A *Mucor pusillus* Mutant Defective in Asparagine-Linked Glycosylation

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A *Mucor pusillus* mutant defective in asparagine-linked glycosylation was found in our stock cultures. This mutant, designated 1116, secreted aspartic proteinase (MPP) in a less-glycosylated form than that secreted by the wild-type strain. Analysis of enzyme susceptibility, lectin binding, and carbohydrate composition indicated that this mutant secreted three glycoforms of MPPs, one of which contained no carbohydrate; the other two had truncated asparagine-linked oligosaccharide chains such as $Man_{0-1}GlcNAc_2$. Further analysis using oligosaccharide processing inhibitors, such as castanospermine, 1-deoxynojirimycin and N-methyldeoxynojirimycin, suggested that MPPs in the mutant were glycosylated through a transfer of the truncated lipid-linked oligosaccharides, $Man_{0-1}GlcNAc_2$, to the MPP protein but not through an aberrant processing. In addition, genetic studies with forced primary heterokaryons indicated that the mutation in strain 1116 was recessive.

Most secreted proteins in filamentous fungi (8, 25, 27, 29), as well as in yeast (22) and mammalian cells (21), are modified by the addition of oligosaccharides to specific asparagine residues (N glycosylation). The early process of N glycosylation, including the synthesis of lipid-linked oligosaccharides, shows great similarities among all eukaryotic organisms (21, 22). Therefore, N-glycosylation mutants of *Saccharomyces cerevisiae* such as strains containing the *alg* (17, 18) or *gls* (14) mutation are very useful for understanding the synthesis of lipid-linked oligosaccharides as well as the topography of these reactions (16, 22). On the other hand, almost no mutants involved in N glycosylation from filamentous fungi have been obtained. Although most N-linked oligosaccharide chains of filamentous fungi such as *Aspergillus* species (8, 13), *Trichoderma reesei* (27), *Mucor pusillus* (25), and *Rhizopus niveus* (29) are the high-mannose-type glycan, like those in *S. cerevisiae*, the late processing reactions that presumably occur in the Golgi apparatus of these fungi seem to be different from those of the yeast species (25). In *S. cerevisiae*, further elongation of several mannosyltransferases occurs (11). The filamentous fungus is therefore an alternative model system for studying the synthesis and regulation of N glycosylation.

The zygomycete fungus, *M. pusillus*, secretes characteristic aspartic proteinase with a high milk-clotting activity and a relatively low proteolytic activity (4, 5). This proteinase, called *M. pusillus* pepsin (MPP), is widely used as a milk coagulant in industrial cheese production. Our previous studies using the MPP gene (31) in the hosts *S. cerevisiae* (3) and *Aspergillus oryzae* (25) showed that the MPP produced in these hosts contained two high-mannose-type glycans at the asparagine-linked glycosylation sites Asn-79–Ile–Thr and Asn-188–Asn–Thr. On the other hand, we also showed that the commercial MPP produced by the industrial strain of *M. pusillus* contained almost no carbohydrates (3, 35), while wild-type strains such as *M. pusillus* IFO4578 had two high-mannose-type glycans (25). On the basis of these observations, we examined the extent of glycosylation of MPPs secreted by our laboratory stock strains and found a glycosylation-defective mutant of *M. pusillus*,

designated 1116. In this report, we describe the characterization of the glycosylation mutant of *M. pusillus* 1116.

MATERIALS AND METHODS

Materials. *M. pusillus* F27 (4), an F27 Arg$^-$ mutant requiring arginine, and 1116 were our laboratory stock strains. Strain 1116 was previously isolated in our laboratory as a mutant that produced MPP with a high milk-clotting activity. *M. pusillus* 1116R3 was a spontaneous mutant which could grow on minimal medium (10). An *M. pusillus* 1116R3 Nia$^-$ mutant defective in nitrate assimilation was isolated as a mutant resistant to chlorate (470 mM) on minimal medium containing glutamate as a sole nitrogen source (32). For classification of mutants, chlorate-resistant clones were tested for growth on minimal medium containing nitrate, nitrite, ammonia, hypoxanthine, or glutamate as the sole nitrogen source (32). Fungal strains were routinely cultured on malt-glucose agar medium (2% [wt/vol] malt extract [Difco], 2% [wt/vol] glucose, 0.1% [wt/vol] Bacto Peptone [Difco]).

