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YEAST-DERIVED VIRUS-LIKE PARTICLES AS COMPONENTS OF NOVEL VACCINE PROTOTYPES

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CONTENTS

ABBREVIATIONS

aa – amino acid(s)
AOX – alcohol oxidase
AEC – anion-exchange chromatography
BMGY – buffered complex glycerol medium
CP – coat protein
CBB – Coomassie brilliant blue
DEAE – diethylaminoethanol
DHAP – dihydroxyacetone phosphate
DNA – deoxyribonucleic acid
FMD – format dehydrogenase
HBV – human hepatitis B virus
HBc – hepatitis B virus core protein
HBe – hepatitis B virus e protein
HBs – hepatitis B virus S protein
MALDI - matrix assisted laser desorption/ionization
MOX – methanol oxidase
MW – molecular weight
OD – optical density
PAAG – polyacrylamide gel
PCR – polymerase chain reaction
PEG – polyethylene glycol
RNA – ribonucleic acid
RDB – regeneration dextrose medium
SDS-PAGE – sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SEC – size-exclusion chromatography
TEF – translation elongation factor
YEPD – yeast extract peptone dextrose medium

SUMMARY

Heterologous expression of viral structural genes often leads to formation of non-infectious virus-like particles (VLPs), which exhibit morphology, antigenicity, and cell tropism similar to the native viruses. In recent years, VLPs have been used for a wide range of applications, both in basic and applied research, but especially in vaccine development. Non-modified (wild-type) or chimeric (exposing defined antigenic epitopes) VLPs with packaged nanomaterials are recently evaluated as components of novel vaccines and vaccine prototypes.

In this work, yeast expression system was adapted for generation of (i) the bacteriophage (phage) VLPs composed from RNA phage coat proteins (CPs) of different origin and (ii) hepatitis B virus (HBV) core (nucleocapsid) particles arranged from the HBV core protein (HBc). Seven different phages were tested for their CP gene expression and VLP formation in yeast *S. cerevisiae* and *P. pastoris*, namely, coliphages Qβ, SP, fr, GA, *Acinetobacter* phage AP205, *Pseudomonas* phage PP7 and *Caulobacter* phage φCb5. In all cases except phage SP VLP formation was detected although synthesis level was very different. The highest synthesis was observed for phage PP7 VLPs (about 8 mg from 1 g of wet cells), and it was found also scalable for fermentation conditions. This was the first report of abovementioned phage VLP production in yeast.

High-level fermentor production was established for HBc VLPs in *P. pastoris* and rapid purification scheme was developed leading to outcome of 3 mg protein from 1 g of wet cells. Mass spectrometry analysis and direct polyacrylamide gel staining revealed the HBc as a phosphoprotein. This was the first report of HBc phosphorylation in yeast.
Another two promising yeast expression systems have been successfully introduced in laboratory practice, namely, *Kluyveromyces lactis* and *Hansenula polymorpha*. A set of new vectors for expression in yeast *H. polymorpha* was constructed. For testing capacity of the new vectors, HBe and HBs genes were used. Synthesis of HBs in *H. polymorpha* resulted in formation of 22 nm particles.

IMPORTANCE, AIM AND OBJECTIVES OF THE STUDY

Generation of pure and functionally active proteins is one of the main challenges of the modern biotechnology. These proteins can be then used for diagnostic and structural purposes as well as for development of novel vaccines and vaccine prototypes. Due to the different origin and high diversity of proteins, it is important to establish a broad host range for their successfull expression.
Only minor part of eukaryotic proteins can be functionally produced in bacterial cells. Yeast as the simpliest eukaryotic organism offers an attractive alternative to quite expensive and non-effective mammalian cell culture systems. Cultivation of yeast cells is relatively simple and material expenses are comparable with bacterial systems. Another benefit of the yeast system is the lack of bacterial endotoxins which need to be removed prior human use. Thus, handling with yeast expression systems is important to obtain functional proteins of different origin.
With development of new expression strategies, the number of purified yeast-derived proteins dramatically increases. Among them, significant part comprises viral structural proteins being capable of self-assembly in VLPs morfologically and immunologically indistinquishable from native viruses or their shells. Modern commercial vaccines against HBV and human papillomavirus have been formulated from yeast-derived VLPs. A number of VLP-based studies are now under different clinical stages of investigation.

The main aim of this work was to establish a broad-range yeast expression system in Laboratory of Protein Engineering of BMC to obtain preparative amounts of VLPs for downstream applications. To achieve this goal, the following objectives have been proposed:

- generation of system for intracellular synthesis of VLPs in yeast *Saccharomyces cerevisiae* and *Pichia pastoris*,
- optimization of expression conditions and comparison of effectivity,
- adaptation of yeast VLP producer strains for large-scale fermentation,
- development of VLP purification methods from yeast and characterization of the final product,
- construction of new vectors for expression in yeast *Hansenula polymorpha*

For particular investigation, two main VLP targets have been selected: (i) the bacteriophage capsids composed from RNA phage coat proteins of different origin and (ii) HBV core (nucleocapsid) particles arranged from HBc. Other HBV proteins were also used for experiments involving secretion studies (HBe) and testing capacity of new yeast vectors (HBs).

Construction of expression vectors was done by standard cloning techniques. In handling with yeast, well-described methods were combined with novel experimental findings for shortening of time-consuming protocols in many cases. A lot of experimental work has been performed to establish optimal protocols for yeast transformation, selection of superproducer clones and optimization of expression conditions. Besides standard analytic laboratory methods, a new approaches were developed to scale-up the process to preparative amounts (fermentation and cell disruption with the *French press* unit). For fractionation of proteins, classical chromatography methods were combined with novel FPLC techniques. Obtained bacteriophage and HBc VLPs could be further used for a number of applications including structural studies, packaging of nanomaterials, coupling of epitopes and gene delivery.

These thesis are written as a review of five original publications, where papers 1-4 deal with phage-derived VLPs while paper 5 describes generation of HBc particles in yeast.

GENERAL INTRODUCTION

Nowadays, highly immunogenic VLPs generated by heterologous expression of viral structural genes have become a powerful tool for vaccine development. In addition to being effective vaccines against the corresponding virus from which they were derived, VLPs can also be used to present foreign epitopes to the immune system (Jennings and Bachmann, 2008, Buonaguro *et al*., 2011). This approach might be combined with the packaging of selected genes, drugs and proteins inside VLPs (see Xu *et al*., 2006, Kaczmarczyk *et al*., 2011, Ma *et al*., 2012). Furthermore, targeted VLPs can be generated by including specific peptide ligands as "molecular addresses" on the particle surface (Yildiz *et al*., 2011).

The RNA bacteriophages (phages) are small viruses with a simple organization. Their *T*=3 icosahedral shell is composed from 180 copies of the CP and one copy of the maturation protein that encapsidate approximately 3,500 nucleotide long genomic RNA. These phages were first isolated from *Escherichia coli* (Loeb and Zinder, 1961), but later were also found in *Caulobacter* (Schmidt, 1966), *Pseudomonas* (Bradley, 1966) and *Acinetobacter* (Coffi, 1995). Icosahedral capsids of RNA phages have attracted the attention of scientists as promising carriers of foreign epitopes and nucleic acids. A special interest has been devoted to group III phage Qβ VLPs. Bacterially expressed Qβ VLPs have been explored for chemical coupling of desired peptides to surface-exposed lysine residues. To increase their immunogenicity, such chimeric VLPs can be further engineered by loading them with short synthetic DNA sequences (Storni *et al*., 2004). Several Qβ phage-derived therapeutic vaccine candidates against nicotine addiction and hypertension have entered clinical trials (Cornuz *et al*., 2008, Tissot *et al*., 2008). Another set of vaccine prototypes targeting West Nile and influenza virus infections has been recently constructed using chimeric phage AP205 VLP platform (Spohn *et al*., 2010, Tissot *et al*., 2010).

Another very promising VLP carrier is the HBV core protein which forms the inner shell (nucleocapsid) of the HBV. Heterologously expressed HBc spontaneously assembles into the particles that are routinely used for HBV diagnostics worldwide (Stahl *et al*., 1982). The ability of HBc carrier to provide inserted epitopes with T-cell help (Fehr *et al*., 1998) and preferential priming of Th1 cells, without any requirement for adjuvants (Milich *et al*., 1997), stimulates the development of a broad range of vaccine prototypes on the basis of HBc VLPs (Pumpens *et al*., 2008). Recently, HBc

has attracted special interest in medicinal nanotechnology as a putative packager of organic and inorganic compounds, including stimulatory oligonucleotides, low molecular weight drugs, and magnetic particles.

For vaccine development, *E. coli*-derived VLPs need to be purified from contamination of bacterial endotoxins, which is costly and time consuming process. Alternatively, VLPs could be produced in "endotoxin-free" organisms, such as yeast, which has been regarded as generally safe for human use. Up to now, a large number of structural genes from mammalian viruses have been expressed in yeast resulting in formation of VLPs (Valenzuela *et al*., 1982, Sasnauskas *et al*., 1999; Samuel *et al*., 2002; Slibinskas *et al*., 2004; Juozapaitis *et al*., 2007). The yeast expression system has been used successfully to produce licensed prophylactic vaccines against human HBV (see Pumpens *et al*., 2008) and human papillomavirus (HPV; Bryan, 2007).

Saccharomyces cerevisiae is perhaps the most useful species of yeast, having been instrumental to winemaking, baking and brewing since ancient times. It is one of the most intensively studied eukaryotic model organisms in molecular and cell biology, much like *Escherichia coli* as the model bacterium (Pausch *et al*., 2005). A large number of *S. cerevisiae* expression systems has been constructed. The first human vaccines against HBV and HPV were produced namely in *S. cerevisiae*. Discovery of the **methylotrophic yeasts** being able to obtain all the carbon and energy needed for growth from methanol, has led to construction of heterologous gene expression systems driven by strong methanol-inducible promoters in a number of methylotrophic yeast strains, including ***Pichia pastoris***, ***Hansenula polymorpha*** *(Pichia angusta)*, *Pichia methanolica*, and *Candida boidinii*. Increasing industrial and academic use of these expression systems allowed the heterologous production of a large number of proteins including enzymes, antibodies, cytokines, plasma proteins, and hormones. Some of the advantages of these systems include (i) cheap synthetic salt-based media for growing the yeast, (ii) strong and tightly regulated promoters induced by methanol and repressed by glucose or ethanol, and (iii) the fact that the processes of protein folding, secretion, and other functions in these yeasts are similar in many respects to the same processes in higher eukaryotes (Gellissen, 2000). One of the most prominent yeast expression systems provided by *Invitrogen* is based on the methylotrophic *P. pastoris* strain, which has been used successfully to produce more than 500 proteins both for basic laboratory research and industrial manufacturing (Macauley-Patrick *et al*., 2005; Böer *et al*., 2007). Another highly effective yeast expression platform for the production of recombinant proteins on an industrial scale is based on methylotropic yeast *H. polymorpha* (Gellissen, 2002) and is now provided by *Artes Biotechnology*. Recently, *New England Biolabs* has developed yeast ***Kluyveromyces lactis*** expression system generally for secretion of proteins. *K. lactis* is not methylotropic but instead can utilize lactose as a sole carbon source. In this system, heterologous genes are expressed under strong inducible *LAC4* promoter (van Ooyen *et al*., 2006).

MATERIALS AND METHODS

Yeast strains

Saccharomyces cerevisiae
AH22 *MATa leu2 his4*
DC5 *MATa leu2 his3*
FH4C wild type
YPH499 *ura3–52 lys2–801*^{amber} *ade2–101*^{ochre} *trp1–Δ63 his3–Δ200 leu2–Δ1* (Stratagene)
S. cerevisiae strains AH22, DC5 and FH4C as well as the expression plasmid pFX7 was a kind gift of Dr. K. Sasnauskas (Institute of Biotechnology, Lithuania)
Pichia pastoris **GS115** *his4* (*Invitrogen*)
Kluyveromyces lactis **GG799** wild type (*New England Biolabs*)
Hansenula polymorpha **NCYC 495** *leu1-1* (Kind gift of Dr. A. Sibirny, Institute of Cell Biology, Ukraine)

Restriction enzymes, markers and kits

All the enzymes, protein and DNA MW markers as well as kits used in this work were purchased from Fermentas (Vilnius, Lithuania) unless otherwise indicated.

Expression vectors

Basic vectors for this study are listed in Table 1. These vectors were used for cloning of target genes described in Table 2.

Table 1. Basic expression vectors

Plasmid	Selection markers (*E.coli*/yeast)	Integration site(s)	Promoter(s) for expression in yeast	Signal sequence	Reference/ manufacturer
pFX7	Ap/*FDH1*	-	*GAL10-PYK1*	-	Sasnauskas *et al*., 1999
pESC-URA	Ap/*URA3*	-	*GAL1, GAL10*	-	*Stratagene*
pPIC3.5K	Ap/*HIS4*	*AOX1, HIS4*	*AOX1*	-	*Invitrogen*
pPIC9K	Ap/*HIS4*	*AOX1, HIS4*	*AOX1*	αMF	*Invitrogen*
pKLAC1	Ap/*amdS*	*LAC4*	*LAC4*	αMF	*New England Biolabs*
pKLAC1_ malE	Ap/*amdS*	*LAC4*	*LAC4*	αMF	*New England Biolabs*
pHIPX2	Em/*LEU2*	*MOX*	*MOX*	-	Faber *et al*., 1994
pTEF1/Zeo	Ap/Zeo	-	-	-	*Invitrogen*
pHaTEF	Ap/Zeo	HaRNA	*TEF*	-	This work
pHaMOX	Ap/Zeo	HaRNA	*MOX*	-	This work
pHaFMD	Ap/Zeo	HaRNA	*FMD*	-	This work

Selection markers encoding antibiotic resistance genes: Ap, ampicillin, Zeo, zeocin, Em, erithromycin. HaRNA, ribosomal RNA locus from *H. polymorpha* genome.

