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Synthesis of the measles virus nucleoprotein in yeast *Pichia pastoris* and *Saccharomyces cerevisiae*

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Abstract

The development of a simple, efficient and cost-effective system for generation of measles virus nucleoprotein might help to upgrade reagents for measles serology. The gene encoding measles nucleoprotein was successfully expressed in two different yeast genera, *Pichia pastoris* and *Saccharomyces cerevisiae*, respectively. Both yeast genera synthesized a high level of nucleoprotein, up to 29 and 18% of total cell protein, in *P. pastoris* and *S. cerevisiae*, respectively. This protein is one of most abundantly expressed in yeast. After purification nucleocapsid-like particles (NLPs) derived from both yeast genera appeared to be similar to those detected in mammalian cells infected with measles virus. A spontaneous assembly of nucleoprotein into nucleocapsid-like particles in the absence of the viral leader RNA or viral proteins has been shown. Compartmentalisation of recombinant protein into large compact inclusions in the cytoplasm of yeast *S. cerevisiae* by green fluorescence protein (GFP) fusion has been demonstrated. Sera from measles patients reacted with the recombinant protein expressed in both yeast genera and a simple diagnostic assay to detect measles IgM could be designed on this basis.

Keywords: Measles; Expression; Nucleocapsid-like particles; *Saccharomyces cerevisiae*; *Pichia pastoris*

1. Introduction

Measles virus, belonging to the *Morbillivirus* genus of a family *Paramyxoviridea*, is an enveloped virus, which encapsidates a 16 kb negative strand RNA genome (for review, Griffin, 2001). Mature particles are assembled from six viral-encoded proteins. Two glycoproteins, the hemagglutinin and the fusion protein, are the major protein constituents of the envelope. A third protein, a matrix protein, lines the interior surface of the viral envelope and is believed to link glycoproteins to the internal viral proteins. The nucleoprotein (MeN), the major internal protein, appears to be responsible for viral genome packaging and formation of replication complexes along with other proteins. Two additional proteins, namely, the

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phosphoprotein and large protein or RNA polymerase, are associated with nucleocapsid and are involved both in replication and transcription of viral RNA (Spehner et al., 1997).

Viral nucleocapsid proteins usually elicit a strong long-term humoral immune response in patients and experimental animals. Therefore, measles diagnosis can be based upon the detection of antibodies specific to MeN (Warnes et al., 1994). Due to advances in gene expression technology, production of recombinant viral proteins in both eukaryotic and prokaryotic host systems has become easier and more efficient. The ease with which genetic modification can be carried out, the yield of recombinant proteins and manifestation of post-translation modification often govern the choice of host systems. Yeasts are unicellular eukaryotic micro-organisms capable of performing eukaryotic processing on the expressed polypeptides and are easy to manipulate genetically. Since yeast represent eukaryotes, their intracellular environment is more suitable for a correct folding of eukaryotic virus proteins (Romanos et al., 1992; Faber et al., 1995). Data on homologous and heterologous virus-like particles (VLPs) formation in yeast have continued to increase (Valenzuela et al., 1982; McAleer et al., 1984; Miyanohara et al., 1986; Cregg et al., 1987; Jacobs et al., 1989; Janowitz et al., 1991; Hofmann et al., 1996; Sasnauskas et al., 1999, 2002; Gedvilaite et al., 2000; Hale et al., 2002; Samuel et al., 2002; Slibinskas et al., 2003).The yeast expression system has also been used to produce licensed recombinant vaccine for human hepatitis B virus and represented the world's first vaccine with an antigen prepared by genetic engineering (for review: McMahon and Wainwright, 1993). Our aim was to construct efficient expression systems for generating MeN as nucleocapsid-like particles (NLPs) in two different yeast genera to be used in measles serology. In this paper we have described MeN expression in *Pichia pastoris* and compared it to *Saccharomyces cerevisiae*.

2. Materials and methods

2.1. Strains, media and transformation

Bacterial recombinants were screened in *Escherichia coli* DH5αF′ cells. Strains *P. pastoris* GS115 *his4* (Invitrogen, Groningen, The Netherlands) and *S. cerevisiae* AH22 *MATa leu2 his4* were used for expression. Transformation of *P. pastoris* GS115 *his4* was performed by electroporation (Bio Rad, Gene Pulser) according to Cregg and Russell (1998). *P. pastoris* His^+ transformants were selected on a minimal agar medium (0.67% YNB, 2% glucose). Transformants with a high copy number were selected on YEPD-agar plates containing 0.5–1.5 mg ml^{-1} antibiotics G418 (Amresco, Solon, USA). The copy number of inserted expression cassettes was determined according to Romanos et al. (1998). Transformation of yeast *S. cerevisiae* cells was performed according to standard procedures (Sambrook and Russell, 2001). Selection of transformants resistant to formaldehyde was carried out on YEPD (yeast extract 1%, peptone 2%, and dextrose 2%) agar supplemented with 5 mmol formaldehyde as earlier described (Sasnauskas et al., 1992). *S. cerevisiae* transformants were grown in YEPD medium supplemented with 5 mmol formaldehyde or in YEPG induction medium (yeast extract 1%, peptone 2%, and galactose 3%).

2.2. Cloning of measles virus N gene into yeast vectors

All DNA manipulations were performed according to standard procedures (Sambrook and Russell, 2001). Enzymes and kits for DNA manipulations were purchased from Fermentas UAB (Vilnius, Lithuania). The MeN gene was amplified by PCR from cDNA after extraction and reverse transcription of reconstituted Priorix vaccine, containing the measles Schwarz strain (GlaxoSmithKline, UK), (GenBank accession no. AF266291). Primers used in amplification of the MeN included a *Spe*I site for subcloning into the yeast vectors, a single ATG codon in the forward primer and a stop TAA codon in the reverse primer. The following primers with the *Spe*I sites (in bold) and the start and stop codons (underlined) were used.