Castanospermine, 1-deoxynojirimycin, and N-methyldeoxynojirimycin were purchased from Takara Shuzo Co., Kyoto, Japan. Peroxidase-concanavalin A (ConA) and peroxidase-wheat germ agglutinin (WGA) were purchased from Seikagaku Kogyo, Tokyo, Japan.

Purification of MPP. MPPs from *M. pusillus* strains were purified as previously described (25). Protein contents in the purified MPP preparations were estimated by measuring A_{280}, with $A^{1\%} = 10$ (19).

Enzymatic digestion. Purified MPP was digested with endo-β-N-acetylglucosaminidase H (endo H) (Seikagaku Kogyo) and peptide-N-glycosidase F (PNGase F) (Takara Shuzo) according to the methods of Tarentino et al. (30) and Chu (9), respectively.

Carbohydrate composition. Purified MPP was hydrolyzed at 100°C for 12 h with 2.5 N trifluoroacetic acid. Carbohydrate compositions were determined by high-pH anion-exchange chromatography with a pulsed amperometric detection system (Dionex BioLC) (15).

Extraction of intra- and extracellular fractions. *M. pusillus* strains were cultured in YPD medium (1% [wt/vol] yeast extract [Difco], 2% [wt/vol] Bacto Peptone [Difco], 2% glucose) at 30°C for 3 days. After removal of the cells by filtration

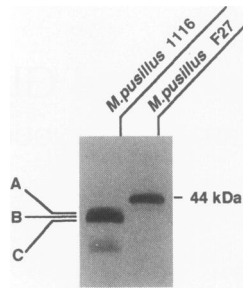* Corresponding author. Phone: 81-03-3812-2111, ext. 5147. Fax: 81-03-3812-0544.

FIG. 1. Western immunoblotting analysis of culture filtrates from *M. pusillus* F27 and 1116. Both strains were cultured in YPD medium at 30°C for 3 days. Both culture filtrates (0.4 ml) were concentrated by ethanol precipitation (2.5 volumes) and then subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting with rabbit anti-MPP antibodies (35). The A, B, and C forms of MPP are indicated.

FIG. 2. Enzymatic analysis of purified MPPs. Purified MPPs (1 μg of each) were digested with endo H or PNGase F according to the method of Tarentino et al. (30) or Chu (9), respectively, and subjected to SDS-polyacrylamide gel electrophoresis. MW, molecular mass standard.

through a glass filter, the supernatant was concentrated by using a Centricut (Kurabo) and then used as an extracellular fraction. The remaining cells were resuspended in extraction buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1% sodium dodecyl sulfate [SDS]) and homogenized in a mortar with sea sand. The homogenate was centrifuged at $3,000 \times g$, and then the supernatant was concentrated by using a Centricut and used as an intracellular fraction.

Generation of forced primary heterokaryons. Protoplast fusion of *M. pusillus* was carried out by the methods of Ohnuki et al. (26) and van Heeswijck (33), with minor modifications. Sporangiospores of *M. pusillus* were inoculated into 50 ml of YPD medium (pH 4.5) at 0.5×10^7 to 1×10^7/ml and germinated at 30°C with shaking for 11 h. After the germlings had been washed twice with 0.5 M sorbitol, they were suspended in 10 ml of enzyme solution (2 mg of Novozym 234 [Novo Nordisk, Bagsvaerd, Denmark] per ml, 1 mg of chitinase [Sigma] per ml, and 1.5 mg of chitosanase [Wako Chemicals, Osaka, Japan] per ml in 10 mM sodium phosphate buffer containing 0.5 M sorbitol, pH 6.5). After incubation at 26.5°C for 3 h with gentle shaking, protoplasts were washed twice with 0.5 M sorbitol and resuspended in 0.5 M sorbitol at 1×10^7/ml. Portions of 1×10^6 protoplasts from two different strains were mixed and then centrifuged at $800 \times g$ for 5 min at room temperature. The protoplast pellets were resuspended in 0.5 ml of polyethylene glycol solution (40% polyethylene glycol 3350 [Sigma], 50 mM $CaCl_2$, 10 mM MOPS [morpholinepropanesulfonic acid] buffer [Sigma], pH 6.3) and incubated at room temperature for 15 min. After the addition of 3 ml of 0.5 M sorbitol, suitably diluted suspensions were mixed with 1% (wt/vol) agar minimal medium (10) containing 0.5 M sorbitol and 10 mM $NaNO_3$ and plated onto the same minimal medium, pH 3.0. Heterokaryons appeared after 3 days of incubation at 30°C.