Table 2. Created expression vectors

Basic vector	Expressed gene	GeneBank acc. No/Reference	Construction
pFX7	fr CP	X15031; Adhin *et al*., 1990	pFX-fr^{Sc}
pFX7	GA CP	X03869; Inokuchi *et al*., 1986	pFX-GA^{Sc}
pFX7	Qβ CP	PQM99039; Kozlovska *et al*., 1993	pFX-Qβ^{Sc}
pFX7	SP CP	X07489; Inokuchi *et al*., 1988	pFX-SP^{Sc}
pFX7	AP205 CP	AF334111; Klovins *et al*., 2002	pFX-AP^{Sc}
pFX7	PP7 CP	X80191; Olsthoorn *et al*., 1995	pFX-PP7^{Sc}
pFX7	φCb5 CP	HM066936; Kazaks *et al*., 2011	pFX-Cb^{Sc}
pPIC3.5K	fr CP	X15031; Adhin *et al*., 1990	pPIC-fr^{Pp}
pPIC3.5K	GA CP	X03869; Inokuchi *et al*., 1986	pPIC-GA^{Pp}
pPIC3.5K	Qβ CP	PQM99039; Kozlovska *et al*., 1993	pPIC-Qβ^{Pp}
pPIC3.5K	SP CP	X07489; Inokuchi *et al*., 1988	pPIC-SP^{Pp}
pPIC3.5K	AP205 CP	AF334111; Klovins *et al*., 2002	pPIC-AP^{Pp}
pPIC3.5K	PP7 CP	X80191; Olsthoorn *et al*., 1995	pPIC-PP7^{Pp}
pPIC3.5K	φCb5 CP	HM066936; Kazaks *et al*., 2011	pPIC-Cb^{Pp}
pPIC3.5K	HBc	X02496; Bichko *et.al*., 1985	pPIC-HBc^{Pp}
pPIC9K	HBe	X02496; Bichko *et.al*., 1985	pPIC-HBe^{Pp}
pPIC3.5K	HBs	X02496; Bichko *et.al*., 1985	pPIC-HBs^{Pp}
pKLAC1	HBc	X02496; Bichko *et.al*., 1985	pKLAC-HBc^{Kl}
pKLAC1	HBe	X02496; Bichko *et.al*., 1985	pKLAC-HBe^{Kl}
pTEF1/Zeo	HBc	X02496; Bichko *et.al*., 1985	pHaMOX-HBc^{Hp}
pTEF1/Zeo	HBc	X02496; Bichko *et.al*., 1985	pHaTEF-HBs^{Hp}
pTEF1/Zeo	HBs	X02496; Bichko *et.al*., 1985	pHaMOX-HBs^{Hp}
pTEF1/Zeo	HBs	X02496; Bichko *et.al*., 1985	pHaFMD-HBs^{Hp}

(^{Sc}) *S. cerevisiae* expression plasmids, (^{Pp}) *P. pastoris* expression plasmids, (^{Kl}) *K. lactis* expression plasmids, (^{Hp}) *H. polymorpha* expression plasmids

Yeast transformation

Two types of shuttle vectors were used: replicative (for expression in *S. cerevisiae*) and integrative (for expression in *P. pastoris*, *K. lactis* and *H. polymorpha*). Replicative vectors contain yeast replication origin and can maintain in cells as episomes. Integrative vectors should be linearized before transformation in a way to achieve their integration into yeast genome through the homologous recombination.

Transformation of S. cerevisiae and K. lactis

Transformation was done according to standard lithium acetate/PEG transformation procedure (Gietz *et al*., 1992) with some modifications. Yeast cultures of *S. cerevisiae and K. lactis* were grown in non-selective YEPD medium (1% yeast extract, 2% peptone, 2% dextrose) overnight without shaking until optical density OD_{590} 1-1.2. Cells were harvested by centrifugation at 2000 *g* for 4 min, washed once with 1 volume (initial culture) ice cold dH_2O, resuspended in 0.02 vol of 0.1 M lithium acetate and incubated for 15 min on ice. 60 μL aliquots of competent cells were prepared. For the transformation reaction, following components were added to one aliquot of cells (in the indicated order):

240 μL 50% PEG 4000,

36 μL 1 M lithium acetate,

15 μL DNA (2-5 μg) containing bacterial RNA,

dH_2O until 400 µL.
Transformation mix was incubated for 1 hour at 30°C following heat shock for 30 min at 42°C. 1 mL of YEPD medium was added and mixture was incubated overnight at 30°C. Transformants were selected on selective YEPD agar plates containing 10 mM formaldehyde (*S. cerevisiae*) or 5 mM acetamide (*K. lactis*). Transformants became visible approximately after 2-3 days.

Transformation of H. polymorpha and P. pastoris
H. polymorpha and *P. pastoris* cultures were grown in non-selective YEPD medium without shaking overnight at 37°C (*H. polymorpha*) or 30°C (*P. pastoris*) until OD_{590} 0.8-1.2. Cells were harvested by centrifugation at 2000 *g* for 4 min, resuspended in 0.2 volume (initial volume) of 50 mM potassium phosphate buffer (6.6 mM K_2HPO_4, 43.4 mM KH_2PO_4, pH 7.5) containing 25 mM dithiotreitol and incubated for 30 min at 37°C or 30°C (*H. polymorpha* or *P. pastoris*, respectively). Subsequently, the cells were washed twice with 1 vol of ice-cold electroporation buffer (270 mM sucrose, 10 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.5). Finally, cells were resuspended in 0.005 vol of electroporation buffer. 20 µL of prelinearized DNA (5-10 µg) containing bacterial RNA was mixed with 80 µL of competent cell suspension into prechilled 1 mm electroporation cuvette and incubated for 15 min on ice. 1200 V electric pulse was gived to DNA/cell mixture, following addition of 1 mL YEPD (room temperature) and incubation 1 h at 37°C or 30°C (*H. polymorpha* or *P. pastoris*, respectively). In a case of *P. pastoris*, transformation mixture was washed with dH_2O and spread on selective RDB (1% ammonium sulphate, 0.34% yeast nitrogen base, 2% dextrose, $4x10^{-5}$ biotin, 0.005% amino acid solution without histidine) agar plates. In a case of *H. polymorpha*, transformation mixture was directly spread on YEPD agar plates containing 20 µg/mL zeocin. Plates were incubated at 37°C or 30°C, respectively, and transformants were selected approximately after 2-3 days.

Selection of clones containing multiple expression units
For *K. lactis*, cultivation of transformants on acetamide plates resulted in naturally selected multicopy clones with more than 50% probability. Presence of multiple expression units was verified by PCR according to the protocol of the manufacturer.
For *H. polymorpha* and *P. pastoris*, multicopy clones were selected as follows: transformants were pooled by washing from agar plates with ~2 mL of YEPD medium and then spread on selective YEPD agar plates containing 50-100 µg/mL zeocin (*H. polymorpha*) or 1.0-4.0 mg/mL geneticin (*P. pastoris*). Higher resistance to appropriate antibiotics typically correlated with more integrated expression units.

Isolation of yeast genomic DNA

10 optical units of yeast cells (at A=590 nm) were mixed with 500 µL lysis buffer (100 mM Tris-HCl, 50 mM EDTA, 1% SDS, pH 8.0) containing 25 µg RNase A and 0.3 volumes of glass beads (425-600 µm). After vortexing 2 min, 275 µL 7 M ammonium acetate pH 7.0 was added and mixture was incubated 5 min at 65°C and subsequently 5 min on ice. 500 µL of chloroform was added, vortexed and spun down for 2 min at top speed. 500 µL of supernatant was precipitated with 1 mL isopropanol for 5 min at -20°C and spun down for 10 min at top speed. Pellet was washed with 70% ethanol, air-dried and dissolved in 50 µL dH_2O.

Southern blotting

To estimate the approximate expression cassette copy number in *P. pastoris*, chromosomal DNA from selected transformants was digested with *Bgl*II and separated by agarose gel electrophoresis. After transferring to a nitrocellulose membrane, DNA was hybridized with a biotin-labeled *HIS4*-specific probe (600-bp *Kpn*I restriction fragment from the pPIC3.5K) using the BiotinDecaLabel™ DNA Labeling Kit. The reaction was further processed and developed by the Biotin Chromogenic Detection Kit.
Alternatively, for simplified dot-blot assay, 5 µL of genomic DNA was directly transferred to a nitrocellulose membrane. After DNA denaturation in buffer (1.5 M NaCl, 0.5 M NaOH), the reaction was processed as described above.

Yeast cultivation in flasks

To prepare a start culture (an inoculum), an individual colony was picked in 5 mL selective YEPD medium and incubated at 30°/37°C overnight without shaking. Further cultivation was done in 2 L Erlenmeyer flasks with 200 ml of medium on *Infors* shaker in strain-depending conditions.

S. cerevisiae
Start culture was diluted in YEPD medium containing 3% (w/v) galactose and 2.5 mM formaldehyde until OD_{590} ~0.05. Flasks were incubated at 29-30°C on a shaker with 200 rpm. After every 24 h solid galactose was added to 2%. The cells were cultivated overall for 72 h, with final OD_{590} 12–14.
For expression in strain YPH499, individual transformants were cultivated in liquid synthetic galactose (2% galactose, 0.67% yeast nitrogen base, 0.13% aa dropout powder without uracil) for up to 72 h, until OD_{590} reached 6-7.

K. lactis
Expression in *K. lactis* was basically done according to the protocol of the manufacturer (www.neb.com) with the following minor modifications: start culture was diluted in YEPG medium (1% yeast extract, 2% peptone, 3% galactose) until OD_{590} ~0.05 and incubated at 30°C on a shaker with 250 rpm. The cells were cultivated overall for 48-72 h.

P. pastoris and *H. polymorpha*
Expression in *P. pastoris* and *H. polymorpha* was basically done according to the *P. pastoris* protocol of the manufacturer (www.invitrogen.com) with the following minor modifications: start culture was diluted until OD_{590} ~0.05 in BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0 combined from 13.2 mM K_2HPO_4 and 86.8 mM KH_2PO_4, 1.34% yeast nitrogen base, $4x10^{-5}$% biotin, 1% glycerol) and incubated at 30°C (*P. pastoris*) or 37°C (*H. polymorpha*) on a shaker with 250 rpm. After 24 h at OD_{590} 6–8 expression was induced with addition of 1% methanol. Each subsequent day, methanol was added to a final concentration of 2%. The cells were harvested 72 h (*P. pastoris*) or 48 h (*H. polymorpha*) after induction.

Yeast *P. pastoris* cultivation in fermentor

A volume of 500 mL of seed material was used to inoculate 4.5 L of BMGY in a fermentor. Fermentation conditions: a 10-L fermentor (Bioflo 410, New Brunswick Scientific) was filled with 4.5 L BMGY (with glycerol concentration 40 g/L) and 0.5 L seed material in BMGY. Non-enriched air was used throughout the fermentation. The dissolved oxygen was set at 20%, the aeration rate on the first day was up to 1 vol/min, and the stirring speed was up to 1200 rpm, while the incubation temperature was 30 °C, and the pH was controlled with 25% (v/v) NH_4OH to keep the pH above 5.0. After the glycerol exhaustion at 18 h, 20 mL of 50% glycerol and 10 mL of 100% methanol was added. One hour later, the methanol supply was set at a rate of 3.0 mL/L/h, and the air supply was increased to 5 vol/min. Cultivation continued for 92 h with methanol feeding adjusted to a consumption rate of 6.8 g/L/h. Antifoam M30 (Serva) was used to prevent extensive foam formation. Cells were harvested at 3000 *g* for 10 min at 4°C. After washing once with dH_2O, the cell pellet was stored at −80°C until use.

Analytical methods

SDS-PAGE and Western blotting

Preparation of yeast protein extracts for SDS-PAGE was done essentially as described by Kushnirov (2000), with minor modifications. Briefly, 4 optical units of yeast cells were resuspended in 200 µL 0.1 M NaOH and incubated for 5 min at room temperature. The cells were pelleted and resuspended in 250 µL SDS sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-ME, 0.01% bromphenol blue), boiled for 6-8 min and pelleted again.

Protein separation by SDS–PAGE (with a 4% stacking and 15% separating PAAG) was carried out in a Tris-glycine buffer (400 mM Tris-HCl, 182 mM glycine, 0.1% SDS), according to standard protocols. To visualize protein bands, the gels were stained with CBB G-250 (0.005% G-250, 2% trichloroacetic acid, 20% ethanol). Alternatively, separated proteins were electrophoretically transferred onto nitrocellulose membranes and detected by immunoblotting with the specific monoclonal antibodies and the anti-mouse IgG peroxidase conjugate.

To detect phosphorylated proteins, PAAG was stained directly with the ProQ® Diamond phosphoprotein gel stain (*Invitrogen*) according to the manufacturer's instructions. Full-length HBc molecules as well as their C-terminal domains resulting from proteinase K cleavage were analyzed. For the proteinase K reaction, VLPs were treated for 10 min at 95 ºC in buffer containing 1% SDS and 2% β-mercaptoethanol followed by the addition of proteinase K and incubation for 5 min at 50 ºC.

Preparation of yeast lysates

For analytic purposes (e.g., Ouchterlony's immunodiffusion and protein solubility test), 20 optical units of yeast cells were resuspended in 200 µL lysis buffer (typically, 20 mM Tris-HCl, pH 8.0, containing 1 mM PMSF). 0.3 volumes of glass beads (425-600 µm) were added and the mixture was homogenized by vortexing 6 times per 30 s, with 1 min chilling on ice after each cycle. The soluble fraction was separated by centrifugation for 7 min at 13,400 *g*.

For preparative purposes (e.g., VLP purification), yeast cells were resuspended in lysis buffer (1 g of cells per 4 mL lysis buffer) and disrupted with a French press (3

cycles, 20,000 psi). The soluble fraction was separated by centrifugation for 30 min at 15,500 *g*.

Electron microscopy and masspectrometry

For electron microscopy, the protein samples were adsorbed on carbon-formvar-coated copper grids and negatively stained with 1% uranyl acetate aqueous solution. The grids were examined with a JEM-1230 electron microscope (JEOL Ltd., Tokyo, Japan) at 100 kV.

For whole protein analysis in mass spectrometry, 2 µl of purified VLPs at 1 mg/mL concentration in 20 mM Tris-HCl, pH 8.0, was mixed with 2 µl 10% trifluoracetic acid and 2 µl DHAP. For tryptic digestion of proteins, the CBB-stained band was excised from SDS-PAAG, incubated for 2x1 h in 0.2 M ammonium bicarbonate and 50% acetonitrile at 30 °C and incubated for 2x20 min in 100% acetonitrile at room temperature. A volume of 0.1 mg/mL trypsin (Sigma, proteomics grade) in 1 mM HCl was mixed 1:1 with 50 mM ammonium bicarbonate in 10% acetonitrile. Gel pieces were covered with the trypsin solution so that the gel absorbed approximately two thirds of the solution's volume. The gel was further incubated for 3 hours at 30 °C. A volume of 2 µl of buffer covering the gel pieces was mixed with 2 µl 10% trifluoracetic acid and 2 µl DHAP solution (15 mg/mL DHAP in 75% ethanol, 2.5 mM diammonium hydrogen cytrate). All samples were analyzed on a Bruker Daltonics Autoflex MALDI-TOF mass spectrometer.

Characterization of VLPs

The endotoxin level in the protein samples was determined by a *Limulus* amoebocyte lysate (LAL) Pyrogent® Plus test kit according to the manufacturer's protocol (BioWhittaker, Walkersville, MD).

Protein samples were subjected to native 1% agarose gel electrophoresis in TAE buffer (pH 8.4) for about 0.5 h at 5 V/cm. Nucleic acids in agarose gels were visualized by ethidium bromide staining. Protein concentration was determined by formula $OD(A_{260})/8$, which has been used to determine concentrations of native phages. The purity of protein samples was estimated by densitometric analysis of the CBB-stained PAAG.

RESULTS AND DISCUSSION
I Expression of bacteriophage CP genes in yeast

1. Assembly of bacteriophage Qβ virus-like particles in yeast *Saccharomyces cerevisiae* and *Pichia pastoris* (paper 1)

Phage Qβ CP gene was the first our object which has been used as a standard for yeast expression studies. Two shuttle vectors were generated: pFX-Qβ and pPIC-Qβ for expression in *S. cerevisiae* and *P. pastoris*, respectively. Three *S. cerevisiae* strains (AH22*,* DC5 and FH4C) were compared for their synthesis capacity. For expression in *P. pastoris*, five clones were selected among ~800 transformants with increased resistance against geneticin (G418). Synthesis of Qβ and VLP assembly were analyzed by SDS-PAGE, immunoblotting and by Ouchterlony's double radial immunodiffusion with rabbit polyclonal anti-Qβ antibodies (Fig. 1).

