα	V	+	+	+	+	+	−	−	+	±	−	−

Growth of the cell patches was scored as strong (+), weak (±), or none (−). Strains are less resistant to vanadate (mM, as indicated) and hygromycin (μg/ml, as indicated) on minimal medium or if they have auxotrophic requirements.

*Revertants to growth occur with a high frequency.

†Colony has a dark green color.

‡Colony has a yellow color.

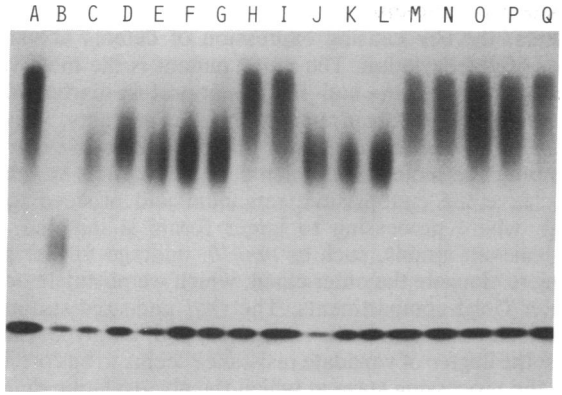

FIG. 2. Complementation between *vrg* and *mnn* mutants based on invertase mobility. Lanes: A and Q, X2180 wild type; B, *mnn9;* C, *mnn8;* D, *mnn10;* E, *vrg2;* F and G, *vrg2* × *mnn10* diploids; H and I, *vrg2* × *mnn8* diploids; J, *vrg3;* K and L, *vrg3* × *mnn8* diploids; M and N, *vrg3* × *mnn10* diploids; O and P, *vrg2* × *vrg3* diploids. Complementation is illustrated between *vrg2* and *mnn8*, *vrg3* and *mnn10*, and *vrg2* and *vrg3*, but not between *vrg2* and *mnn10* or *vrg3* and *mnn8*. Similar analysis was applied to all *vrg* mutants.

Table 3. Cell surface phenotype and morphology of yeast strains

Strain	Antiserum α1 → 3	Antiserum α1 → 6	Alcian blue dye binding	Morphology
X2180 *MAT***a**	+	−	4+	NC
*mnn2 MAT***a**	±	++	2+	NC
*mnn4 MAT***a**	+	−	0	NC
*mnn9 MAT***a**	±	−	2+	C
*vrg6 MAT*α	±	−	2+	C
*mnn8 MAT***a**	±	−	3+	SC
*vrg3 MAT*α	±	−	3+	SC
*mnn10 MAT***a**	±	−	3+	SC
*vrg2 MAT*α	±	−	3+	SC
*vrg4 MAT*α	±	±	1+	C
*vrg5 MAT*α	±	±	1+	C
*vrg1 MAT*α	++	−	4+	SC
*vrg7 MAT*α	++	−	4+	SC
*vrg8 MAT*α	++	−	4+	SC

Agglutination of whole cells by α1 → 3- or α1 → 6-specific antiserum varied from very strong (++) to none (−). The binding of dye varied from very strong (4+) to none (0). The cells grew with a clumpy (C), slightly clumpy (SC), or nonclumpy (NC) morphology.

serum. Two of the mutants gave a significant reaction with α1 → 6-specific serum, which is typical of the *mnn2* strain and indicates exposure of some of the α1 → 6-linked outer chain backbone. In all instances, the changes in carbohydrate structure inferred from the serum agglutination assays agreed with the changes in alcian blue dye binding activity. Thus, loss of branching in the outer chain that would reduce reaction with the α1 → 3-specific serum and enhance reaction with the α1 → 6-specific serum, as in the *vrg4* class, would also reduce the available sites for phosphorylation and thereby the dye binding activity (8). Conversely, strains such as *vrg1* that show a strong α1 → 3-specific reaction, indicative of outer chain branching, are also strong dye binders. Al-

Table 2. Anomeric proton chemical shifts and structures of oligosaccharides

Strain	Structure	H-1 chemical shift (δ) D	C	B	A_α	A_β
mnn1 *mnn9* or *mnn1* *vrg6*	(A) αM(→2)αM(→6)αM(→6)βM(→4)αβGNAc	5.035	5.085, 5.110			
		5.125	4.870	4.771	5.236	4.710
	αM(→6)αM (αM(→2), αM(→2))		5.125	5.325		
	αM(→2), αM(→2), αM(→2)		5.054	5.288		
	αM(→2)			5.046		
mnn1 *vrg1*	(B) αM(→2)αM(→6)αM(→6)βM(→4)αβGNAc	5.038	5.088, 5.110			
		5.122	4.871	4.772	5.237	4.715
	αM(→6)αM(→6)αM (αM(→2), αM(→2))	4.910	4.910	5.335		
	αM(→2)			5.288		
	αM(→2)			5.046		
	(C) αM(→2)αM*(→6)αM(→6)βM(→4)αβGNAc†	5.042	5.085, 5.110			
		5.124	4.868	4.770	5.234	4.710
	αM(→6)αM(→6)αM (αM(→2), αM(→2))	5.124	4.906	5.325		
	αM, αM(→2)	5.045		5.286		
	αM(→2)			5.042		

Residues A–D are indicated. M, Man; GNAc, GlcNAc.

*About half of the oligosaccharide fraction had an extra α1 → 2-mannose attached to this mannose, in which instance the signals at δ5.085/5.110 are shifted downfield to δ5.376.