Reverse (5′→3′) TT **ACT AGT** <u>TTA</u> GTC TAG AAG ATT TCT GTC ATT GTA CAC.

Forward (5′→3′) TT **ACT AGT** ACA <u>ATG</u> GCC ACA CTT TTA AGG AGC TT.

Bands corresponding to the MeN gene were gel-purified and cloned into pCR® 2.1 TOPO®

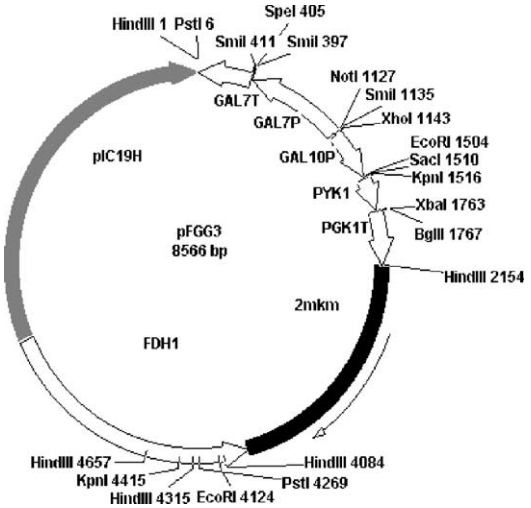


Fig. 1. Expression vector pFGG3. 2 mkm–1.74 kb fragment of yeast 2 μm plasmid; FDH1—*FDH1* gene of *Candida maltosa*, conferring resistance to formaldehyde (Sasnauskas et al., 1992); pIC19H—bacterial plasmid; GAL7T—*S. cerevisiae GAL7* gene transcription terminator (381 bp); GAL7P—*S. cerevisiae GAL7* gene promoter (−1 to −716 nt); GAL10P—*GAL10* gene UAS sequence (−511 to −138 nt); PYK1—*S. cerevisiae PYK1* gene promoter (−271 to −4 nt); PGK1—fragment of *S. cerevisiae PGK1* gene transcription terminator sequence (387 bp).

(Invitrogen), sequenced and used for cloning into the *Avr*II site of the *P. pastoris* vector pPIC3.5 K (Invitrogen) under control of methanol inducible *AOX1* promoter. The resulting plasmid pPIC3.5-MeN was linearized with restriction endonuclease *Bgl*II and used for electroporation into *P. pastoris* GS115 (Cregg and Russell, 1998). *P. pastoris* transformants were selected on medium, containing antibiotic G418 (Amresco, USA) as previously described (Romanos et al., 1998). In case of *S. cerevisiae*, the MeN gene was cloned into the *Spe*I site of the *S. cerevisiae* expression vector pFGG3 under control of *GAL7* promoter (Fig. 1). The resulting plasmid pFGG3-MeN was used for transformation of yeast *S. cerevisiae* strain AH22 as described previously (Samuel et al., 2002).

2.3. Determination of *P. pastoris* expression cassette copy number in transformants

Determination of a copy number was carried out exactly as recommended in *Pichia* protocols (Romanos et al., 1998; Clare et al., 1991). In order to determine the expression cassette copy number in *P. pastoris*, total DNA from selected transformants were isolated and Southern blot analysis was used to determine the chromosomal structure of integrated cassette DNA. Chromosomal DNA was digested with *Bgl*II and hybridised with a *HIS4*-specific probe. The resulting Southern blots revealed a 2.7 kb band corresponding to the *HIS4* gene and a larger one equivalent to the *HIS4*-containing *Bgl*II fragment of expression vector. As expected, a majority of the transformants were single copy transformants and exhibited two bands of similar intensity. A single-copy transformant, identified by Southern blot analysis, was used as a control. Densitometer scan of blot was used to determine the precise copy numbers. Copy number was calculated by normalising the intensity of 2.7 kb *Bgl*II band corresponding to a single copy of *HIS4* to the larger one corresponding to the inserted cassettes. Data presented in Table 1 represent an average of three independent hybridisation experiments.

Table 1
MeN expression level in *P. pastoris* transformants with a different copy number of the gene

P. pastoris clones	Copy number	Expression level[a]
2 vk	1	3.8 ± 1.1
10 dk	4 ± 1	12.0 ± 1.3
12 dk	4 ± 1	12.4 ± 2.1
8 dk	10 ± 1	29.4 ± 2.8
7 dk	13 ± 2	26.6 ± 2.7
2 dk	80 ± 10	22.4 ± 3.2
13 dk	80 ± 10	17.9 ± 3.8

[a] A level of expression was determined after induction for 96 h. Levels are given as a percentage of total cell protein.

2.4. Construction of C-terminal green fluorescence protein (GFP) fusion of MeN

A unique *Bsp*1407I site in position 1551 nt of the MeN coding sequence was used for in frame cloning of full length green fluorescence protein coding sequence from the plasmid pEGFP-1 (Clonthech, Palo Alto, CA, USA). The fused gene was inserted into the *Xba*I site of *S. cerevisiae* expression vector pFX7 (Sasnauskas et al., 1999). Fluorescence of yeast cells was examined with an Olympus microscope.