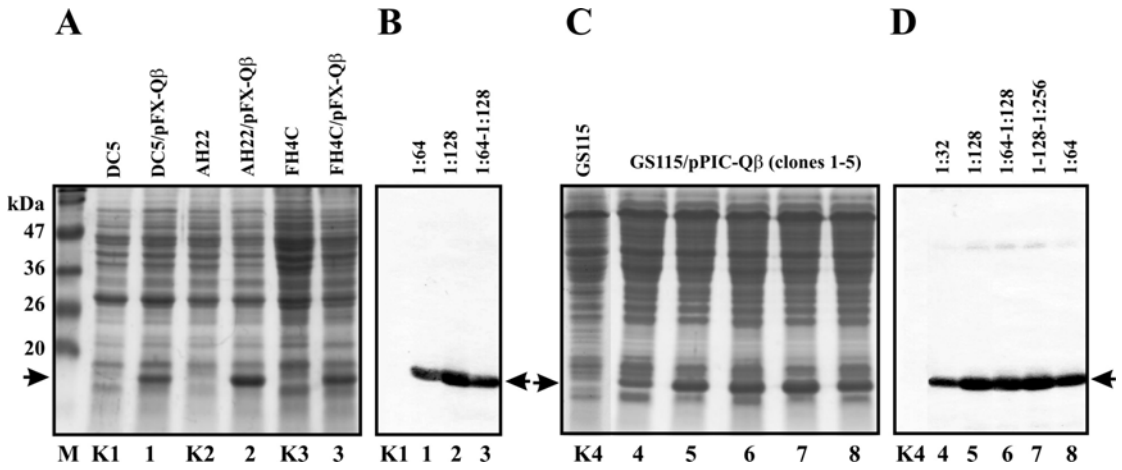

Figure 1. Expression of Qβ CP in *S. cerevisiae* and *P. pastoris*, estimated by CBB-stained 15% SDS-PAAG (A and C) and Western blotting with rabbit polyclonal anti-Qβ antibody (B and D). K1, K2, K3 in (A) represent non-transformed *S. cerevisiae* DC5, AH22, and FH4C cells, and 1–3—the same strains transformed with pFX-Qβ, respectively. (C) Qβ CP expression in *P. pastoris* clones with single (lane 4) and multiple (lanes 5–8) gene insertions. K4, non-transformed *P. pastoris* cells. The lower labels in (B) and (D) correspond to those in (A) and (C), respectively. The Ouchterlony immunodiffusion titres are indicated on the top of respective lines in Western blot. Arrows show the position of the Qβ CP.

Production of Qβ CP was slightly better in the *S. cerevisiae* AH22 and FH4C strains as shown by SDS-PAGE and immunoblotting. Ouchterlony double radial immunodiffusion visible immunoprecipitation formed lines at dilution up to 1:64, but lysates from the *S. cerevisiae* AH22 and *S. cerevisiae* FH4C strains - up to 1:128 (see Fig. 1A and B). Significant differences in the expression level of *P. pastoris* transformants were found. Most of clones reacted in immunodiffusion with anti-Qβ antibody at dilution 1:32, however, variations from 1:64 up to 1:256 were observed for clones with higher G418 resistance, most probably representing multicopy Qβ CP gene insertions (Fig. 1C and D).

For purification of Qβ VLPs, proteins in the supernatant were precipitated with ammonium sulfate and fractionated on a Sepharose CL-4B gel filtration column. Qβ CP purification resulted about a 90% purity level of the target protein produced in both yeasts (Fig. 2A). Electron microscopy demonstrated well-assembled Qβ VLPs in the purified material, both from *S. cerevisiae* and *P. pastoris* cells (Fig. 2B and C). The presence of nucleic acid in yeast-derived VLPs was confirmed by native agarose gel electrophoresis (Fig. 2D and E). Production reached 3.6±0.3 mg/1 g of wet cells for *S. cerevisiae* and 4.9±0.6 mg for *P. pastoris*; attaining 15–20% and 20–30% of the Qβ VLP production level in *E. coli*, respectively (see Kozlovska *et al*., 1993).

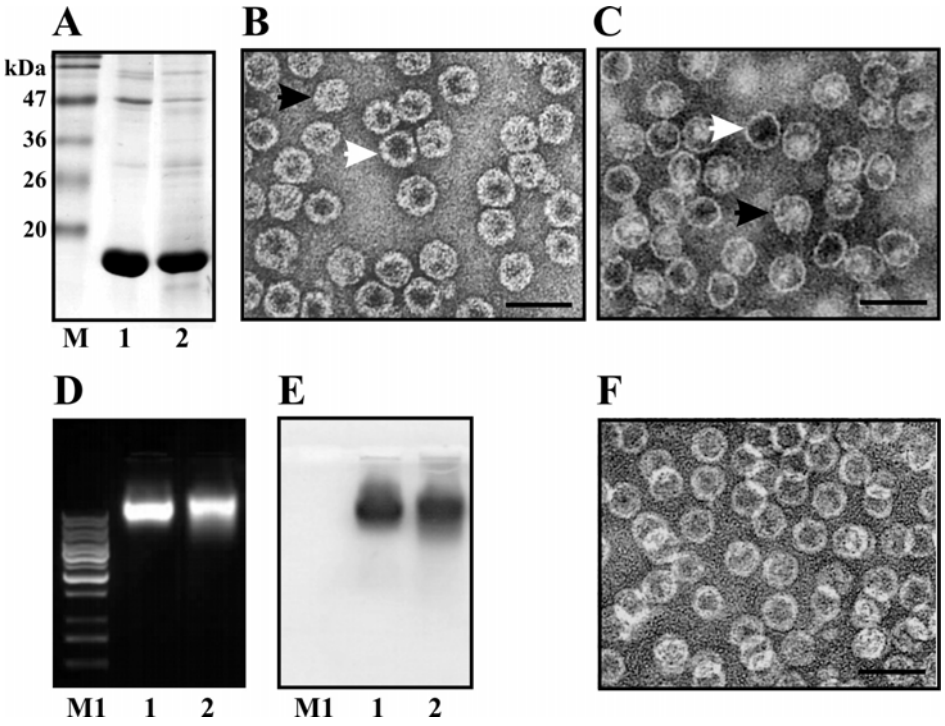

Figure 2. Characterization of yeast-derived Qβ VLPs. Particles after purification from *S. cerevisiae* (lane 1) and *P. pastoris* (lane 2) were analyzed in CBB-stained 15% SDS-PAAG (A) by electron microscopy (B and C) and in native 1% agarose gel (D and E) stained with ethidium bromide (D) and subsequently with CBB G-250 (E). (F) *E. coli*-derived Qβ VLPs. "Empty" (free of nucleic acid) and "full" (containing nucleic acid) particles are marked with white and black arrows, respectively. Bar, 50 nm.

To compare the properties of the Qβ VLPs from different hosts, mice were immunized with *S. cerevisiae*-, *P. pastoris*-, or *E. coli*-derived VLPs. Sera obtained were analyzed for their reactivity with the Qβ VLPs purified from all three hosts in direct ELISA and Ouchterlony immunodiffusion. Both assays suggested identical antigenicity and immunogenicity of the recombinant Qβ particles. Furthermore, the length of heterologously expressed Qβ CPs was compared also by MALDI technique, confirming the lack of the first methionine in all three cases.

In conclusion, both yeast hosts were found suitable for production of Qβ VLPs in similar amounts. Although expression in *P. pastoris* resulted in ~30% higher output of VLPs, *S. cerevisiae*-based expression is much more faster and convenient. Based on data obtained by us and Vilnius group (Sasnauskas *et al*., 1999, Samuel *et al*., 2002, Slibinskas *et al*., 2004), *S. cerevisiae* strain AH22 was selected as the most promising host for expression of viral structural genes. Along with the paper describing the expression of MS2-like particles (Legendre and Fastrez, 2005), this was one of the first reports on generation of phage VLPs in yeast.

2. High-level expression and purification of bacteriophage GA virus-like particles from yeast *Saccharomyces cerevisiae* and *Pichia pastoris* (paper 2)

An important drawback of repetitive vaccination with chimeric VLPs might be their limited effectiveness due to the presence of neutralizing antibodies against capsid protein induced after the first application (Da Silva *et al*., 2001). Additionally, different VLPs display different properties regarding their stability, tolerance to foreign amino acid insertions and chemical modifications, ease of *in vitro*

reconstruction and ability to pack foreign materials during assembly. Therefore, technologies to generate a broad spectrum of carrier VLPs need to be developed. To extend investigations of phage VLP generation in yeast, the phage GA CP gene was cloned and expressed in a similar way as described above for Qβ. Plasmids pFX-GA and pPIC-GA were generated for expression in *S. cerevisiae* AH22 and *P. pastoris*, respectively. Legendre and Fastrez (2005) reported two-gene yeast expression system for packaging functional RNAs within MS2 VLPs. To test this possibility also for phage GA VLPs, the GA CP gene was cloned in pESC-URA vector, resulting in a pESC-GA plasmid. This vector contains also the second promoter for co-expression with an additional gene of interest. For expression of the latter construct, the special *S. cerevisiae* strain YPH499 was used. The resulting expression units are schematically presented in Fig. 3.

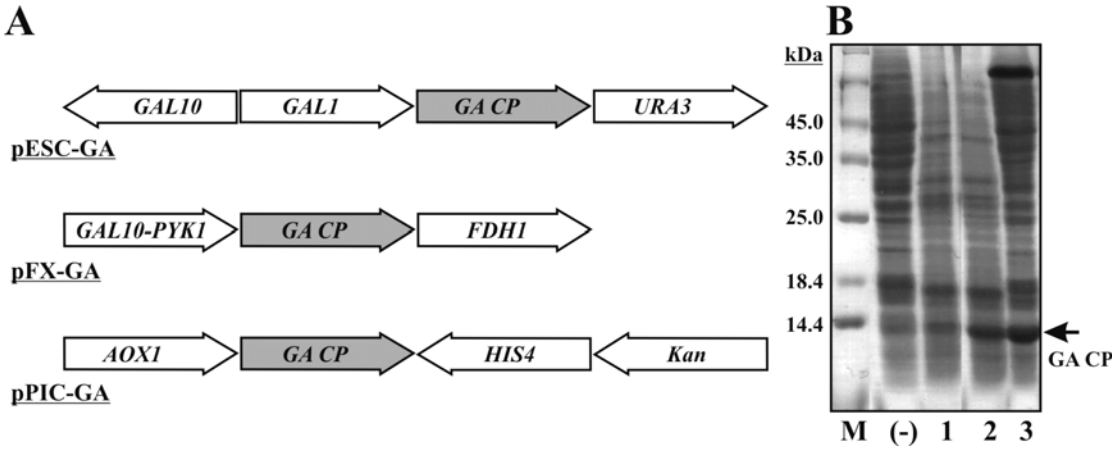

Figure 3. Expression of the GA CP gene in yeast. A, schematic presentation of the vectors used. The relative direction of genes and promoters is indicated by arrows. *URA3*, *HIS4*, and *FDH1* encode for genes used as primary markers for selection of yeast transformants, while the *Kan* gene allows secondary screening of *P. pastoris* for high-copy integrants. B, CBB-stained PAAG demonstrating the total synthesis level of GA CP. M, protein molecular weight marker, (-), non-transformed *P. pastoris* cells as a negative control. Lanes 1, 2 and 3 represent cell lysates from strains YPH499, AH22, and GS115, respectively. Accumulation of GA CP is indicated by an arrow.

Total synthesis of GA CP was monitored by CBB-stained PAAG (Fig. 3B) showing well-detectable accumulation of ~13.6 kDa protein in strains AH22 and GS115, while in strain YPH499 the production was significantly lower. After cell distruption, the soluble proteins were salt-precipitated and fractionated by SEC similarly as described for phage Qβ. VLP-containing fractions were detected by CBB-stained PAAG and immunodiffusion. The overall purity of the VLP samples in PAAG can be estimated as 80 to 90% (Fig. 4A). Electron microscopy confirmed the formation of icosahedral phage GA-like particles in all three cases (Fig. 4B). As in case with Qβ, the selected *P. pastoris* clone ensured slightly higher production than *S. cerevisiae* strain AH22 (Table 3).

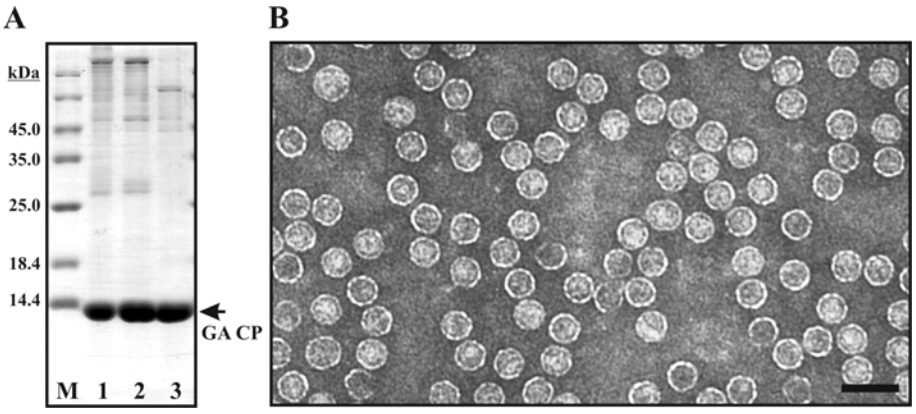

Figure 4. Characterization of purified GA VLPs. A, CBB-stained PAAG of the final product after size exclusion chromatography. M, protein MW marker. Lanes 1, 2 and 3 demonstrate the purity of VLPs obtained from strains YPH499, AH22, and GS115, respectively. B, direct evidence of VLP formation by electron microscopy. Only VLPs purified from yeast *P. pastoris* are presented. Scale bar: 50 nm.

3. Specificity of packaging mRNAs in bacteriophage GA virus-like particles in yeast *Saccharomyces cerevisiae* (paper 3)

Despite the rather small production of GA CP observed under *GAL1* promoter in pESC-URA, this vector contains another *GAL10* promoter located in the opposite orientation, allowing co-expression of two genes for protein-protein or protein-nucleic acid interaction studies in *S. cerevisiae*. We attempted to produce GA VLPs *in vivo* with a specified RNA content in a similar way as Legendre and Fastrez (2005) demonstrated with MS2 coat protein in *S. cerevisiae*. To produce mRNAs for packaging, three different genes were cloned under *GAL10* promoter (see Fig. 3A). After co-expression of two genes, VLPs were purified in each case. RNA content analysis of the produced capsids in agarose gel electrophoresis revealed the existence of RNAs of predicted lengths, along with the mRNA of GA CP. Importantly, yeast provides a source of eukaryotic, 5'-capped and 3'-poly(A)-tailed mRNAs, which is attractive for further goals to test phage VLPs as RNA packaging and delivery tools to mammalian cells. Also, the absence of bacterial endotoxins in yeast preparations simplifies the capsid purification procedures for this purpose.