RESULTS

Isolation of a mutant strain defective in N glycosylation. A total of 20 of our laboratory stock strains of *M. pusillus* were cultured in YPD liquid medium at 30°C for 3 days, and the secreted MPPs in the culture broth were analyzed by Western blotting (immunoblotting) with the anti-MPP antibody (35). The survey identified strain 1116, a derivative of strain F27, which secreted MPP with a lower molecular mass than that (44 kDa) of the MPP secreted by the wild-type strain (Fig. 1). Strain 1116 showed slower growth but secreted more MPPs
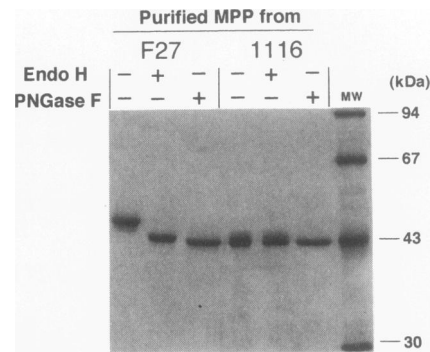
than strain F27 (data not shown). Western blotting analysis indicated that this strain secreted three forms of MPPs (Fig. 1, MPP-A, -B, and -C). It was unlikely that this lower level of glycosylation was due to the presence of some contaminating glycosidases, because no glycosidase activity was detected in the strain 1116 culture broth (data not shown).

Carbohydrate moiety of the MPP secreted by strain 1116. We purified MPPs secreted by strain 1116 and the wild-type strain, F27, and then both purified MPPs were digested by endoglycosidases such as endo H and PNGase F. Both endo H and PNGase F cleaved the oligosaccharide chains of MPP produced by F27 (Fig. 2), consistent with the idea that the oligosaccharide chains of MPP secreted by the wild-type *M. pusillus* strain were high-mannose-type glycans, as described previously (25). On the other hand, in the case of MPPs from strain 1116, PNGase F could cleave the small oligosaccharides of the A and B forms of MPPs, but endo H caused no changes in the molecular mass of the MPPs (Fig. 2). The minimum oligosaccharide chain susceptible to cleavage by endo H is at least three mannose (Man) and two *N*-acetylglucosamine (GlcNAc) residues (30), while that susceptible to PNGase F cleavage is only two GlcNAc core units (9). It is therefore concluded that the A and B forms of MPPs from strain 1116 contain at least a chitobiose core unit plus 0 to 3 mannose residues.

We further separated the C form from the A and B forms of MPPs by Mono Q anion-exchange column chromatography (25) and analyzed their carbohydrate moieties by using ConA and WGA lectins. The purified C form and the mixture of the A and B forms of MPPs from strain 1116 as well as from F27 were subjected to SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting using peroxidase-ConA and WGA lectins (Fig. 3). As shown in Fig. 3, the MPP from F27 was bound by ConA but not by WGA, which showed that the wild-type MPP contained high-mannose-type oligosaccharides. On the other hand, MPP-A and -B from 1116 were bound by WGA but not by ConA, and both lectins did not bind to MPP-C. For binding, ConA lectin requires the presence of at least two α-linked mannoses with free hydroxyl groups at C-3, -4, and -6, and WGA lectin can bind strongly to two core GlcNAc residues and weakly to $Man_1GlcNAc_2$, but not to $Man_{5 \sim 8}GlcNAc_2$ or $GlcNAc_1$ (9). Accordingly, the oligosaccharide chains of MPP-A and -B from strain 1116 were thought to be $Man_1GlcNAc_2$ and $GlcNAc_2$, respectively.
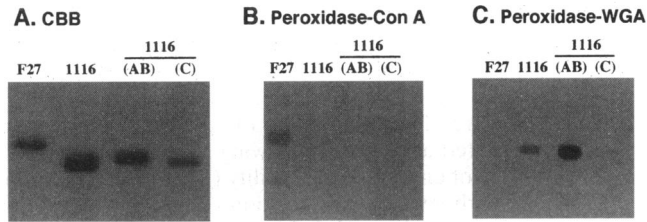
We also analyzed the carbohydrate composition of these