2.5. Expression and purification of nucleocapsid-like particles from yeast

Selected *P. pastoris* GS115 [pPIC3.5-MeN] transformants with up to 80 integrated copies of the MeN gene, under control of *AOX1* promoter and transcription terminator, were inoculated into 250 ml of BMG medium (100 mmol potassium phosphate, pH 6, 1.34% YNB, 4 $\mu g\,ml^{-1}$ biotin, 1% glycerol) in a 2.5 l flask and grown at 28 °C with shaking at 250 revolutions per minute (series 25 shaker, New Brunswick Scientific, Edison, NJ). Induction of *P. pastoris* was performed under Mut^+ conditions essentially as described by the manufacturers, except that the yeast cells were resuspended in BMM medium (100 mmol potassium phosphate, pH 6, 1.34% YNB, 4 $\mu g\,ml^{-1}$ biotin, 0.5% methanol) to an absorbance ($A_{600\,nm}$) of 2 and the culture supplemented with methanol at 12 h intervals. The cells were harvested after induction for 96 h and stored at −70 °C (Cregg et al., 1987).

The procedure used for expression of MeN protein in *S. cerevisiae* cells was similar to that earlier described for mumps proteins (Samuel et al., 2002; Slibinskas et al., 2003). Briefly, *S. cerevisiae* cells harbouring pFGG3-MeN were inoculated into YEPD media supplemented with 5 mmol formaldehyde, grown overnight and re-inoculated into YEPG induction media and cultured at 28 °C for 24 h. After 24 h induction we observed a slow decline of MeN protein level and it coincided with the exhaustion of galactose in the medium (data not shown). The cells were harvested by centrifugation and stored at −70 °C. Five gram of each recombinant clone of yeast biomass were suspended in PBS containing 10 mmol EDTA and 1 mmol PMSF and after disruption by homogenisation in the presence of glass beads were used for purification. The MeN protein was purified by successive ultracentrifugations through 30% sucrose cushion (100,000 × *g*, 3 h) followed by ultracentrifugation in CsCl gradient ranging from 1.23 to 1.38 $g\,ml^{-1}$ (36 h, 100,000 × *g*) performed twice. CsCl gradient fractions were analysed by SDS-PAGE. After the initial centrifugation, fractions were collected and analysed for the presence of protein by SDS-PAGE. Fractions containing protein with the molecular weight corresponding to MeN protein (∼60 kDa) were pooled, diluted with 1.31 $g\,ml^{-1}$ CsCl and ultracentrifugation repeated. Fractions containing MeN were pooled and dialysed against PBS for EM and Western blot analysis (Samuel et al., 2002; Slibinskas et al., 2003). The buoyant density of fractions was determined with a refractometer.

2.6. SDS-PAGE and Western blotting

Samples were boiled in a reducing sample buffer and gel electrophoresis was run in SDS-Tris-glycine buffer. Proteins were stained by the addition of Coomassie brilliant blue. After SDS PAGE electrophoresis proteins were transferred to nitrocellulose membrane Hybond^{TM} ECL (Amersham, UK) as described in Sambrook and Russell (2001). The blots were blocked with 5% milk in PBS for 2 h. The blocking solution was removed and the blots were incubated with diluted measles positive human serum. Horseradish peroxidase (HRP)-labelled anti-human IgG conjugates (Dako, UK) for detection of specific antibody-binding were used. The blots were stained with the TMB substrate (Fermentas UAB, Vilnius, Lithuania).

2.7. Determination of expression level of MeN protein in yeast lysates

The percentage of MeN present in the cell lysates was determined by densitometric scanning of the Coomassie brilliant blue stained protein bands fractionated by SDS-PAGE, using the Herolab E.A.S.Y. 429K (Germany) device. Analysis was conducted with the Easy Win 32 software supplied with the instrument. At least three scans of electrophoresed yeast lysates were used in the estimation of expressed protein. The percentage of the recombinant MeN as a total of cell lysate proteins was calculated using only the main band of 60 kDa. Degradation products (57 and 47 kDa bands, see Fig. 2) were not included in the calculation of expression level. The percentage of yeast proteins co-migrating in SDS-PAGE with the MeN band was determined in comparison to control samples of yeast containing vector without the MeN gene after induction under the same conditions. The quantity of MeN protein in yeast lysates was finally calculated by subtracting intensities of co-migrating protein bands, assuming that the expression level of these proteins is proportional in both control and MeN producing clones.