†A small amount of the homolog with an additional α1 → 6-mannose in the outer chain was also present, which increased the signal at δ4.906.

though Kanik-Ennulat and Neff (4) have noted that vanadate-resistant mutants may show enhanced protein phosphorylation, the dye binding property of intact cells reflects phosphorylation of the mannoprotein carbohydrate chains, not of serine or threonine in the protein chains.

It is of interest that the oligosaccharides from the *vrg1* mannoprotein lack internal $\alpha 1 \rightarrow$ 2-branching of the short outer chain part, although some chains are terminated by $\alpha 1 \rightarrow$ 2-linked mannose (Table 2, oligosaccharide C), the so-called "stop signal" (11). Because this strain does not carry the *mnn2* mutation, the lack of outer chain branching is unexpected. Therefore, the result suggests that the *vrg1* invertase may bypass the compartment in which both elongation and branching normally occur or that branching normally occurs only after the outer chain is further elongated. The oligosaccharides of this strain, like those of the *mnn9* strain, also retain much of the $\alpha 1 \rightarrow$ 2-linked mannose that is normally removed in the endoplasmic reticulum by mannosidase I, which could mean that the *vrg1* mutation also affects endoplasmic reticulum function.

Our previous studies (6, 8, 9) on the *mnn9* strain have shown that both the invertase and the cell wall mannoproteins are altered in a similar fashion. In contrast, whereas the *vrg1* invertase has a *mnn9*-like phenotype, the cell-wall mannoprotein of this *vrg1* strain has the wild-type phenotype in that the cells react strongly with $\alpha 1 \rightarrow$ 3-specific serum and with alcian blue dye (Table 3). The results suggests that the processing of the periplasmic enzyme invertase is affected by the mutation, whereas a large part of the cell-wall mannoprotein is unaffected or is altered in a different manner. Similarly, the *vrg4* invertase migrates on gel electrophoresis like a *mnn9* invertase, but the cell surface has some of the antigenic and dye binding proteins of a *mnn2* strain, again suggesting that the invertase and the cell wall mannoproteins are affected differently by the *vrg4* mutation. These anomalies are consistent with the view that the different proteins are processed through different compartments and that the effect of the mutation is dependent on the compartment in which the mutation is expressed.

Willsky *et al.* (2) found that vanadate resistance was not associated with a change in the ability of the mutant cells to transport phosphate or vanadate, but they concluded that the data supported a model in which orthovanadate was converted to toxic forms by a multistep process and that a defect in any step could confer resistance. Such a resistance mechanism could lead to the changes we observe in protein glycosylation if it involved a general change in the electrogenic potential of the cell that also affected Golgi function. That vanadate resistance should be accompanied by an increased sensitivity to hygromycin B, an antibiotic that inhibits protein synthesis in yeast at the concentrations we have used (12), is suggestive. Perhaps relevant are the observations that mutants selected for resistance to hygromycin B show defects in the plasma membrane ATPase (13), and the phenotype is "closely correlated with a depolarization of cellular membrane potential" (14), whereas vanadate-resistant mutants have normal or increased levels of the same enzyme activity (4).

Our results demonstrate that vanadate resistance can result from mutations in several different genes that lead to different, although related, glycosylation phenotypes. Because all of the mutants we obtained show glycosylation defects, it is possible that a single protein that is the target of vanadate sensitivity can be altered to a resistant state by any significant change in its glycosylation resulting from any of several defects. Such a protein could be one involved in vanadate uptake, secretion, or detoxification. Alternatively, it is possible that a multisubunit complex, which is universally involved in protein sorting in the Golgi, is the vanadate target and that the complex can become vanadate-insensitive by a mutation in any of the subunits, thereby causing expression of defects at various stages of glycosylation. The *mnn9* mutant is the most vanadate-resistant isolate and its mannoprotein carbohydrate chains are the most truncated of the *mnn* mutants. We conclude that the defect leading to this phenotype is expressed soon after the molecules, which carry $Man_8GlcNAc_2$ chains (15), leave the endoplasmic reticulum and proceed to the Golgi, where processing to larger forms is initiated. The less-resistant strains, such as *mnn10*, undergo further processing to elongate the outer chain, which we postulate occurs in later Golgi compartments. The *vrg1* and *vrg4* strains are intermediate in vanadate resistance and in glycosylation. Thus, the degree of vanadate resistance seems to be correlated with the processing stage at which the glycosylation defect is expressed. Unexplained, however, is the fact that the glycosylation-defective *mnn2* and *mnn3* mutants are more sensitive to vanadate than the parent strain.

Several mutations that perturb the yeast secretory pathway have been shown to affect protein glycosylation. The lethal *sec14* (secretion defective) mutation affects glycosylation of carboxypeptidase Y (16), and the defect in this strain has been attributed to a mutation in a phospholipid exchange protein that may be involved in the recycling of membrane lipids from the Golgi to the endoplasmic reticulum (17). Rudolph *et al.* (18) have reported that the nonlethal *pmr1* (plasma membrane ATPase related) mutation, which is postulated to affect a Ca^{2+}-ATPase pump, leads to underglycosylation of invertase. The electrophoretic mobility of this altered invertase is similar to that of the *mnn9* mutant, although *pmr1* is not allelic to *mnn9*. Finally, the *erd1* (endoplasmic reticulum retention defective) mutation, which affects glycoprotein processing in the Golgi, also leads to formation of a *mnn9*-like invertase (19). All of these mutations probably affect energy-dependent processes that could be potential targets for inhibition by vanadate.

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