4. Yeast-expressed bacteriophage-like particles for packaging of nanomaterials (paper 4)

Here, were investigated CP genes from phages fr, SP, AP205, PP7 and φCb5 for generation of respective VLPs in yeast. Consequently, expression level was compared in *S. cerevisiae* and *P. pastoris*, using the episomal and integrative vectors, respectively. Synthesis of phage CPs in all cases was well detectable by SDS-PAGE, however, amount of target protein varied significantly (Fig. 5A and B). Generally, higher synthesis of phage CPs was observed in *P. pastoris* clones containing multiple integration units. Especially effective *P. pastoris* producer strains have been selected for phage PP7 and φCb5 CPs (Fig. 5B). However, for phage fr and SP CPs slightly better production appeared in *S. cerevisiae*. Our previous experience with expression of viral structural proteins in *P. pastoris* has led to conclusion that more multicopy clones should be analyzed to select the best producer strains. This is supported by novel data as superproducer clones were successfully obtained for phage φCb5 and PP7 VLPs (Fig. 1C and D).

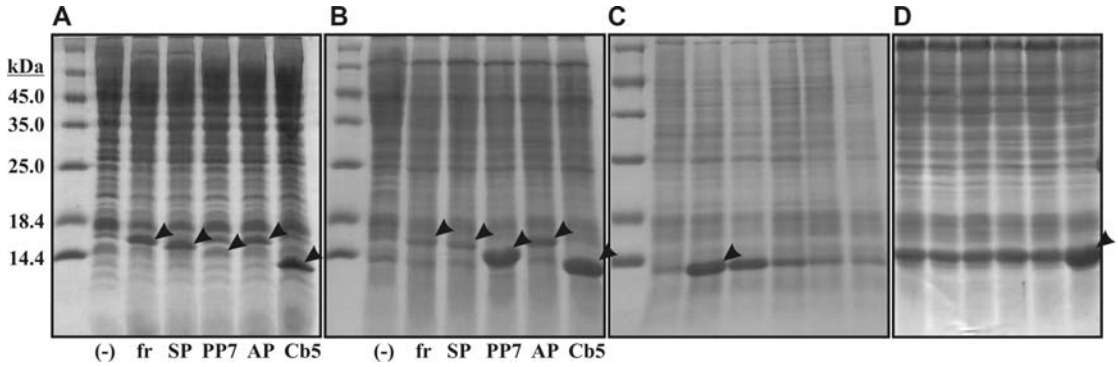

Figure 5. CBB-stained SDS-PAAG demonstrating synthesis of phage CPs in yeast *S. cerevisiae* (A) and *P. pastoris* (B). Panels C and D show selection of *P. pastoris* superproducer clones for phage φCb5 and PP7 CPs, respectively. Target proteins and selected producer clones are marked by arrowheads.

Purification procedure of phage-like particles was evaluated to obtain material with more than 90% purity. This included three major steps: (i) lysate clarification and first SEC on Sepharose 4 Fast Flow column, (ii) AEC on DEAE column, and (iii) final polishing on SEC HiLoad 16/600 Superdex 200 column. Interestingly, phage φCb5 VLPs are salt sensitive and easily dissociate to dimers when applied to strong anion exchangers. The process is reversible with a high effectivity and can be used for *in vitro* packaging of functional RNAs and other nanomaterials (e.g., gold nanoparticles) within φCb5 VLPs. This is important finding as the other phage-derived VLPs dissociate to dimers only under denaturating conditions which makes them problematic for *in vitro* packaging experiments.

Table 3. Summary of phage VLP synthesis, solubility and outcome in yeast

Strain	Expression[a]	Solubility[b]	VLP yield [c]
$Q\beta^{Sc}$	+++	+++	3-4*
$Q\beta^{Pp}$	+++	+++	4.5-5.5*
GA^{Sc}	+++	+++	1.5-2*
GA^{Pp}	+++	+++	2.5-3*
fr^{Sc}	++	+++	0.4-0.6
fr^{Pp}	+	+++	0.2-0.3
SP^{Sc}	+	-	ND
SP^{Pp}	+	+	ND
$PP7^{Sc}$	+	+++	0.6-0.8
$PP7^{Pp}$	+++	+++	6-8
$AP205^{Sc}$	+	+++	0.4-0.6
$AP205^{Pp}$	++	+++	1-1.5
$\varphi Cb5^{Sc}$	++	+++	2-4
$\varphi Cb5^{Pp}$	+++	+++	5-7

(^{Sc}) *S. cerevisiae* strains, (^{Pp}) *P. pastoris* strains. ([a]) total synthesis level of target proteins estimated by CBB-stained PAAG as follows: +++, high-level expression exceeding 10% of total cell proteins, ++ well-detectable synthesis reaching 5-10% of total cell proteins, + detectable synthesis below 5% of total cell proteins. ([b]) solubility of target proteins estimated by CBB-stained PAAG as follows: +++, more than 50% of CP in soluble fraction, +, below 50% of CP in soluble fraction, -, no CP in soluble fraction. ([c]) VLP yield was calculated in mg per 1 g of wet cells. (*), VLP yield was calculated after single SEC column. ND, not detected.

In all cases majority of target protein appeared in soluble fraction and total synthesis amount correlated well with amount of target protein in soluble fraction with the sole exception for phage SP CP, which was found completely insoluble when expressed in *S. cerevisiae* and partially soluble in *P. pastoris.* The data about generation of phage VLPs in yeast are summarized in Table 3. One of the highest synthesis was observed for phage PP7 VLPs and it was the first phage object which was successfully adapted for large-scale fermentation. Although production in fermentor was slightly lower than in flask, it was still high enough for efficient purification (Fig. 6A). Electron microscopy confirmed purity of final phage fr, PP7, AP205, and φCb5 VLP preparations (Fig. 6B). Up to now, this is the first report of abovementioned phage VLP production in yeast.

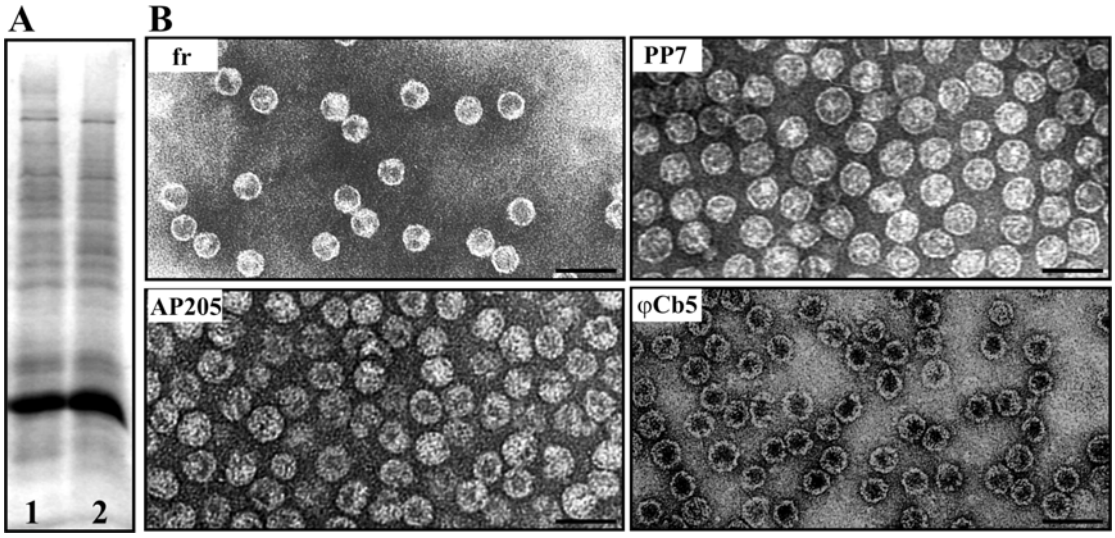

Figure 6. A, comparison of phage PP7 CP synthesis in fermentor (lane 1) and in flask (lane 2). B, electron microscopy of purified phage VLPs. Scale bar: 50 nm.

II Expression of HBV protein genes in yeast

5. Highly efficient production of phosphorylated hepatitis B core particles in yeast *Pichia pastoris* (paper 5)

Although expression of the *HBc* gene in yeast cells including *P. pastoris* has been described by several authors (Miyanohara *et al*., 1986, Rolland *et al*., 2001, Li *et al*., 2007), the majority of published HBc VLP purification methods remain either too complicated and time-consuming or non-effective due to the high costs and/or low output of the target protein. HBc gene was cloned in pPIC3.5K vector and transformed in *P. pastoris*. Among ~2000 transformants, selected clones were analyzed for their HBc synthesis level by SDS–PAGE (Fig. 7A).

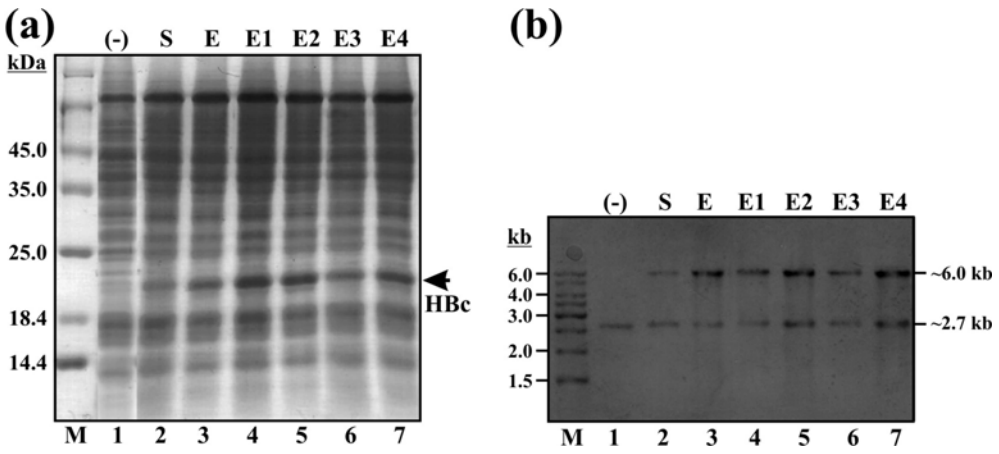

Figure 7. Correlation between HBc gene expression level and the amount of integration units in individual *P. pastoris* subclones estimated by CBB-stained PAAG (a) and Southern blotting (b). Lane 1, negative control, non-transformed *P. pastoris* cells. Lanes 2–7, *P. pastoris* clones harboring single (S; lane 2) and multiple (E–E4; lanes 3–7) HBc gene insertions. Only a part of the multicopy clones is shown to demonstrate deviation in expression level.

Clone E1 was chosen as the best producer for further investigations. Interestingly, in Southern blotting (Fig. 7B) appeared that higher number of integrated expression units doesn't always correlate with higher production of target protein. This is consistent with data from other authors for expression of other viral structural genes in *P. pastoris* (Slibinskas *et. al.*, 2004).

For HBc producer E1 fermentation conditions were established. Production level of HBc was practically the same as in flasks but the final biomass reached 163 g/L. A rapid and effective method of HBc VLPs was developed. To make purification cost-effective and fast, we aimed to avoid many commonly used protein purification steps such as centrifugation in a sucrose gradient, affinity and size-exclusion chromatography, filter-concentration, and dialysis. After cell disruption with the French press, majority of HBc was found in soluble fraction. As the HBc VLPs are sufficiently thermostable, the defined combination of heat/pH treatment allowed precipitating most of contaminating proteins (Fig. 8A-C). HBc precipitation with ammonium sulfate efficiently concentrated protein and also removed majority of non-specific nucleic acid from the HBc preparation. Finally, DEAE Sepharose column removed most of remaining impurities as well as aggregated VLP material (Fig. 8D). An output of HBc VLPs reached 3 mg from 1 g of wet cells.

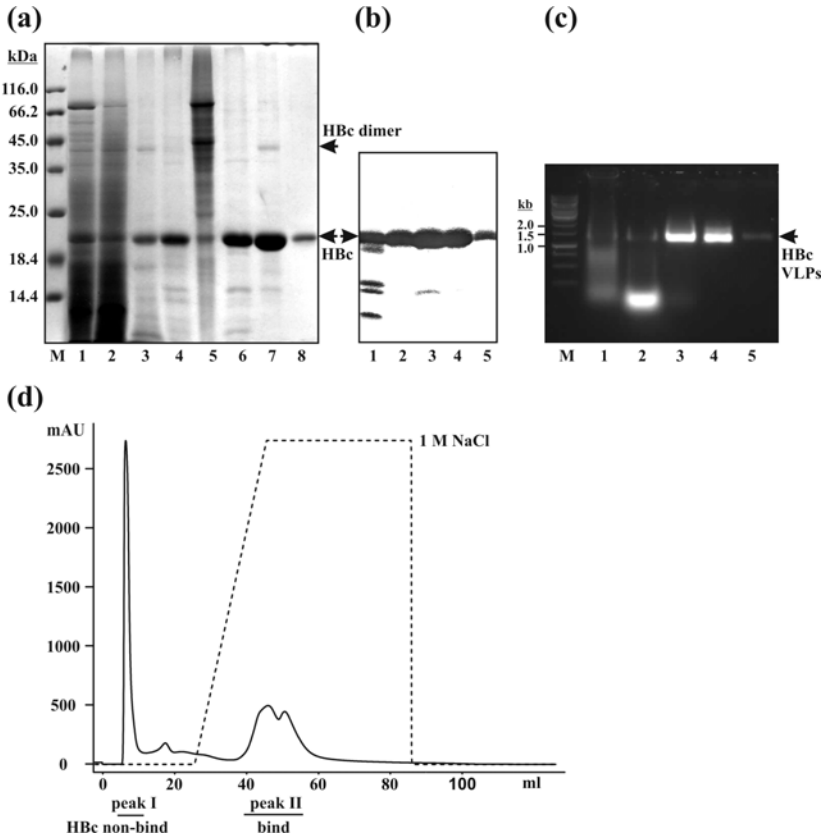

Figure 8. Main purification steps for HBc VLPs. (a) CBB-stained PAAG illustrating HBc purity in protein samples. Lanes 1 and 2, soluble and unsoluble fractions, respectively. Lanes 3 and 4, soluble fraction after heat treatment at pH 6.2 and 8.0, respectively. Lane 5, heat-precipitated proteins from supernatant at pH 8.0. Lane 6, ammonium sulfate precipitate. Lanes 7 and 8, proteins from AEC peaks I and II, respectively. (b) and (c) Western blotting and native agarose gel electrophoresis, showing HBc degradation pattern and presence of nucleic acids, respectively. Lanes 1 and 2, non-treated and heat-treated cell supernatants, respectively. Lane 3, dissolved ammonium sulfate precipitate. Lanes 4 and 5, proteins from AEC peaks I and II, with column flow-through and bound material, respectively (d).