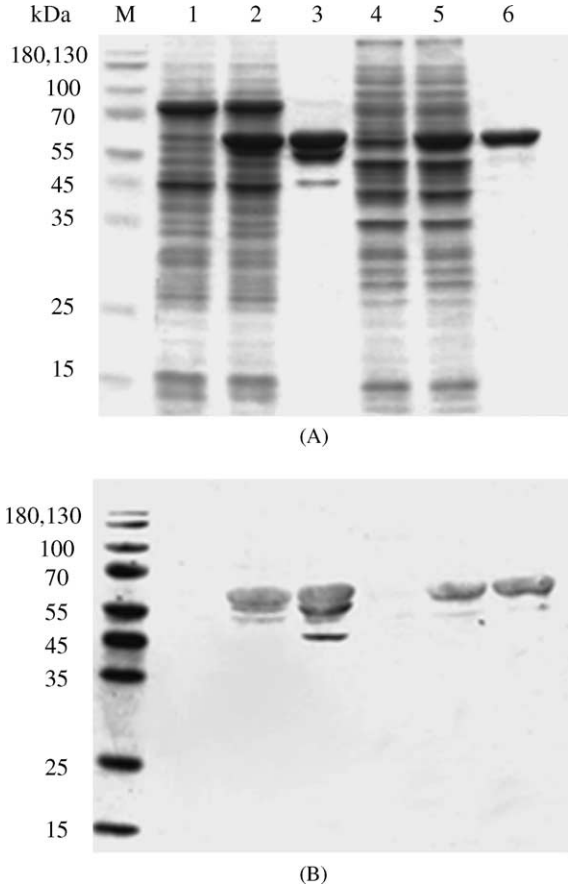


Fig. 2. (A) SDS-PAGE and (B) Western blot analysis of yeast lysates and CsCl-ultracentrifugation purified yeast expressed MeN. Five microgram of purified MeN from *P. pastoris* (lane 3, A and B) and *S.cerevisiae* (lane 6, A and B) or 20 μg of yeast lysates (lanes 1, 2, A and B—*P. pastoris*, lanes 4, 5, A and B—*S. cerevisiae*) were separated in a 12% SDS-PAGE and stained Coomassie brilliant blue. Separated proteins were analysed by Western blot using measles positive human serum. Lane 1, A and B, *P. pastoris* [pPIC3.5K] lysate; lane 2, A and B, *P. pastoris* [pPIC3.5-MeN]; lane 3, A and B, CsCl purified MeN from *P. pastoris*; lane 4, A and B, *S. cerevisiae* [pFGG3]; lane 5, A and B, *S. cerevisiae* [pFGG3-MeN]; lane 6, A and B, CsCl purified MeN from *S. cerevisiae*. M—prestained protein molecular mass marker (Fermentas UAB, Vilnius, Lithuania) 180, 130, 100, 70, 55, 45, 35, 25 and 15 kDa.

2.8. Electron microscopy

After purification by CsCl centrifugation suspensions of MeN particles were placed on 400-mesh carbon coated palladium grids. Samples were stained with 2% aqueous uranyl acetate solution and examined with the JEM-100S electron microscope.

2.9. Measles IgM capture enzyme immunosassay (EIA)

A measles IgM capture EIA was developed using purified MeN from *P. pastoris* and *S. cerevisiae* as described previously for mumps IgM captue EIA (Samuel et al., 2002) and partially evaluated for measles IgM EIA (Samuel et al., 2003). A panel of sera from acute measles cases (10) and from controls (18) were tested in the developed measles IgM EIA to determine the relative reactivity of MeN expressed in the two yeast genera.

3. Results and discussion

3.1. Synthesis of MeN in yeast P. pastoris and S. cerevisiae

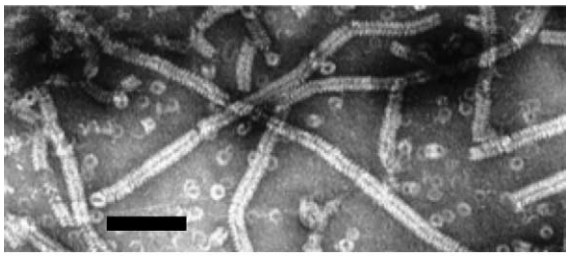
Previously it was demonstrated that the recombinant measles nucleoprotein after expression in animal and insect cells formed NLPs (Spehner et al., 1991; Fooks et al., 1993; Warnes et al., 1994, 1995). In our study formation of measles NLPs in different yeast genera, *P. pastoris* and *S. cerevisiae*, respectively, has been compared. For the MeN protein expression in *P. pastoris* we used the invitrogen (Groningen, The Netherlands) expression system available commercially. The MeN gene was inserted in the plasmid pPIC3.5 K under control of methanol inducible *AOX1* gene promoter, resulting in the plasmid pPIC3.5-MeN. SDS-PAGE analysis of crude lysates of *P. pastoris* and *S.* cerevisiae, containing differing copy numbers of integrated pPIC3.5-MeN (1–80) after growth in the methanol medium or harbouring plasmid pFGG3-MeN after induction with galactose, respectively, revealed the presence of an additional protein band corresponding to the molecular weight of MeN (60 kDa) (Fig. 2A, lanes 2 and 5). No bands of the corresponding molecular size were observed in crude lysates of yeast cells, harbouring integrated vector pPIC3.5 K or vector pFGG3, in *P. pastoris* or *S. cerevisiae*, respectively (Fig. 2A, lanes 1 and 4).

3.2. Purification of yeast derived MeN in CsCl gradient and EM analysis

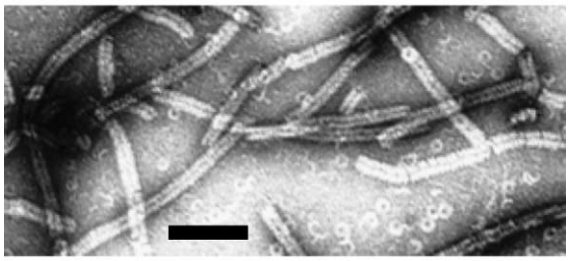
The assembly of MeN into nucleocapsid-like structures was analysed by density-gradient centrifugation and EM. Yeast cells were disrupted with glass beads

and analysed after centrifugation through 30% sucrose cushion. The MeN protein was found in pellets (data not shown), consistent with its large size and multimeric organisation. Further, ultracentrifugation of the resolubilised pellet in CsCl gradient and analysis revealed MeN in fractions with buoyant density of 1.31 g ml^{-1}, which is characteristic for most virus-like particles or NLPs (Thorne and Dermott, 1976). Fractions of this buoyant density contained highly purified N protein with only minor contaminants present as observed in SDS-PAGE. The SDS-PAGE analysis of purified protein after centrifugation in CsCl revealed a band of 60 kDa (Fig. 2A, lanes 3 and 6), which was also observed in the crude lysates (Fig. 2A, lanes 2 and 5). MeN derived from different yeast genera exhibited different levels of degradation. The yeast lysates obtained after induction and CsCl gradient purified protein from both *P. pastoris* and *S. cerevisiae* expression systems revealed a predominant protein band corresponding to 60 kDa (Fig. 2A, lanes 2, 3 and 5, 6). However, the MeN in lysates and the CsCl purified protein from *P. pastoris* showed evidence of additional protein bands of ∼57 and ∼45 kDa (Fig. 2A, lanes 2, 3). The low molecular weight bands were clearly visible in Western blot (Fig. 2B, lanes 2, 3) suggesting that degradation of MeN in *P. pastoris* occurs during growth and purification. In contrast the degradation of the MeN from *S. cerevisiae* was negligible (Fig. 2, A and B, lanes 5 and 6). A number of reports have described the degradation, attributed to proteolysis in some cases, of MeN expressed in *E. coli*, baculovirus, mammalian systems and in measles virus infected cells (Warnes et al., 1994). In comparison to other expression systems used for MeN expression, both yeast systems employed in the present study were extremely stable with minimal degradation.