FIG. 3. Lectin binding analysis of purified MPPs. Purified MPPs A, B, and C (1 μg of each) as indicated immediately above the gels were subjected to SDS-polyacrylamide gel electrophoresis (panel A) and analyzed by lectin blotting using peroxidase-ConA (panel B) or WGA (panel C) lectins. CBB, Coomassie brilliant blue.
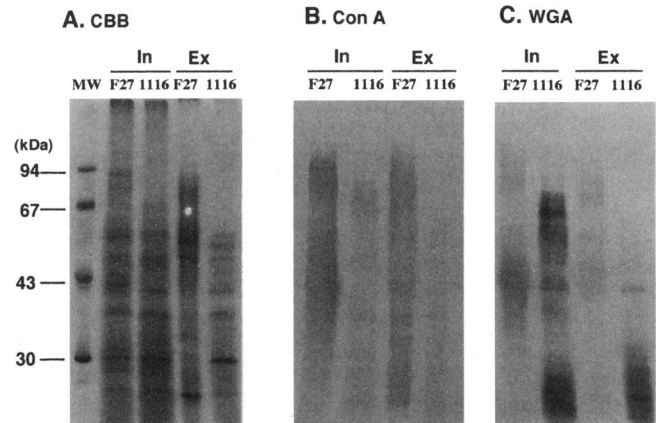

FIG. 4. Lectin binding analysis of intracellular (In) and extracellular (Ex) fractions from strains *M. pusillus* F27 and 1116. Each fraction was subjected to SDS-polyacrylamide gel electrophoresis (A) and analyzed by lectin blotting using peroxidase-ConA (B) or WGA (C) lectins. MW, molecular mass standard. CBB, Coomassie brilliant blue.

MPPs. As shown in Table 1, MPP from F27 contained nearly four (moles per mole of protein) GlcNAc and eight to nine (moles per mole of protein) mannose residues. In addition, endo H-treated MPP contained only two (moles per mole of protein) GlcNAc residues, indicating that the wild-type MPP had only two high-mannose-type oligosaccharide chains. Since the mixture of MPP-A and -B forms from 1116 contained 1.7 (moles per mole of protein) GlcNAc and 0.7 (moles per mole of protein) mannose residues, it was confirmed that the oligosaccharide chains of the MPP-A and -B forms were $Man_{0\sim1}GlcNAc_2$. Moreover, the MPP-C form from 1116 was found to contain no carbohydrate, which was consistent with the results of enzymatic and lectin binding analyses.

Glycosylation in strain 1116. As described above, strain 1116 secreted three glycoforms of MPPs, two of which had the truncated oligosaccharide chains consisting of $Man_{0\sim1}GlcNAc_2$ and one of which had no carbohydrates. To further analyze glycosylation in strain 1116, the crude extracts of intracellular and extracellular fractions of both the F27 and 1116 strains were analyzed by lectin blotting. As shown in Fig. 4, ConA lectin bound to both intra- and extracellular fractions from F27 but weakly bound to those from strain 1116. On the other hand, WGA lectin bound to fractions from 1116 but weakly to those from F27. These results indicated that not only MPP but also all other N-linked glycoproteins of strain 1116 had truncated oligosaccharide chains exposing GlcNAc residues. We therefore concluded that strain 1116 was defective in N glycosylation.

Effects of inhibitors of N-linked oligosaccharide processing. In mammalian cells and the yeast *S. cerevisiae*, N glycosylation starts with the synthesis of a lipid-linked oligosaccharide ($Glc_3Man_9GlcNAc_2$-pyrophosphoryl-dolichol) and its transfer to a nascent polypeptide chain in the recognition sequence Asn-X-Ser/Thr in the endoplasmic reticulum (21). After its transfer to protein, the core oligosaccharide is modified by processing enzymes such as glucosidase I and II and manno-

sidase sequentially, to form a $Man_8GlcNAc_2$-linked protein (21). In the case of the *M. pusillus* 1116 mutant defective in N glycosylation, the oligosaccharide chain linked to the MPP protein was very small, composed of only the GlcNAc core unit or the unit plus one mannose residue. To determine whether this lower glycosylation was due to a defect in the synthesis of a lipid-linked oligosaccharide and its subsequent transfer to protein or an aberrant processing by some glycosidase, we analyzed the effects of oligosaccharide-processing inhibitors such as castanospermine, 1-deoxynojirimycin, and *N*-methyldeoxynojirimycin. A previous study (12) showed that castanospermine and *N*-methyldeoxynojirimycin inhibited glucosidase I and 1-deoxynojirimycin inhibited both glucosidase I and II. In addition, Elbein et al. (13) reported that N-linked oligosaccharides of proteins secreted by *Aspergillus fumigatus* in the presence of castanospermine had $Glc_3Man_{7\sim8}GlcNAc_2$ structures, presumably due to the specific inhibition of glucosidase I, whereas those found in the absence of castanospermine were $Man_{8\sim9}GlcNAc_2$ structures. Because the oligosaccharide chains of MPP secreted by F27 were assumed to be $Man_5GlcNAc_2$, as judged from the results of determination of the carbohydrate composition (Table 1), the MPP secreted from the castanospermine-treated F27 cells was expected to have a higher molecular mass because of the blocking of removal of three glucose residues. As expected, the addition of castanospermine in the culture medium of the wild-type strain F27 resulted in an increase of the molecular mass of secreted MPP (Fig. 5). *N*-Methyldeoxynojirimycin and 1-deoxynojirimy-