Electron microscopy confirmed quality of purified VLPs (Fig. 9A). The endotoxin level in the final product was less than 100 EU/mg of pure protein, which makes it attractive both for *in vitro* and *in vivo* applications. The length of *E. coli*- and yeast-derived HBc was compared by MALDI-TOF mass spectrometry which revealed MW 21,136 and 21,324 Da, respectively (Fig. 9B). It was assumed that the difference is due to some post-translational modification that does not occur in *E. coli*. To investigate whether our yeast-produced protein is phosphorylated, we subjected full-length HBc molecules to SDS–PAGE and performed phosphoprotein and CBB staining (Fig. 9C and D). The results clearly indicated that the yeast-produced HBc is indeed phosphorylated. Until now, this is the first report of HBc phosphorylation in yeast.

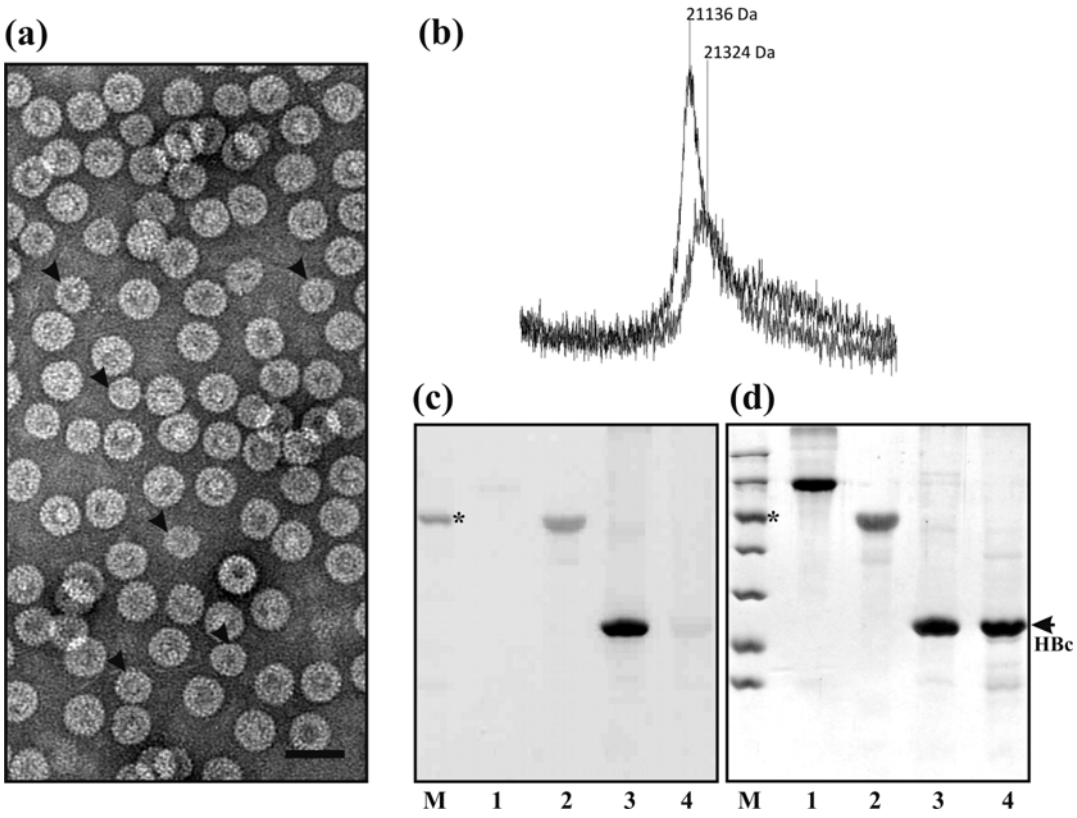

Figure 9. (a) Electron microscopy of purified HBc VLPs. Some of the smaller $T = 3$ quasi-symmetry particles are marked by arrowheads. Scale bar: 50 nm. Comparison of full-length yeast and *E. coli*-derived HBc molecules by mass spectrometry (b), and by denaturing SDS–PAAG stained directly with phosphoprotein stain (c), and subsequently with CBB (d). BSA and pepsin were loaded as negative and positive controls (lanes 1 and 2, respectively). Lanes 3 and 4 represent *P. pastoris*- and *E. coli*-derived HBc, respectively. Phosphoprotein ovalbumin is marked by asterisk.

6. Expression of HBV genes in other yeasts

Since commercial vectors for *H. polymorpha* are not available, it was decided to create a flexible modular vector set by analogy with CoMed vector system (Steinborn et al., 2006). First of all, a 3.5 kb genomic fragment encoding rRNA locus has been isolated from *H. polymorpha* genome according to Klabunde *et al*., (2002) and cloned in pTEF/Zeo1 vector using *Bgl*II and *Nhe*I restriction sites, resulting in pTEF-rRNA vector. Then, *AOX* transcription terminator sequence from pPIC3.5K was isolated and cloned using *Sal*I and *Pae*I restriction sites. Finally, promoter encoding sequences were cloned using *Eco*RI and *Bam*HI restriction sites, resulting in pHaTEF, pHaMOX and pHaFMD plasmids (Fig. 10A). Appropriate promoter sequences were PCR-amplified from pTEF/Zeo1, pHIPX2 and *H. polymorpha* genome, respectively.

Hepatitis B virus structural protein genes (*HBc* and *HBs*) have been cloned in particular vectors to test their efficiency. The same genes along with the *HBe* gene as secretion model were tested in other yeast systems, namely, *P. pastoris* and *K. lactis*. Results are summarized in Table 4. Among four HBs constructs tested, synthesis of target protein was detected only in *H. polymorpha* under MOX promoter. Part of HBs was solubilized in detergent-containing buffer and subjected to electron microscopy. Presence of typical 22 nm particles was detected (Fig. 10B). In the same system, expression of HBc was also detected although synthesis was considerably lower than in *P. pastoris* (Table 4). As to secretion of HBe, only traces of expression was

observed in *P. pastoris*. Evaluation of *S. cerevisiae* and *H. polymorpha* systems for secretion purposes is currently in progress.

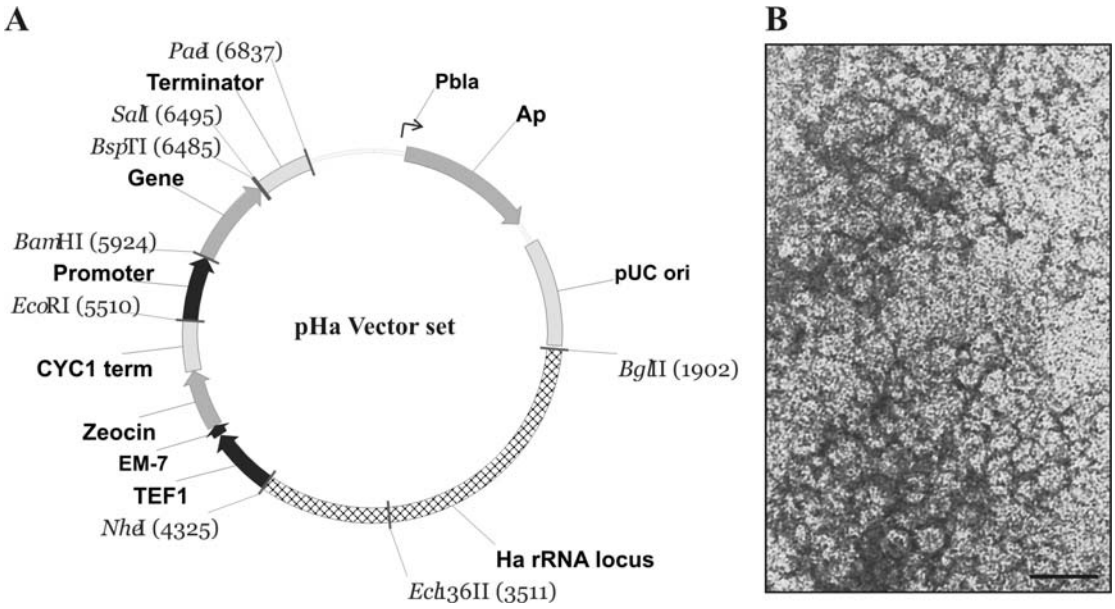

Figure 10. A, pHa vector set for gene expression in *H. polymorpha*. Individual modules (promoter, gene, transcription terminator, integration marker) are flanked with unique restriction sites to facilitate cloning procedures. For integration in rRNA locus, plasmid is linearised with *Ecl*136II. B, electron microscopy of HBs particles isolated from *H. polymorpha*. Bar: 50 nm.

Table 4. Expression of hepatitis B virus genes in different yeasts

Construction	Expression	Synthesis	Solubility
pPIC-HBc^{Pp}	Intracellular	+++	+
pPIC-HBe^{Pp}	Secretion	+	+
pPIC-HBs^{Pp}	Intracellular	-	-
pKLAC-HBc^{Kl}	Intracellular	-	-
pKLAC-HBe^{Kl}	Secretion	-	-
pHaMOX-HBc^{Hp}	Intracellular	++	+
pHaTEF-HBs^{Hp}	Intracellular	-	-
pHaMOX-HBs^{Hp}	Intracellular	++	+
pHaFMD-HBs^{Hp}	Intracellular	-	-

(^{Pp}) *P. pastoris* expression plasmids, (^{Kl}) *K. lactis* expression plasmids, (^{Hp}) *H. polymorpha* expression plasmids. Synthesis level of target proteins was estimated by CBB-stained PAAG as follows: +++, high-level expression reaching 5-10% of total cell proteins, ++, well-detectable synthesis below 5% of total cell proteins, +, target protein detectable only in concentrated lysate, -, no detectable protein. Solubility was estimated by CBB-stained PAAG as follows: +, at least 50% of protein in soluble fraction, -, no target protein in soluble fraction.

CONCLUSIONS

- Bacteriophage coat proteins can be produced and self-assembled in yeast cells. Synthesis level is highly variable and phage-dependent.
- Correctly folded HBc VLPs can be produced in yeast *P. pastoris* at high level and in *H. polymorpha* – at moderate level.
- Expression level in methylotrophic yeasts is not directly dependent on the number of integrated expression units.
- *P. pastoris*-expressed HBc appears as phosphoprotein.
- *P. pastoris* generally ensures higher VLP outcome than *S. cerevisiae*, with a single exception for bacteriophage fr.
- Purification procedures can be developed to obtain target VLPs at more than 90% purity.
- Fermentation conditions can be established for high-level production in yeast *P. pastoris* for both phage and HBc VLPs.
- Yeast-expressed VLPs can be applied for downstream applications, for example, packaging of nanomaterials.
- A convenient vector system has been generated for gene expression in *H. polymorpha.* Among yeast systems tested, *H. polymorpha* appeared as most promising host for HBs expression.
- Systematic screening of expression systems is needed to obtain synthesis of each particular protein.

THE LIST OF ORIGINAL PUBLICATIONS

1. Freivalds J., Dislers A., Ose V., Skrastina D., Cielens I., Pumpens P., Sasnauskas K., Kazaks A**.** (2006) Assembly of bacteriophage Qβ virus-like particles in yeast *Saccharomyces cerevisiae* and *Pichia pastoris*. *J Biotechnol* **123**, 297–303

2. Freivalds J., Rūmnieks J., Ose V., Renhofa R., Kazāks A. (2008) High-level expression and purification of bacteriophage GA virus-like particles from yeast *Saccharomyces cerevisiae* and *Pichia pastoris*. *Acta Universitatis Latviensis*, Biology **745**, 75–85

3. Rūmnieks J., **Freivalds J.**, Cielēns I., Renhofa R. Specificity of packaging mRNAs in bacteriophage GA virus-like particles in yeast *Saccharomyces cerevisiae*. (2008) *Acta Universitatis Latviensis*, Biology 745, 145–154

4. Freivalds J., Kotelovica S., Voronkova T., Ose V., Tars K., Kazaks A. Yeast-expressed bacteriophage-like particles for packaging of nanomaterials. *Mol Biotechnol* 2013 Jul 13, *In press*. DOI 10.1007/s12033-013-9686-0

5. Freivalds J., Dislers A., Ose V., Pumpens P., Tars K., Kazaks A. (2011) Highly efficient production of phosphorylated hepatitis B core particles in yeast *Pichia pastoris. Protein Expr Purif* **75**, 218–224

CONFERENCE THESIS

Freivalds J., Dislers A., Kazāks A. (2005) Heterologous expression of viral structural genes in yeast *Saccharomyces cerevisiae* and *Pichia pastoris*. Abstr. International Biology Students Conference, Riga, April 10-16, p22

Freivalds J., Grīgs O. (2010) Hepatīta B kor-antigēna fermentatīva iegūšana raugā *Pichia pastoris* un baktērijās *Escherichia coli*: tehnoloģisku attīrīšanas shēmu salīdzinājums. Molekulārās bioloģijas sekcija 9. februāris. LU 68. konferences programma, p30

Freivalds J., Kazaks A. (2011) Yeast cells allow high-level synthesis and assembly of bacteriophage-like particles. *4th Annual protein congress and vaccines congress*, London, UK, April 4–5

Freivalds J., Kazaks A. (2012) Yeast-expressed RNA bacteriophage-like particles for packaging of nanomaterials. Virus-like particle & nano-particle vaccines. Cannes, France, November 28–30

PATENT

Kazaks A., **Freivalds J.**, Pumpens P., Dislers A. (2008) Recombinant strain of yeast Pichia pastoris for obtaining nucleocapsids of human hepatitis B virus (subtype *ayw*). Patent LV 13740 (B) IPC1-7: C07K14/39; C12N15/09

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Participation in scientific projects:
Grants from Latvian Council of Sciences
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05.1631 "Combined use of *E. coli, S. cerevisiae and P. pastoris* expression systems to obtain recombinant proteins in functional form" (2005-2008)
09.1294 „Structural investigations of virus like particles" (2009-2012)

Latvian State Research Program 07-VP2.6 "Technology of virus-like particles for recombinant vaccine prototypes" (2005-2009)

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REFERENCES

Adhin M.R., Avots A., Berzin V., Overbeek G.P., van Duin J. (1990) Complete nucleotide sequence of the group I RNA bacteriophage fr. *Biochim Biophys Acta* **1050**, 104–109.

Bichko V., Pushko P., Dreilina D., Pumpen P., Gren E. (1985) Subtype *ayw* variant of hepatitis B virus. DNA primary structure analysis. *FEBS Lett* **185**, 208–212.

Böer E., Steinborn G., Kunze G., Gellissen G. (2007) Yeast expression platforms. *Appl Microbiol Biotechnol* **77**, 513–523.

Bradley D.E. (1966) The structure and infective process of a *Pseudomonas aeruginosa* bacteriophage containing ribonucleic acid. *J Gen Microbiol* **45**, 83–96.

Bryan J.T. (2007) Developing an HPV vaccine to prevent cervical cancer and genital warts. *Vaccine* **25**, 3001–3006.

Buonaguro L., Tagliamonte M., Tornesello M.L., Buonaguro F.M. (2011) Developments in virus-like particle-based vaccines for infectious diseases and cancer. *Expert Rev Vaccines* **10**, 1569–1583.

Coffi H. (1995) Lysotypie des *Acinetobacter*. Thèse de Maîtrise dès Sciences, Laval University, Québec, Canada.

Cornuz J., Zwahlen S., Jungi W.F., Osterwalder J., Klingler K., van Melle G., Bangala Y., Guessous I., Müller P., Willers J., Maurer P., Bachmann M.F., Cerny T. (2008) A vaccine against nicotine for smoking cessation: a randomized controlled trial. *PLoS One* **3**, e2547.