Analysis by transmission EM of negatively stained MeN from both yeast genera purified in CsCl gradient or even obtained after concentration through sucrose cushion exhibited a classic herring bone morphology (Fig. 3A and B). Such structures had an overall diameter of 20–22 nm and a central core approximately 4.5–5 nm in diameter. The average length of rods derived from both yeast genera ranged in size and depended on the purification procedures. The longest rods were observed after sucrose centrifugation, and each additional step of purification shortened the length of rods. These structures were similar to those



(A)



(B)

Fig. 3. Electron micrographs of negatively stained CsCl gradient ultracentrifugation purified nucleocapsids isolated from (A) *P. pastoris* and (B) *S. cerevisiae*. Scale-bar: 100 nm.

previously observed in mammalian cells infected with measles virus and insect expression system (Thorne and Dermott, 1976; Rima, 1983; Fooks et al., 1993; Griffin, 2001). The morphology of proteins expressed in *P. pastoris* and in *S. cerevisiae*, respectively, was similar as demonstrated by EM. Furthermore, the rods identified by electron microscopy were identical to the MeN isolated from both yeast genera despite the partial degradation observed on SDS-PAGE analysis in the samples from *P. pastoris* after centrifugation in CsCl gradient (Fig. 2A, lane 3). Probably, both degraded and undegraded forms of MeN were present within yeast derived NLPs and were maintained by intra- and inter-subunit interactions. Formation of NLPs occurs in the absence of other viral proteins in both yeast expression systems. If host cells proteins are involved in formation of NLPs, they appear to be conserved in both yeast and mammalian cells. A wide distribution of the rods length was found both in naturally occurring infection and recombinant yeast cells.

3.3. Yield and stability of MeN particles

Densitometric scanning of protein bands enabled the estimation of the quantity of MeN expressed

compared to other yeast cell proteins. Recombinant protein was expressed at high levels of MeN in both yeast genera. Analysis of the level of the MeN protein expression in *P. pastoris* transformants with a differing number of integrated gene copies revealed an increase in protein expression with the number of integrated gene up to 10 copies. Further, increases in the gene copy number led to a reduction in heterologous protein expression. A number of examples exist, where the yield of product has been improved using multiple vector copies, and indeed, the maximal copy number tested was optimal in several cases (Clare et al., 1991). However, it has too-high copy numbers reduce yield, i.e., an optimal rather than maximal copy number is required (Thill et al., 1990; Romanos et al., 1998). The level of MeN expression in *P. pastoris* 8 dk harbouring 10 copies of the inserted gene reached over 29% of total cellular protein (Table 1). Clones, 10 and 12 dk with an equal number of integrated genes expressed similar levels of protein. The dependence of the MeN expression level in *P. pas*toris upon the induction time was similar in all clones tested. The amount of recombinant protein in cell lysates gradually increased during induction for 4 days (approximately from 13% after induction for 24 h to over 29% after induction for 96 h in the case of clone 8 dk).

The level of recombinant protein synthesis in *S. cerevisiae* was less than in *P. pastoris* and was approximately 18% of total cellular protein after 24 h of induction. Several preparative CsCl gradient procedures yielded, approximately 13 and 6 mg of purified NLPs, from *P. pastoris* 8 dk and *S. cerevisiae* transformants per 1 g of wet biomass, respectively. A high yield of purified MeN NLPs indicates a very efficient assembly of NLPs in both yeast genera. Recombinant measles NLPs were stable in CsCl solution at 4 °C. Treatment with 10 mmol EDTA, 10 mmol EGTA or 5 mmol DTT did not cause dissociation of NLPs, thus indicating that the assembled structure did not require divalent ions or disulfide bonds. Dialysed samples were stored in PBS containing 1 mmol EDTA and 40% glycerol at −20 °C.

The yield of MeN greatly exceeded the yields of heterologous proteins in other *Paramyxoviridea* family members. Expression of mumps virus nucleocapsid protein has been recently described in both yeast genera using similar expression schemes (Samuel et al., 2002; Slibinskas et al., 2003). The clone of yeast *P. pastoris* producing the highest level of mumps nucleoprotein also contained about 10 copies of integrated expression cassettes, however the yield of NLPs was about six times less than MeN NLPs obtained from clone 8 dk in this study. The yield of MeN NLPs from *S. cerevisiae* was seven-fold greater than that of mumps virus nucleocapsid protein due to very efficient synthesis and the NLPs assembly of MeN in yeast. Hence, it is difficult to predict the yield of heterologous protein before the experiment. The yield of heterologous protein depends on many factors, such as, stability of mRNA, stability of heterologous protein, “toxicity” of heterologous protein for the host cell, ability to form stable substructures like NLP, which do not interfere with cellular processes and protein quality control, do not raise unfolded protein response or endoplasmic reticulum overcrowded response and other stress reactions (Kaufman, 1999; Wickner et al., 1999).

3.4. Fluorescence analysis of sub-cellular localisation of MeN-GFP protein in S. cerevisiae

To test the sub-cellular localisation and to observe the kinetics of the heterologous MeN protein synthesis in yeast cells, the fusion of MeN and GFP was constructed and expressed in *S. cerevisiae*. Fluorescence analysis of yeast cells under induction demonstrated, that recombinant protein in the cytoplasm of yeast *S. cerevisiae* was compartmentalised in large compact inclusions (Fig. 4). Possibly, a tight self-localisation of recombinant protein into inclusions helps to avoid stress under induction and during posttranslational quality control (Kaufman, 1999). The high-level of expression and stability of the MeN protein may be due to this compartmentalisation. Further, studies are needed to determine if MeN itself provides any specific signal for directing recombinant NLPs to the compact sub-cellular structure.