TABLE 1. Carbohydrate composition of MPPs

MPP source	Carbohydrate composition (mol/mol of protein)				
	Fucose	GalNAc[a]	GlcNAc	Galactose	Mannose
F27	—[b]	—	3.8	—	8.4
F27 (endo H)	—	—	2.0	—	—
1116	—	—	0.9	—	0.5
1116 (AB)	—	—	1.7	—	0.7
1116 (C)	—	—	—	—	—

[a] GalNAc, *N*-acetylgalactosamine.
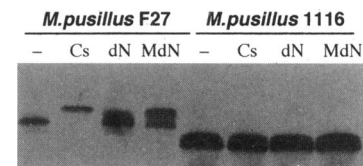[b] —, not detected.

FIG. 5. Effects of N-linked oligosaccharide processing inhibitors on the glycosylation of MPPs. *M. pusillus* F27 and 1116 were cultured at 30°C for 3 days in YPD medium containing 0.5 mM castanospermine (Cs), 1 mM 1-deoxynojirimycin (dN), or 1 mM *N*-methyldeoxynojirimycin (MdN). The protocol for Western immunoblotting is described in the legend to Fig. 1. −, no addition.
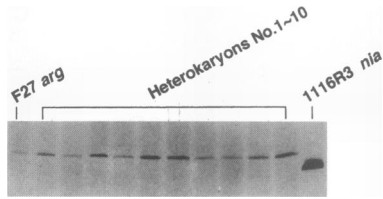
FIG. 6. Western immunoblotting analysis of culture filtrates from heterokaryons. The protocol for Western immunoblotting is described in the legend to Fig. 1.

cin also affected the molecular mass of MPP but yielded smear bands on SDS-polyacrylamide gel electrophoresis, indicating that they partially inhibited glucosidases. In contrast, these inhibitors had no effect on the molecular mass of MPPs secreted by the 1116 mutant (Fig. 5). It is therefore unlikely that the lower glycosylation levels of MPPs secreted by the 1116 mutant were due to aberrant processing by glycosidases. All these observations suggested that the smaller oligosaccharides, $Man_{0\sim1}GlcNAc_2$, were transferred to the MPP protein in strain 1116.

Genetic analysis of the 1116 mutant. To further investigate the 1116 mutant strain by genetic analysis, we formed heterokaryons between the 1116 and F27 strains. Since strain 1116 showed almost no growth on minimal medium, we first isolated a spontaneous mutant strain, designated 1116R3, by selection for the ability to grow on minimal medium. In addition, 1116R3 Nia$^-$, which could not grow on minimal medium containing nitrate as a sole nitrogen source, was isolated as described in Materials and Methods. Strain 1116R3 Nia$^-$ showed slower growth in YPD liquid medium than strain F27 and secreted less-glycosylated MPPs, just like strain 1116 (Fig. 6), thus indicating that this derivative was defective in N glycosylation. Protoplasts of 1116R3 Nia$^-$ and F27 Arg$^-$ could be efficiently fused by the polyethylene glycol-CaCl$_2$ method (number of colonies observed/number of protoplasts $= 3 \times 10^{-3}$ to 4×10^{-3}) (26), and heterokaryons obtained in this way grew well on minimal medium (10) containing nitrate as a sole nitrogen source. No colonies were observed in the case of crosses between the same strain. Ten of the heterokaryons were cultured in liquid minimal medium at 30°C for 5 days, and secreted MPPs in the culture broth were analyzed by Western blotting. As shown in Fig. 6, MPPs secreted by those heterokaryons had the same molecular mass as that secreted by strain F27, thus indicating that the mutation of strain 1116 was recessive to the wild-type F27 strain.