Da Silva D.M., Pastrana D.V., Schiller J.T., Kast W.M. (2001) Effect of preexisting neutralizing antibodies on the anti-tumor immune response induced by chimeric human papillomavirus virus-like particle vaccines. *Virology* **290**, 350–360.

Faber K.N., Haima P., Gietl C., Harder W., Ab G., Veenhuis, M. (1994) The methylotrophic yeast *Hansenula polymorpha* contains an inducible import pathway for peroxisomal matrix proteins with an N-terminal targeting signal (PTS2 proteins). *Proc Natl Acad Sci USA* **91**, 12985–12989.

Gellissen G. (2000) Heterologous protein production in methylotrophic yeasts. *Appl Microbiol Biotechnol* **54**, 741–750.

Gellissen G. (2002) *Hansenula polymorpha*—biology and applications. Wiley-VCH, Weinheim, Germany.

Gellissen G. (2005) Production of recombinant proteins—novel microbial and eukaryotic expression systems. Wiley-VCH, Weinheim, Germany.

Gietz D., St Jean A., Woods R.A., Schiestl R.H. (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res* **20**, 1425.

Inokuchi Y., Takahashi R., Hirose T., Inayama S., Jacobson A.B., Hirashima A. (1986) The complete nucleotide sequence of the group II RNA coliphage GA. *J Biochem* **99**, 1169–1180.

Inokuchi Y., Jacobson A.B., Hirose T., Inayama S., Hirashima A. (1988) Analysis of the complete nucleotide sequence of the group IV RNA coliphage SP. *Nucleic Acids Res* **16**, 6205–6221.

Jennings G.T., Bachmann M.F. (2008) The coming of age of virus-like particle vaccines. *Biol Chem* **389**, 521–536.

Juozapaitis M., Serva A., Zvirbliene A., Slibinskas R., Staniulis J., Sasnauskas K., Shiell B.J., Wang L.F., Michalski W.P. (2007) Generation of henipavirus nucleocapsid proteins in yeast *Saccharomyces cerevisiae*. *Virus Res* **124**, 95–102.

Kaczmarczyk S.J., Sitaraman K., Young H.A., Hughes S.H., Chatterjee D.K. (2011) Protein delivery using engineered virus-like particles. *Proc Natl Acad Sci USA* **108**, 16998-17003.

Kazaks A., Voronkova T., Rumnieks J., Dishlers A., Tars K. (2011) Genome structure of Caulobacter phage phiCb5. *J Virol* **85**, 4628–4631.

Klabunde J., Diesel A., Waschk D., Gellissen G., Hollenberg C.P., Suckow M. (2002) Single-step co-integration of multiple expressible heterologous genes into the ribosomal DNA of the methylotrophic yeast Hansenula polymorpha. *Appl Microbiol Biotechnol* **58**, 797–805.

Kozlovska T.M., Cielens I., Dreilina D., Dislers A., Baumanis V., Ose V., Pumpens P. (1993) Recombinant RNA phage Qβ capsid particles synthesized and self assembled in *Escherichia coli*. *Gene* **137**, 133–137.
Kushnirov V.V. (2000) Rapid and reliable protein extraction from yeast. *Yeast* **16**, 857–860.
Legendre D., Fastrez J. (2005) Production in *Saccharomyces cerevisiae* of MS2 virus-like particles packaging functional heterologous mRNAs. *J Biotechnol* **117**, 183–194.
Li Z.X., Hong G.Q., Hu B., Liang M.J., Xu J., Li L. (2007) Suitability of yeast- and *Escherichia coli*-expressed hepatitis B virus core antigen derivatives for detection of anti-HBc antibodies in human sera. *Protein Expr Purif* **56**, 293–300.
Loeb T., Zinder N.D. (1961) A bacteriophage containing RNA. *Proc Natl Acad Sci USA* **47**, 282–289.
Ma Y., Nolte R.J., Cornelissen J.J. (2012) Virus-based nanocarriers for drug delivery *Adv Drug Deliv Rev* **64**, 811–825.
Macauley-Patrick S., Fazenda M.L., McNeil B., Harvey L.M. (2005) Heterologous protein production using the *Pichia pastoris* expression system. *Yeast* **22**, 249–270.
Miyanohara A., Imamura T., Araki M., Sugawara K., Ohtomo 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.
Olsthoorn R.C., Garde G., Dayhuff T., Atkins J.F., Van Duin J. (1995) Nucleotide sequence of a single-stranded RNA phage from Pseudomonas aeruginosa: kinship to coliphages and conservation of regulatory RNA structures. *Virology* **206**, 611–625.
Pausch M.H., Kirsch D.R., Silverman S.J. (2005) *Saccharomyces cerevisiae*: Applications. eLS. Wiley-VCH.
Pumpens P., Ulrich R., Sasnauskas K., Kazaks A., Ose V., Grens E. (2008) Construction of novel vaccines on the basis of the virus-like particles. Hepatitis B virus proteins as vaccine carriers, in: Y. Khudyakov (Ed.), Medicinal Protein Engineering, CRC Press, pp. 205–248.
Pushko P., Kozlovskaya T., Sominskaya I., Brede A., Stankevica E., Ose V., Pumpens P., Grens E. (1993) Analysis of RNA phage fr coat protein assembly by insertion, deletion and substitution mutagenesis. *Protein Eng* **6**, 883–891.
Rolland D., Gauthier M., Dugua J.M., Fournier C., Delpech L., Watelet B., Letourneur O., Arnaud M., Jolivet M. (2001) Purification of recombinant HBc antigen expressed in *Escherichia coli* and *Pichia pastoris*: comparison of size-exclusion chromatography and ultracentrifugation. *J Chromatogr B Biomed Sci Appl* **753**, 51–65.
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.
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.
Schmidt J.M. (1966) Observations on the adsorption of *Caulobacter* bacteriophages containing ribonucleic acid. *J Gen Microbiol* **45**, 347–353.
Slibinskas R., Samuel D., Gedvilaite A., Staniulis J., Sasnauskas K. (2004) Synthesis of the measles virus nucleoprotein in yeast *Pichia pastoris* and *Saccharomyces cerevisiae*. *J Biotechnol* **107**, 115–124.
Spohn G., Jennings G.T., Martina B.E., Keller I., Beck M., Pumpens P., Osterhaus A.D., Bachmann M.F. (2010) A VLP-based vaccine targeting domain III of the West Nile virus E protein protects from lethal infection in mice. *Virol J* **7**, 146.
Steinborn G., Böer E., Scholz A., Tag K., Kunze G., Gellissen G. (2006) Application of a wide-range yeast vector (CoMed) system to recombinant protein production in dimorphic Arxula adeninivorans, methylotrophic Hansenula polymorpha and other yeasts. *Microb Cell Fact* **5**, 33.
Storni T., Ruedl C., Schwarz K., Schwendener R.A., Renner W.A., Bachmann M.F. (2004) Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic side effects. *J Immunol* **172**, 1777–1785.

Tissot A.C., Maurer P., Nussberger J., Sabat R., Pfister T., Ignatenko S., Volk H.D., Stocker H., Müller P., Jennings G.T., Wagner F., Bachmann M.F. (2008) Effect of immunisation against angiotensin II with CYT006-AngQb on ambulatory blood pressure: a double-blind, randomised, placebo-controlled phase IIa study. *Lancet* **371**, 821–827.
Tissot A.C., Renhofa R., Schmitz N., Cielens I., Meijerink E., Ose V., Jennings G.T., Saudan P., Pumpens P., Bachmann M.F. (2010) Versatile virus-like particle carrier for epitope based vaccines. *PLoS One* **5**, e9809.
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.
van Ooyen A.J., Dekker P., Huang M., Olsthoorn M.M., Jacobs D.I., Colussi P.A., Taron C.H. (2006) Heterologous protein production in the yeast *Kluyveromyces lactis*. *FEMS Yeast Res* **6**, 381–392.
Xu Y.F., Zhang Y.Q., Xu X.M., Song G.X. (2006) Papillomavirus virus-like particles as vehicles for the delivery of epitopes or genes. *Arch Virol* **151**, 2133–2148.
Yildiz I., Shukla S., Steinmetz N.F. (2011) Applications of viral nanoparticles in medicine. *Curr Opin Biotechnol* 22, 901–908.

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Short communication

Assembly of bacteriophage Qβ virus-like particles in yeast *Saccharomyces cerevisiae* and *Pichia pastoris*

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Abstract

Recombinant bacteriophage Qβ coat protein (CP), which has been proposed as a promising carrier of foreign epitopes via their incorporation either by gene engineering techniques or by chemical coupling, efficiently self-assembles into virus-like particles (VLPs) when expressed in *Escherichia coli*. Here, we demonstrate expression and self-assembly of Qβ CP in yeast *Saccharomyces cerevisiae* and *Pichia pastoris*. Production reached 3–4 mg/1 g of wet cells for *S. cerevisiae* and 4–6 mg for *P. pastoris*, which was about 15–20% and 20–30% of the *E. coli* expression level, respectively. Qβ VLPs were easily purified by size-exclusion chromatography in both cases and contained nucleic acid, shown by native agarose gel electrophoresis. The obtained particles were highly immunogenic in mice and the resulting sera recognized both *E. coli*- and yeast-derived Qβ VLPs equally well.

Keywords: Bacteriophage Qβ; Coat protein; Expression; Virus-like particles; Yeast

1. Introduction

The recombinant virus-like particles (VLPs) formed by coat protein (CP) of RNA-bacteriophage fr (Borisova et al., 1987; Kozlovskaya et al., 1988; Pushko et al., 1993) or MS2 (Mastico et al., 1993) of the *Levivirus* genus in the *Leviviridae* family were among the first proposed icosahedron carriers for the presentation of foreign short-sized epitopes on their surface. More recently, MS2 and fr VLPs were found to tolerate longer, 24 and 52 amino acid (aa) insertions, respectively, with retained self-assembly (Heal et al., 1999; Voronkova et al., 2002).

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High-level expression of the CP gene of bacteriophage Qβ from *Allolevivirus* genus of the *Leviviridae* family, which causes the formation of Qβ VLPs, was obtained in *Escherichia coli* (Kozlovska et al., 1993). The native Qβ particle (with quasi $T = 3$ symmetry) contains 180 copies of 133 aa-long CP enveloping a complex of single-stranded genomic RNA and maturation protein, and several molecules of a prolonged CP, 324 aa-long A1 protein, a natural read-through product of the UGA termination codon of the CP gene (Weber and Konigsberg, 1975). The A1 extension was considered a promising target site for foreign insertions, since it contained elements typical for spike-like structures exposed on the surface of particles (Kozlovska et al., 1996). In the presence of the wild-type CP as a helper, the formation of mosaic Qβ capsids with hepatitis B virus (HBV) preS1 or human immunodeficiency virus 1 gp120 epitopes of different length inserted at the A1 extension was demonstrated (Kozlovska et al., 1996; Vasiljeva et al., 1998). Non-mosaic Qβ CP derived VLPs (without A1 extension) have been used as a template for chemical coupling of desired peptides to surface-exposed lysine residues (Storni et al., 2004). Short CpG oligonucleotides, the most promising vaccine adjuvants known to date (for review, Krieg, 2004), were packaged successfully in vitro into chemically-engineered particles resulting in increased VLP immunogenicity and protection of CpG from nucleases (Storni et al., 2004; Schwarz et al., 2005).

For vaccine development, expression systems beside that of *E. coli* are desirable to avoid contamination of bacterial endotoxins. Yeast is an attractive eukaryotic microorganism presenting such an alternative. Up to now, a number of structural genes from mammalian viruses have been expressed in yeast resulting in the formation of VLPs (Valenzuela et al., 1982; Miyanohara et al., 1986; Kniskern et al., 1986; Cregg et al., 1987; Jacobs et al., 1989; Janowitz et al., 1991; Hofmann et al., 1995, 1996; Sasnauskas et al., 2002; Samuel et al., 2002; Slibinskas et al., 2004). The yeast expression system has been used successfully to produce the first licensed HBV vaccine (McAleer et al., 1984). In this work, we demonstrate the formation of recombinant RNA phage VLPs in two different yeasts to open, therefore, a way for further development of a yeast-derived phage VLP technology.

2. Cloning and expression of Qβ CP gene in yeast *S. cerevisiae* and *P. pastoris*

The Qβ CP-encoding gene was PCR-amplified from *E. coli* expression plasmid pQβ10 (Kozlovska et al., 1993) with the forward primer 5′-TT *TCT AGA* ACA **ATG** GCA AAA TTA GAG ACT G-3′ and the reverse primer 5′-T T*AC TAG* ***T*****TA** ATA CGC TGG GTT CAG C-3′ (start and termination codons are shown in bold). For expression in *S. cerevisiae*, the PCR fragment was digested with *Xba*I/*Spe*I (restriction sites are in italics) and cloned under the control of the galactose-inducible promoter in *Xba*I-treated vector pFX7 carrying the formaldehyde resistance gene (Samuel et al., 2002). The resulting pFX-Qβ plasmid was used to transform *S. cerevisiae* strains AH22 *MATa leu2 his4*, and *S. cerevisiae* DC5 *MATa leu2 his3*, as well as the wild-type strain *S. cerevisiae* FH4C, as described earlier (Sasnauskas et al., 2002; Ražanskienė et al., 2004). The transformed clones were selected on agarized YEPD medium supplemented with 3–10 mmol formaldehyde and cultivated as described by Sasnauskas et al. (1999). Transformants were incubated at 30 °C on a shaker in flasks containing YEPD medium supplemented with 3–5 mmol formaldehyde for 20–24 h until optical density OD_{590} reached 6–8. For induction, galactose was added to 3% in medium and cultivation was continued for another 20–24 h, with final OD_{590} 12–14. Production of Qβ CP was slightly better in the *S. cerevisiae* AH22 and *S. cerevisiae* FH4C strains as shown by SDS-PAGE and immunoblotting (Fig. 1A and B). This finding was confirmed by Ouchterlony double radial immunodiffusion with rabbit polyclonal anti-Qβ antibody, where lysate from the DC5 strain formed visible immunoprecipitation lines at dilution up to 1:64, but lysates from the *S. cerevisiae* AH22 and *S. cerevisiae* FH4C strains—up to 1:128 (see Fig. 1B).