Strong correlation between the amount of fused MeN-GFP and native NLPs was observed under different growth conditions. Consequently, fluorescence analysis of the yeast culture during the induction period contributed to the selection of optimal induction time, media and growth conditions to produce a high yield of recombinant protein.

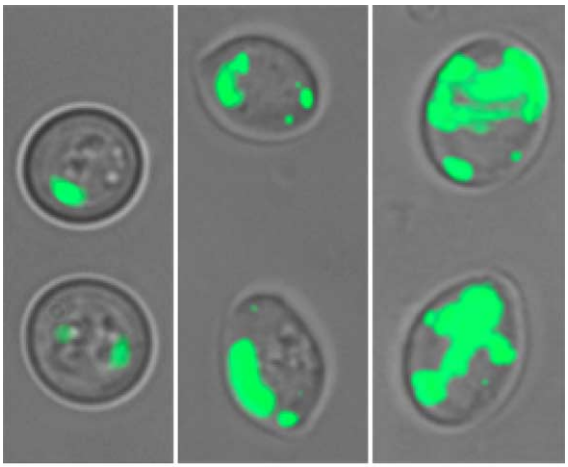


Fig. 4. Synthesis of MeN-GFP fused protein in yeast *S.cerevisiae*. (A) 2 h, (B) 6 h and (C) 16 h after induction.

3.5. Measles IgM capture EIA

After optimisation of the assay using MeN from both yeast genera, the measles IgM assay was used to test a panel of acute measles sera and control sera. Acute measles serum samples gave high optical density readings in the measles IgM capture EIA using MeN from both *P. pastoris* and *S. cerevisae* (mean $OD_{450/620\,nm}$ 1.363; range 0.269–2.459 for *P. pastoris* and mean $OD_{450/620\,nm}$ 1.35, range 0.467–2.082 for *S. cerevisae*) compared to control serum samples (mean $OD_{450/620\,nm}$ 0.060; range 0.032–0.115 for *P. pastoris* and mean $OD_{450/620\,nm}$ 0.096, range 0.048–0.075 for *S. cerevisae*. There was good correlation ($R^2 = 0.97$) between the optical density values obtained with MeN from yeast genera and the panel of sera used in the test.

3.6. Conclusions

Many virus surface and nucleocapsid proteins when expressed in animal host in the absence of other viral gene products have the intrinsic capacity to self-assemble into VLPs (for review Ulrich et al., 1998; Pumpens and Grens, 1999, 2003). We aimed to develop an efficient expression system suitable for generation of measles virus NLPs and determine, whether the MeN protein forms NLPs in yeast without the assistance of other measles virus gene products and RNA.

The yeast system offers many advantages relevant to the synthesis of NLPs. Yeast-derived heterologous proteins are free of toxic contaminations and are excellent tools for developing vaccines, diagnostics and gene delivery systems (McAleer et al., 1984; McMahon and Wainwright, 1993; Stowers et al., 2001; Samuel et al., 2002). The majority of recombinant proteins produced in yeast have been expressed using *S. cerevisiae* as a host system. Nowadays molecular biologists are familiar with this organism and a lot of data has been accumulated on its genetics and physiology. Furthermore, through its use in brewing and baking *S. cerevisiae* is acknowledged as GRAS (generally regarded as safe) organism. However, *S. cerevisiae* has been found to have certain limitations as a host for heterologous protein expression e.g. the yield of product usually is not high, except for a few notable exceptions. Furthermore, because synthesis of a cloned gene product places an additional stress on cells, the use of autonomously replicating expression plasmids has usually been found to result in poor plasmid stability during production runs. A heterologous protein yields maximum of 1–5% of total protein, even with a strong promoter (Valenzuela et al., 1982; Hinnen et al., 1994; Gellissen and Hollenberg, 1999; Dargeviciute et al., 2002; Sasnauskas et al., 2002). Hence, the MeN protein expression described here, together with human hepatitis B core antigen expressed with a yield of 40% of total soluble proteins described by Knickern et al. (1986) belong to the most abundant heterologous proteins expressed in the yeast *S. cerevisiae*. Methylotrophic yeast represents an attractive alternative because they possess strong, tightly regulated promoter elements and allow multiple copy integration of gene of interest, which contributes to its stability. Methylotrophic yeast expression systems based on *AOX* gene regulatory elements were characterised as most efficient among yeast. Moreover, the advanced fermentation technology has been developed for the production of biomass from methanol. Currently, methylotrophic yeast are commercially exploited for the production of foreign proteins. However, only few examples have been described concerning virus like particles formation in methylotrophic yeast (Cregg et al., 1987; Janowitz et al., 1991; Gellissen et al., 1995; Slibinskas et al., 2003).

In this study we have demonstrated, that yeast *P. pastoris* and *S. cerevisiae* are excellent hosts for a high-level production of MeN protein as NLPs. The results with a limited number of serum samples from acute measles cases and controls suggests that *P. pastoris* expressed MeN is likely to be as useful as the *S. cerevisiae* derived MeN described earlier (Samuel et al., 2003). Further, evaluation of the MeN from the two yeast expression systems in sero-diagnosis of measles is presently being conducted and will be reported elsewhere. The measles NLPs described above represent useful tools for the development of new virus detection systems and demonstrate the effectiveness of yeast as a host for generation of recombinant proteins organised in complex structures like human virus NLPs. Since antibodies to MeN are not considered to be protective, it is unlikely that the expressed protein will have use as a potential vaccine candidate. To summarise, we demonstrated the high level of MeN protein expression in both yeast genera, *S. cerevisiae* and *P. pastoris*, respectively.