DISCUSSION

In this study, we found and characterized an *M. pusillus* mutant defective in N glycosylation. This mutant, designated 1116, was found to secrete less-glycosylated MPP (Fig. 1), which had the almost same molecular mass as commercial MPP. Baudys et al. reported (6) that commercial MPP contained two GlcNAc and three mannose residues and probably one fucose residue at the N-glycosylation site, Asn-188. Although the carbohydrate composition of MPPs produced by the 1116 mutant is not in perfect agreement with that of the commercial MPP (Table 1), it is possible that the industrial strain producing commercial MPP is deficient in N glycosylation, as is strain 1116. We previously reported that by endo H digestion or site-directed mutagenesis both commercial MPP and deglycosylated MPP had a higher milk-clotting activity, along with a lower proteolytic activity, than the fully glycosy-

lated MPP (3, 25). These observations led us to assume that the defect in N glycosylation of one industrial strain resulted from the screening among repeatedly mutated *M. pusillus* strains for a mutant showing a high milk-clotting activity. This may also hold true for the isolation of strain 1116, which was previously isolated as a mutant showing a similar property.

The analyses of enzyme susceptibility (Fig. 2), lectin binding (Fig. 3), and carbohydrate composition (Table 1) showed that strain 1116 secreted three glycoforms of MPPs, two of which had truncated N-linked oligosaccharides such as $Man_{0\sim1}Glc$ NAc_2 and one of which contained no carbohydrates. Furthermore, the glucosidase inhibitors such as castanospermine had no effect on the glycosylation of MPPs secreted by strain 1116 (Fig. 5), and the studies with forced primary heterokaryons indicated that the mutation of strain 1116 was recessive. It is therefore unlikely that glycosidases digested the oligosaccharide chains of MPPs in the mutant strain. Considering that the small oligosaccharides such as $Man_{0\sim1}GlcNAc_2$-pyrophosphoryl-dolichol can be transferred to proteins by oligosaccharyltransferase in vitro (23, 24, 28), we assume that the truncated lipid-linked oligosaccharides are directly transferred to protein in the mutant. The presence of two glycoforms of MPP and one nonglycosylated form may reflect the glycosylation efficiency of an oligosaccharyltransferase with these truncated lipid-linked oligosaccharides as the sugar donor (24).

Recent studies on the topography of glycosylation reactions using mammalian cells (1, 2, 20) have suggested that the synthesis of $GlcNAc_2$-pyrophosphoryl-dolichol and Man_1Glc NAc_2-pyrophosphoryl-dolichol occurs on the cytosolic side of the endoplasmic reticulum membrane, and thus these smaller oligosaccharides are not transferred to proteins in vivo. These studies are inconsistent with our idea that the truncated oligosaccharides like $Man_{0\sim1}GlcNAc_2$ were directly transferred to the MPP protein in the *M. pusillus* 1116 mutant. However, the studies with the *alg-1* and *alg-2* mutants of *S. cerevisiae*, where $GlcNAc_2$-pyrophosphoryl-dolichol and $Man_{1\sim2}GlcNAc_2$-pyrophosphoryl-dolichol, respectively, are accumulated in the cells, showed that these smaller oligosaccharides can be transferred to protein in vivo (18). These results suggested that the smaller lipid-linked oligosaccharides like $Man_{0\sim2}GlcNAc_2$-pyrophosphoryl-dolichol can be translocated across the endoplasmic reticulum membrane into the lumen, where they can serve as the sugar donor. Although the *M. pusillus* 1116 mutant is not a conditional mutant, in contrast with the yeast strains containing the *alg-1* or *alg-2* mutation, the present studies with the *M. pusillus* 1116 mutant can support the idea that, when mannosylation on the cytosolic side is blocked, even smaller lipid-linked oligosaccharides like $Man_{0\sim2}GlcNAc_2$-pyrophosphoryl-dolichol can be translocated across the endoplasmic reticulum membrane into the lumen.

The biological roles of N-linked oligosaccharide chains have been studied by various approaches, such as enzymatic removal of sugar chains, alteration of oligosaccharide processing, and elimination of specific glycosylation sites by site-directed mutagenesis (34). Besides these approaches, studies with glycosylation mutants of yeast (22) and mammalian (7) cells have provided much information about not only the functions of carbohydrates but also the pathways of their biosynthesis. As described in this report, the analysis of the *M. pusillus* 1116 mutant may contribute to the studies on the biosynthesis of N-linked oligosaccharides, including the topography of glycosylation reactions. In addition, this mutant might be used as a host strain for producing deglycosylated forms of glycoproteins, which would help in understanding the role of N-linked oligosaccharides. Development of a transformation system of *M. pusillus* is now in progress in our laboratory.

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