For expression of the Qβ CP gene in *P. pastoris*, the Qβ CP-encoding PCR fragment was cloned by blunt-end ligation into *Sna*BI-treated vector pPIC3.5K (Invitrogen, Groningen, The Netherlands), under the control of the *AOX1* promoter. The resulting pPIC-Qβ plasmid after linearization with *Ecl*136II was used for transformation of the *P. pastoris* GS115 *his4* strain by electroporation (Bio-Rad, Gene Pulser), according to Cregg and Russell (1998). Mut^+His^+ transformants were selected on the minimal agar medium (0.67%

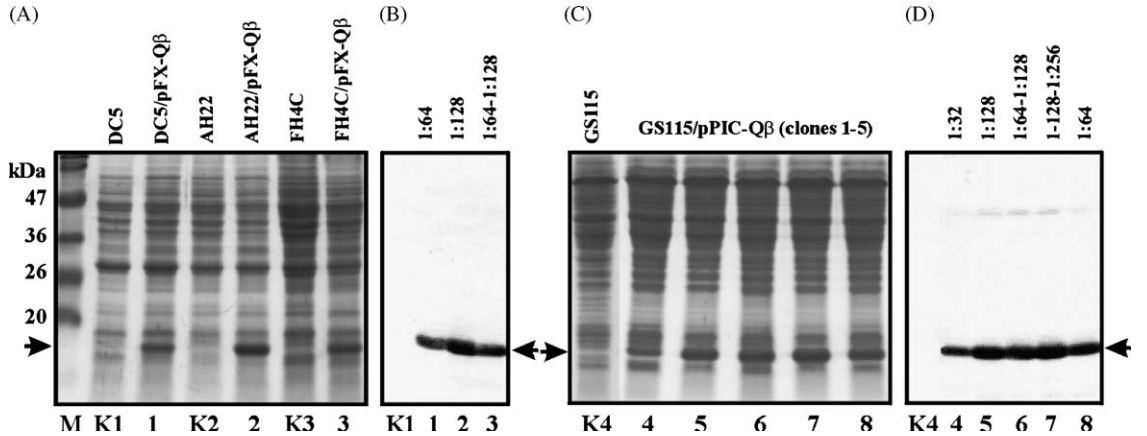

Fig. 1. Expression of Qβ CP in *S. cerevisiae* and *P. pastoris*, estimated by Coomassie-stained 15% SDS-PAGE (A and C) and Western blotting with rabbit polyclonal anti-Qβ antibody (B and D). K1, K2, K3 in (A) represent non-transformed *S. cerevisiae* DC5, *S. cerevisiae* AH22, and *S. cerevisiae* FH4C cells, and 1–3—the same strains transformed with pFX-Qβ, respectively. (C) Qβ CP expression in *P. pastoris* clones with single (lane 4) and multiple (lanes 5–8) gene insertions. K4, non-transformed *P. pastoris* cells. The lower labels in (B) and (D) correspond to those in (A) and (C), respectively. The Ouchterlony immunodiffusion titres are indicated on the top of respective lines in Western blot. Arrows show the position of the Qβ CP. M, molecular mass standards (MBI Fermentas, Vilnius, Lithuania) of 118, 85, 47, 36, 26, and 20 kDa proteins.

YNB, 2% glucose) and more than 800 clones were replica-plated on agarized YEPD medium containing G418 antibiotic (Gibco, UK). Most of the clones were resistant to a G418 concentration of 0.4 mg ml^{-1} representing likely the clones where a single copy of the expression unit was integrated in the yeast chromosome. Clones with a G418 resistance level up to 0.8–1.2 mg ml^{-1} were found at 2–3% frequency, suggesting insertions with increased copy number. Induction of the Qβ CP gene expression in *P. pastoris* was achieved according to the recommendations of manufacturer as follows: selected clones were incubated at 30 °C on a shaker in flasks containing BMGY medium for 20–24 h until OD_{590} reached 4–6, collected by low-speed centrifugation and resuspended in BMMY induction medium with dilution to OD_{590} 1.5. Samples taken each 24 h were analyzed by electron microscopy (EM), Western blotting, and Ouchterlony immunodiffusion. Maximal Qβ CP production and VLP formation were observed at 72 h after induction. Significant differences in the expression level of *P. pastoris* transformants were found (Fig. 1C and D). Most of clones reacted in immunodiffusion with anti-Qβ antibody at dilution 1:32 (Fig. 1C and D, lane 4), however, variations from 1:64 up to 1:256 were observed for clones with higher G418 resistance, most probably representing multicopy Qβ CP gene insertions (Fig. 1C and D, lanes 5–8). The indicated titres were observed in at least three independent expression experiments and their interval is presented if varied between repetitions.

3. Purification and characterization of yeast-derived Qβ VLPs

For analytical screening of clones, 20 optical units of yeast cells were disrupted by bead-beating with glass beads (450–500 μ; Serva) in 100 μl of A lysis buffer (10 mM Tris–HCl, pH 7.6, 50 mM NaCl, 0.01% Triton X-100, 2 mM PMSF), for 8 × 0.5 min. For the purification of VLPs from yeast, 1 g of frozen cells, of either *S. cerevisiae* or *P. pastoris*, were resuspended in 5 ml of the A buffer. After bead-beading (8 × 0.5 min) and following brief sonication, the soluble and insoluble fractions were separated by low-speed centrifugation. Proteins in the supernatant were precipitated at 50% (v/v) saturation of ammonium sulfate for 3 h at 4 °C and the precipitate was collected at 10,000 rpm for 30 min at 4 °C. The precipitate was resuspended in 1–2 ml of the A buffer without Triton X-100 and fractionated on a Sepharose CL-4B gel filtration column (H = 60 cm, V = 75 ml) with elution speed 3 ml h^{-1} at 4 °C. The Qβ CP-containing fractions were identified by SDS-PAGE and proteins were precipitated at 50%

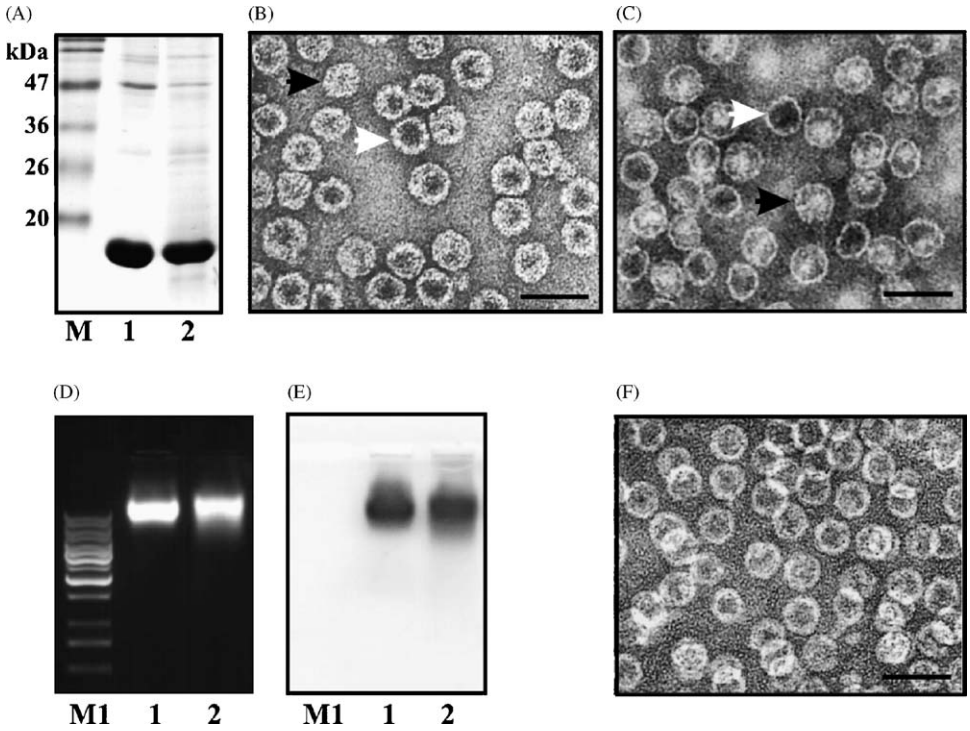

Fig. 2. Characterization of yeast-derived Qβ VLPs. Particles after purification from *S. cerevisiae* (lane 1) and *P. pastoris* (lane 2) were analyzed in Coomassie-stained 15% SDS-PAGE (A) by electron microscopy (B and C) and in native 1% agarose gel (D and E) stained with ethidium bromide (D) and subsequently with Coomassie G-250 (E). (F) *E. coli*-derived Qβ VLPs. "Empty" (free of nucleic acid) and "full" (containing nucleic acid) particles are marked with white and black arrows, respectively. M, protein molecular mass standards. M1, 1 kB DNA ladder (MBI Fermentas, Vilnius, Lithuania): 10,000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, and 250 base pairs. For EM, samples were adsorbed on carbon-formvar coated grids and stained with 2% phosphotungstic acid (pH 6.8); the grids were examined with a JEM 100 C electron microscope (JEOL Ltd., Tokyo, Japan) at an accelerating voltage 80 kV; bar, 50 nm.

saturation of ammonium sulfate overnight at 4 °C. Precipitate was collected at 12,000 rpm for 30 min at 4 °C, dissolved in 1–2 ml of column buffer and extensively dialyzed against the same buffer. This simplified purification protocol ensured about a 90% purity level of the target protein produced in both yeasts, as detected by densitometric analysis of an original SDS-PAGE picture (see Fig. 2A). EM demonstrated well-assembled Qβ VLPs in the purified material, both from *S. cerevisiae* and *P. pastoris* cells (Fig. 2B and C). Production reached 3.6 ± 0.3 mg/1 g of wet cells for *S. cerevisiae* and 4.9 ± 0.6 mg for *P. pastoris*, attaining 15–20% and 20–30% of the Qβ VLP production level in *E. coli*, respectively (see Kozlovska et al., 1993). These data were calculated as an average from three independent purifications. Compared to the *E. coli*-derived Qβ VLPs (Fig. 2F), those from yeasts were more heterogeneous in morphology. It was easy also to differentiate between particles containing nucleic acid (seen as particles with lighter tone centrally) from particles without nucleic acid (black centre; see Fig. 2B and C). The presence of nucleic acid in yeast-derived VLPs was confirmed by native agarose gel electrophoresis (Fig. 2D and E). The large part of VLP-associated nucleic acid was contributed by RNA, as it was lost after RNAse (but not DNAse) treatment (data not shown).

To compare the antigenicity and immunogenicity of the Qβ VLPs from different hosts, BALB/c female mice (five mice per group) were immunised on day 0 with 50 μg of *S. cerevisiae*-, *P. pastoris*-, or *E. coli*-derived VLPs intraperitoneally/subcutaneously (25 μg/25 μg) in complete Freund's adjuvant (Sigma, St. Louis, USA). Sera obtained on day 12 after immunization were analyzed for their reactivity with the Qβ

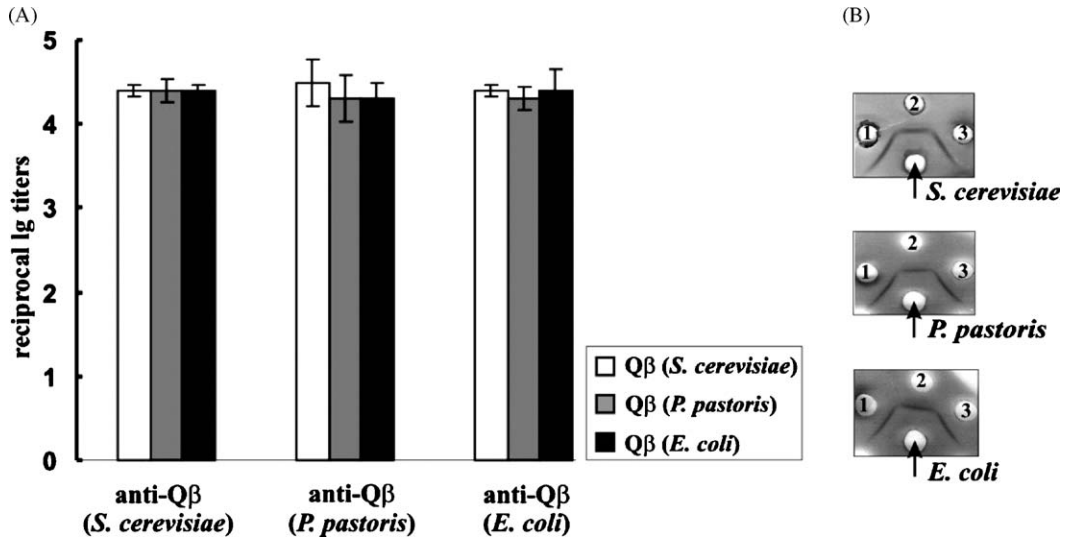

Fig. 3. Antigenicity and immunogenicity of Qβ VLPs. Reactivity of murine anti-Qβ sera with the recombinant Qβ VLPs from different hosts in direct ELISA (A) and in immunodiffusion according to Ouchterlony (B). ELISA plates were coated with particles derived from *S. cerevisiae* (open bars), *P. pastoris* (grey bars) and *E. coli* (black bars) and serially diluted murine sera were added. The titres are expressed as decimal logarithms from the reciprocal of the highest serum dilution required to yield an optical density value three times that of pre-immunised mice. Numbers in (B) represent Qβ VLPs from *S. cerevisiae* (1), *E. coli* (2), and *P. pastoris* (3).

VLPs purified from all three hosts in (i) direct ELISA according to Borisova et al. (1999) (Fig. 3A), and (ii) Ouchterlony immunodiffusion (Fig. 3B). ELISA titres were highly similar and exceeded 10^{-4} in all cases, suggesting identical antigenicity and immunogenicity of the recombinant Qβ particles. Confluence of antigen/antibody precipitation lines in the immunodiffusion test (Fig. 3B) confirmed full antigenic identity of Qβ VLPs from all three hosts.

The length of heterologously expressed Qβ CPs was compared also by matrix assisted laser desorption/ionization (MALDI) technique. The molecular mass of full-length Qβ CP theoretically is 14.3 kDa; since the proteins expressed in *E. coli* lack the first methionine, the mass of CP should be 14.18 kDa. In fact, MALDI technique confirmed the lack of methionine, as the molecular mass for *E. coli*-derived Qβ CP was 14.14 kDa. The same result was obtained for Qβ CPs produced both in *S. cerevisiae* and *P. pastoris*, indicating that yeast-derived Qβ CPs also lack the first methionine. These MALDI data were obtained in three independent measurements.

A paper devoted to expression of MS2-like particles (Legendre and Fastrez, 2005) appeared during preparation of this manuscript. Thus, these results should encourage the further development of yeast-based expression systems for high-level synthesis of phage coats as putative carriers for the genetic fusion and/or chemical coupling of foreign peptides.

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References

Borisova, G., Bundule, M., Grinstein, E., Dreilina, D., Dreimane, A., Kalis, J., Kozlovskaya, T., Loseva, V., Ose, V., Pumpen, P., Pushko, P., Snikere, D., Stankevica, E., Tsibinogin, V., Gren, E.J., 1987. Recombinant capsid structures for exposure of protein antigenic epitopes. Mol. Genet. (Life Sci. Adv.) 6, 169–174.

Borisova, G., Borschukova, O., Skrastina, D., Dislers, A., Ose, V., Pumpens, P., Grens, E., 1999. Behavior of a short preS1 epitope on the surface of hepatitis B core particles. Biol. Chem. 380, 315–324.

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 (NY) 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.

Heal, K.G., Hill, H.R., Stockley, P.G., Hollingdale, M.R., Taylor-Robinson, A.W., 1999. Expression and immunogenicity of a liver stage malaria epitope presented as a foreign peptide on the surface of RNA-free MS2 bacteriophage capsids. Vaccine 18, 251–258.

Hofmann, K.J., Cook, J.C., Joyce, J.G., Brown, D.R., Schultz, L.D., George, H.A., Rosolowsky, M., Fife, K.H., Jansen, K.U., 1995. Sequence determination of human papillomavirus type 6a and assembly of virus-like particles in *Saccharomyces cerevisiae*. Virology 209, 506–518.