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References

Clare, J.J., Romanos, M.A., Rayment, F.B., Rowedder, J.E., Smith, M.A., Paayne, M.M., Sreekrishna, K., Henwood, C.A., 1991. Production of mouse epidermal growth factor in yeast: high-level secretion using *Pichia pastoris* strains containing multiple gene copies. Gene 105, 205–212.

Cregg, J.M., Tschop, J.F., Sillman, C., Siegel, R., Akong, M., Craig, W.S., Buckholtz, R.G., Madden, K.R., Kellaris, P.A., Davis, G.R., Smiley, B.L., Cruze, J., Torregrossa, R., Velicelebi, G., Thill, G.P., 1987. High-level expression and efficient assembly of hepatitis B surface antigen in the methylotrophic yeast, *Pichia pastoris*. Biotechnology (New York) 5, 479–485.

Cregg, J.M., Russell, K.A., 1998. Transformation. In: Higgins, D.R., Cregg, J.M. (Eds.), Pichia Protocols. Methods in Molecular Biology, vol. 103, Humana Press, Totowa, NJ, pp. 27–39.

Dargeviciute, A., Brus-Sjölander, K., Sasnauskas, K., Krüger, D.H., Meisel, H., Ulrich, R., Lundkvist, Å., 2002. Yeast-expressed Puumala hantavirus nucleocapsid protein induces protection in a bank vole model. Vaccines 20, 3523–3531.

Faber, K.N., Harder, W., Geert, A.B., Veenhuis, M., 1995. Review: methylotrophic yeast as factories for the production of foreign proteins. Yeast 11, 1331–1344.

Fooks, A., Stephenson, J.R., Warnes, A., Dowsett, A.B., Rima, B.K., Wilkinson, G.W.G., 1993. Measles virus nucleocapsid protein expressed in insect cells assembles into nucleocapsid-like structures. J. Gen. Virol. 74, 1439–1444.

Gedvilaite, A., Frömmel, C., Sasnauskas, K., Micheel, B., Özel, M., Behrsing, O., Staniulis, J., Jandrig, B., Scherneck, S., Ulrich, R., 2000. Formation of immunogenic virus-like particles by inserting epitopes into surface–exposed regions of hamster polyomavirus major capsid protein. Virology 273, 21–35.

Gellissen, G., Hollenberg, C.P., Janowicz, Z.A., 1995. Gene expression in methylotrophic yeasts. Bioprocess Technol. 22, 195–239.

Gellissen, G., Hollenberg, C.P., 1999. Application of yeast in gene expression studies: a comparison of *Saccharomyces cerevisiae*, *Hansenula polymorpha* and *Kluyveromyces lactis*—a review. Gene 190, 87–97.

Griffin, D., 2001. Measles virus. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, fourth ed. Lippincott Williams & Wilkins Publications, Philadelphia, pp. 1401–1441.

Hale, A.D., Bartkeviciute, D., Dargeviciute, A., Jin, L., Knowles, W., Staniulis, J., Brown, D.W.G., Sasnauskas, K., 2002. Expression and antigenic characterization of the major capsid proteins of human polyomaviruses BK and JC in *Saccharomyces cerevisiae*. J. Virol. Methods 104, 93–98.

Hinnen, A., Buxton, F., Chaudhuri, B., Heim, J., Hottiger, T., Meyhack, B., Pohlig, G., 1994. Gene expression in recombinant yeast. In: Smith, A. (Ed.), Gene Expression in Recombinant Microorganisms. Marcel Dekker, New York, pp. 121–193.

Hofmann, K.J., Neeper, M.P., Marcus, H.Z., Brown, D.R., Muller, M., Jansen, K.U., 1996. Sequence conservation within the major capsid protein of human papillomavirus (HPV) type 18 and formation of HPV-18 virus-like particles in *Saccharomyces cerevisiae*. J. Gen. Virol. 77, 465–468.

Jacobs, E., Rutgers, T., Voet, O.P., Dewerchin, M., Cabezon, T., de Wilde, M., 1989. Simultaneous synthesis and assembly of various hepatitis B surface proteins in *Saccharomyces cerevisiae*. Gene 80, 279–291.

Janowitz, A.Z., Melber, K., Merckelbach, A., Jacobs, E., Harford, N., Comberbach, M., Hollenberg, C.P., 1991. Simultaneous expression of the S and L surface antigens of hepatitis B, and formation of mixed particles in the methylotrophic yeast, *Hansenula polymorpha*. Yeast 7, 431–443.

Kaufman, R.J., 1999. Stress signalling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational control. Genes. Dev. 13, 1211–1233.

Knickern, P.J., Hagopian, A., Montgomery, D.L., Burke, P., Dunn, N.R., Hofmann, K.J., Miller, W.J., Ellis, R.W., 1986. Unusually high-level expression of a foreign gene (hepatitis B virus core antigen) in *Saccharomyces cerevisiae*. Gene 46, 135–141.

McAleer, W.J., Buynak, E.B., Maigetter, R.Z., Wampler, D.E., Miller, W.J., Hilleman, M.R., 1984. Human hepatitis B vaccine from recombinant yeast. Nature 307, 178.

McMahon, B., Wainwright, R., 1993. Protective efficacy of hepatitis B vaccines in infant's children and adults. In: Ellis,

R. (Ed.), Hepatitis B vaccine in clinical practice. Dekker, New York, pp. 243–261.

Miyanohara, A., Imamura, T., Araki, M., Sugawara, K., Otomo, N., Matsubara, K., 1986. Expression of hepatitis B virus core antigen gene in *Saccharomyces cerevisiae*: synthesis of two polypeptides translated from different initiation codons. J. Virol. 59, 176–180.

Pumpens, P., Grens, E., 1999. Hepatitis B core particles as a universal display model: a structure-function basis for development. FEBS Lett. 442, 1–6.

Pumpens, P., Grens, E., 2003. Artificial gene for chimeric virus-like particles. In: Khudakov, Y.E., Fields, H.A. (Eds.), Artificial DNA. Methods and Applications. CRC Press, New York, pp. 249–327.

Rima, B.K., 1983. The proteins of morbilliviruses. J. Gen. Virol. 64, 1205–1219.

Romanos, M.A., Scorer, C.A., Clare, J.J., 1992. Foreign gene expression in yeast: a review. Yeast 8, 423–488.

Romanos, M., Scorer, K., Sreekrishna, K., Clare, J., 1998. The generation of multicopy recombinant strains. In: Higgins, D.R., Cregg, J.M. (Eds.), Pichia protocols. Methods in Molecular Biology, vol. 103, Humana Press, Totowa, NJ, pp. 55–72.

Sambrook, J., Russell, D.W., 2001. Molecular cloning, A Laboratory Manual, third ed. CSHL Press, Cold Spring Harbor, New York.

Samuel, D., Sasnauskas, K., Jin, L., Beard, S., Zvirbliene, A., Gedvilaite, A., Cohen, B., 2002. High level expression of recombinant mumps nucleoprotein in *Saccharomyces cerevisiae* and its evaluation in mumps IgM serology. J. Med. Virol. 66, 123–130.

Samuel, D., Sasnauskas, K., Jin, L., Gedvilaite, A., Slibinskas, R., Beard, S., Žvirblienë, A., Artimos, S., Staniulis, J., Cohen, B., Brown, D., 2003. Development of a measles specific IgM ELISA for use with serum and oral fluid using recombinant measles nucleoprotein produced in *Saccharomyces cervisiae*. J Clin. Virol. 28, 121–129.

Sasnauskas, K., Jomantiene, R., Januška, A., Lebediene, E., Lebedys, J., Janulaitis, A., 1992. Cloning and analysis of a *Candida maltosa* gene, which confers resistance to formaldehyde in *Saccharomyces cerevisiae*. Gene 122, 207–211.

Sasnauskas, K., Buzaite, O., Vogel, F., Jandrig, B., Razanskas, R., Staniulis, J., Scherneck, S., Krüger, D.H., Ulrich, R., 1999. Yeast cells allow the high-level expression and formation of polyomavirus—virus like particles. Biol. Chem. 380, 381–386.

Sasnauskas, K., Bulavaite, A., Hale, A., Jin, L., Knowles, W., Gedvilaite, A., Dargeviciute, A., Bartkeviciute, D., Zvirbliene, A., Staniulis, J., Brown, D.W.G., Ulrich, R., 2002. Generation of recombinant virus-like particles of human and non-human polyomaviruses in yeast *Saccharomyces cerevisiae*. Intervirology 45, 308–317.

Slibinskas, R., Zvirblienë, A., Gedvilaite, A., Samuel, D., Jin, L., Beard, S., Staniulis, J., Brown, D., Sasnauskas, K., 2003. Synthesis of mumps virus nucleocapsid protein in yeast *Pichia pastoris*. J. Biotechnol. 103, 43–49.

Spehner, D., Kim, A., Drillien, R., 1991. Assembly of nucleocapsid-like structures in animal cells infected with a vaccinia virus recombinant encoding the measles virus nucleoprotein. J. Virol. 65, 6296–6300.

Spehner, D., Drillien, R., Howley, P.M., 1997. The assembly of the measles virus nucleoprotein into nucleocapsid-like particles is modulated by the phosphoprotein. Virology 232, 260–268.

Stowers, A.W., Zhang, Y., Shimp, R.L., Kaslow, D.C., 2001. Structural conforms produced during malaria vaccine production in yeast. Yeast 18, 137–150.

Thill, G.P., Davis, G.R., Stillman, C., Holtz, G.C., Brierley, R., Engel, M., Buckholthz, R., Kinney, J., Provow, S., Vedvick, T., Siegel, R.S., 1990. Positive and negative effects of multi-copy integrated expression vectors on protein expression in *Pichia pastoris*. In: Heslot, H., Davies, J., Florent, J., Bobishon, L., Durand, G., Parnasse, L. (Eds.), Proceedings of the Sixth International Symposium on Genetics of Micro-organisms, vol. II. Societe Francaise de Microbiologie, Paris, pp. 477–490.

Thorne, H.V., Dermott, E., 1976. Circular and elongated linear forms of measles virus nucleocapsid. Nature (Lond.) 264, 473–474.

Ulrich, R., Nassal, M., Meisel, H., Kruger, D.H., 1998. Core particles of hepatitis B virus as carrier for foreign epitopes. Adv. Virus Res. 50, 141–182.

Valenzuela, P., Medina, A., Rutter, W.J., Ammerer, G., Hall, B.D., 1982. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. Nature 298, 347–350.

Warnes, A., Fooks, A.R., Stephenson, J.R., 1994. Production of measles nucleoprotein in different expression systems and its use as a diagnostic reagent. J. Virol. Methods 49, 257–268.

Warnes, A., Fooks, A.R., Dowsett, A.B., Wilkinson, G.W., Stephenson, J.R., 1995. Expression of the measles virus nucleoprotein gene in *Escherichia coli* and assembly of nucleocapsid-like structures. Gene 160, 173–178.

Wickner, S., Maurizi, M.R., Gottesman, S., 1999. Posttranslational quality control: folding, refolding, and degrading proteins. Science 286, 1888–1893.