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.

Kniskern, 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.

Kozlovskaya, T.M., Pushko, P.M., Stankevich, E.I., Dreimane, A.J., Snikere, D.J., Grinstein, E.E., Dreilina, D.E., Vejina, A.E., Ose, V.P., Pumpen, P.P., Gren, E.J., 1988. Genetically engineered mutants of the coat protein of RNA-containing bacteriophage fr. Mol. Biol. 22, 731–740, in Russian.

Kozlovska, T.M., Cielens, I., Dreilina, D., Dislers, A., Baumanis, V., Ose, V., Pumpens, P., 1993. Recombinant RNA phage Qβ capsid particles synthesized and self-assembled in *Escherichia coli*. Gene 137, 133–137.

Kozlovska, T.M., Cielens, I., Vasiljeva, I., Strelnikova, A., Kazaks, A., Dislers, A., Dreilina, D., Ose, V., Gusars, I., Pumpens, P., 1996. RNA phage Qβ coat proteins as a carrier for foreign epitopes. Intervirology 39, 9–15.

Krieg, A.M., 2004. Antitumor application of stimulating toll-like receptor 9 with CpG oligodeoxynucleotides. Curr. Oncol. Rep. 6, 88–95.

Legendre, D., Fastrez, J., 2005. Production in *Saccharomyces cerevisiae* of MS2 virus-like particles packaging functional heterologous mRNAs. J. Biotechnol. 117, 183–194.

Mastico, R.A., Talbot, S.J., Stockley, P.G., 1993. Multiple presentation of foreign peptides on the surface of an RNA-free spherical bacteriophage capsid. J. Gen. Virol. 74, 541–548.

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.

Miyanohara, A., Imamura, T., Araki, M., Sugawara, K., Ohtomo, 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.

Pushko, P., Kozlovskaya, T., Sominskaya, I., Brede, A., Stankevica, E., Ose, V., Pumpens, P., Grens, E., 1993. Analysis of RNA phage fr coat protein assembly by insertion, deletion and substitution mutagenesis. Protein Eng. 6, 883–891.

Ražanskienė, A., Geldmacher, A., Schmidt, J., Ritzi, A., Niedrig, M., Lundkvist, Å., Krüger, D., Meisel, H., Sasnauskas, K., Ulrich, R., 2004. High yields of stable and highly pure nucleocapsid proteins of different hantaviruses can be generated in the yeast *Saccharomyces cerevisiae*. J. Biotechnol. 111, 319–333.

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.

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.

Schwarz, K., Meijerink, E., Speiser, D.E., Tissot, A.C., Cielens, I., Renhof, R., Dishlers, A., Pumpens, P., Bachmann, M.F., 2005. Efficient homologous prime-boost strategies for T cell vaccination based on virus-like particles. Eur. J. Immunol. 35, 816–821.

Slibinskas, R., Samuel, D., Gedvilaite, A., Staniulis, J., Sasnauskas, K., 2004. Synthesis of the measles virus nucleoprotein in yeast *Pichia pastoris* and *Saccharomyces cerevisiae*. J. Biotechnol. 107, 115–124.

Storni, T., Ruedl, C., Schwarz, K., Schwendener, R.A., Renner, W.A., Bachmann, M.F., 2004. Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic side effects. J. Immunol. 172, 1777–1785.

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.

Vasiljeva, I., Kozlovska, T., Cielens, I., Strelnikova, A., Kazaks, A., Ose, V., Pumpens, P., 1998. Mosaic Qβ coats as a new presentation model. FEBS Lett. 431, 7–11.

Voronkova, T., Grosch, A., Kazaks, A., Ose, V., Skrastina, D., Sasnauskas, K., Jandrig, B., Arnold, W., Scherneck, S., Pumpens, P., Ulrich, R., 2002. The highly flexible and immunodominant C-terminal region of hamster polyomavirus VP1 exposed on chimeric bacteriophage fr capsids induced a strong VP1-specific antibody response in mice. Viral Immunol. 15, 627–643.

Weber, K., Konigsberg, W., 1975. Proteins of the RNA phages. In: Zinder, N.D. (Ed.), RNA Phages. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 51–84.

II

High-level expression and purification of bacteriophage GA virus-like particles from yeast *Saccharomyces cerevisiae* and *Pichia pastoris*

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High-level expression and purification of bacteriophage GA virus-like particles from yeast *Saccharomyces cerevisiae* and *Pichia pastoris*

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Abstract

The recombinant virus-like particles (VLPs) generated by heterologous expression of RNA bacteriophage coat protein genes have been proposed as promising carriers of foreign epitopes and nucleic acids for development of novel vaccines and gene therapy tools. Here, we investigated the possibility to produce bacteriophage GA coat protein-derived VLPs in yeast *Saccharomyces cerevisiae* and *Pichia pastoris*. To optimize growth conditions, three expression systems have been explored: GAL1 and GAL10 promoter-directed expression in *S. cerevisiae* as well as AOX1 promoter-directed expression in *P. pastoris*. Synthesis of GA coat protein and formation of VLPs was observed in all three cases. GA VLPs were purified by a single size-exclusion chromatography step till 80 to 90 % of homogeneity. The final amount of purified VLPs varied between 0.6 to 2.0 mg per 1 g of cells for *S. cerevisiae*, while expression in *P. pastoris* resulted in VLP yield of up to 3 mg from the same amount of cells. The recombinant VLPs obtained may be further used for exposition of foreign epitopes on their surface via chemical coupling and/or packaging of immunostimulatory DNA sequences internally.

Key words: bacteriophage GA, coat protein, expression, virus-like particles, yeast.

Introduction

The RNA bacteriophages (phages) are small viruses with a simple organization. Their T = 3 icosahedral shell is composed of 180 copies of the coat protein (CP) and one copy of the maturation protein that encapsidates approximately 3,500-nucleotide-long genomic RNA. These phages were first isolated from *Escherichia coli* (Loeb, Zinder 1961), but later were also found in *Caulobacter* (Schmidt 1966), *Pseudomonas* (Bradley 1966) and *Acinetobacter* (Coffi 1995). To date, the coliphages have been classified into four groups based on their serological and physicochemical properties. Groups I and II with MS2 and GA phages as the type species are quite similar and are collectively called group A. Phages Qb and SP, members of groups III and IV, respectively, together form group B (Furuse 1987).

The molecular biology of the RNA phages has been extensively studied (van Duin 1999; Weber 1999). In addition, high-resolution X-ray structures of several RNA phages have been determined (Valegård et al. 1990; Liljas et al. 1994; Golmohammadi et al. 1996; Tars et al. 1997; Tars et al. 2000). These data together with the observation that phage CPs alone in

absence of the viral nucleic acid are able to form non-infectious virus-like particles (VLPs) in *E. coli* (Kozlovska et al. 1993; Pushko et al. 1993) have made icosahedral phage shells attractive as objects for gene and protein engineering manipulations. Thus, recombinant VLPs formed by CPs of group-I RNA phages fr and MS2 have been successfully used for presentation of foreign protein sequences on their surface via genetic fusion (Mastico et al. 1993; Heal et al. 1999; Voronkova et al. 2002). However, steric factors limit the length of peptides that can be added to the CP while still preserving its ability to self-assemble into VLPs. Alternatively, the desired peptides can be chemically coupled to surface-exposed lysine residues (Jegerlehner et al. 2002).

Recently, we and others have demonstrated the potential of yeast cells as a host for producing properly folded phage MS2 and Qb CP-derived VLPs (Legendre, Fastrez 2005; Freivalds et al. 2006). Here, we continue our investigations to show the formation of group-II RNA phage GA VLPs in two different yeasts to therefore extend a way for further development of a yeast-derived phage VLP technology.

Materials and methods

Strains and plasmid constructions

The GA CP-encoding gene was amplified with desired oligonucleotide primers by polymerase chain reaction (PCR) from *Escherichia coli* expression plasmid pGA-355-24 (I. Cielēns, personal communication). The CP sequence encoded by this plasmid differs from that deposited in GenBank (Acc. No. X03869; Inokuchi et al. 1986) at positions 59 and 79 and is identical to that published by Tars et al. (1997). Construction details are summarized in Table 1. For expression in *S. cerevisiae* strain YPH499, the appropriate PCR fragment was digested with *Bam*HI/*Hind*III and cloned in pESC-URA vector, resulting in a pESC-GA plasmid. For expression in the *S. cerevisiae* strain AH22, the PCR fragment was digested with *Xba*I/*Bgl*II and cloned in pFX-Qb, resulting in a pFX-GA plasmid. The *P. pastoris* expression plasmid pPIC-GA was generated by cloning of the respective PCR fragment into pPIC3.5K vector using *Bam*HI and *Sna*BI restriction sites. PCR and cloning procedures were carried out using standard molecular biology protocols (Sambrook et al. 1989).

Yeast transformation and expression conditions

S. cerevisiae strains AH22 and YPH499 were transformed with pFX-GA and pESC-GA, respectively, using standard lithium acetate/polyethylene glycol procedure as decribed by Gietz et al. (1992). The YPH499/pESC-GA transformants were selected on uracil-free agarized synthetic dextrose (SD) minimal medium according to manufacturer's protocol. For expression, individual transformants were cultivated in liquid synthetic galactose (SG) medium for up to 72 h, until OD590 reached 6 - 7.

Transformed AH22/pFX-GA clones were selected on agarized rich YEP medium containing 2 % glucose (YEPD medium) supplemented with 10 mM formaldehyde. Individual transformants were then incubated in liquid YEPD medium supplemented with 5 mM formaldehyde for 20 to 24 h until optical density OD_{590} reached 6 - 8. For induction, the cells were collected by low-speed centrifugation and resuspended in YEP medium with 3 % galactose (YEPG medium), and cultivation was continued for another 20 - 24 h, with final OD_{590} 10 - 14.

Electroporation of *P. pastoris* with the Ecl136II-linearized pPIC-GA plasmid and selection of clones containing multiple integrations of expression cassette into yeast chromosome were performed as described by Freivalds et al. (2006). GA CP gene expression in *P. pastoris* was achieved according to recommendations of the manufacturer. Briefly, selected clones were incubated in BMGY medium for 20 to 24 h until OD_{590} reached 4 - 6; then the cells were collected by low-speed centrifugation and resuspended in BMMY induction medium and cultivated for 72 h. All cultivations were performed in 500 mL flasks with 100 mL of expression media at 30 °C on a rotary shaker either at 200 rpm (*S. cerevisiae*) or at 250 rpm (*P. pastoris*). The cells were collected by low-speed centrifugation, washed with distilled water and stored at –20 °C until use.

Purification of GA VLPs

For purification of GA VLPs, 1 g of yeast cells was resuspended in 4 mL of lysis buffer (20 mM Tris-HCl, 5 mM EDTA, 0,65 M NaCl, 1 mM PMSF, pH = 7.8). To disrupt the cells, suspension was applied to the French press (three strokes, 20 000 psi). The unsoluble cell debris was separated by centrifugation (1 h, 15 500 g) and discarded. Soluble supernatant proteins were concentrated by addition of solid ammonium sulfate to 60 % of saturation and incubation overnight at 4 °C. After centrifugation 20 min at 8000 g, the proteins were solubilized into 1 mL of lysis buffer without PMSF and loaded onto a Sepharose CL4B gelfiltration column (V = 90 mL, h = 110 cm), with the buffer flow rate approximately 1.0 mL h^{-1}, and 1.5 mL fractions were collected. All of the purification steps were performed at 4 °C.

Protein content in cell and protein samples was analyzed in denaturating polyacrylamide gels (PAAG), with 4 % stacking and 15 % separating gel, according to standard protocols. To vizualize protein bands, the gels were stained with Coomassie Brilliant Blue (CBB). Ouchterlony's double radial immunodiffusion with cell lysates was performed using rabbit polyclonal anti-GA antibodies. VLP electrophoresis in 1 % native agarose gels was performed in TAE buffer (pH 8.4) for about 1 h at a constant 90 mA current. VLPs were concentrated

Table 1. Description of vectors and strains used for expression of phage GA CP gene in yeast *S. cerevisiae* and *P. pastoris*. Cloning sites in forward (Fw) and reverse (Rv) primers are underlined. Start and termination codons of the GA CP gene are shown in bold

Strain/genotype	Vector	Primers (5' _ 3')	Construct
S. cerevisiae YPH499	pESC-URA (Stratagene)	Fw: CAGGATCC**ATG**GCAACTTTACGCAGTTTCGT	pESC-GA
MATa ura3-52 lys2-801_amber		Rv: TGAAGCT**TA**CGCGTAGAAGCCACTCTG	
ade2-101_ochre trp1-_63 his3-_200 leu2-_1			
S. cerevisiae AH22	pFX-Q_ (Samuel et al. 2002;	Fw: TTTCTAGAACAA**ATG**GCAACTTTACGCAGTTTCG	pFX-GA
MATa leu2 his4	Freivalds et al.,2006)	Rv: TTAGATCT**TA**CGCGTAGAAGCCACTCTG	
P. pastoris GS115	pPIC3.5K (Invitrogen)	Fw: TTGGATCCACC**ATG**GCAACTTTACGCAG	pPIC-GA
his4		Rv: TTTACGTA**TTA**CGCGTAGAAGCCACTC	

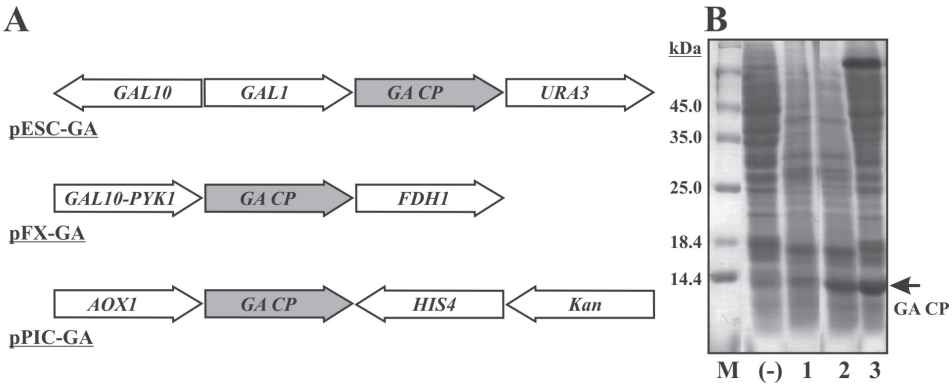

Fig. 1. Expression of the GA CP gene in yeast. A, schematic presentation of the vectors used. The relative direction of genes and promoters is indicated by arrows. *URA3*, *HIS4*, and *FDH1* encode for genes used as primary markers for selection of yeast transformants, while the Kan gene allows secondary screening of *P. pastoris* for high-copy integrants. B, CBB-stained PAAG demonstrating the total synthesis level of GA CP. M, protein molecular weight marker, (-), non-transformed *P. pastoris* cells as a negative control. Lanes 1, 2 and 3 represent cell lysates from strains YPH499, AH22, and GS115, respectively. Accumulation of GA CP is indicated by an arrow.

by dialysis against storage buffer (50 % glycerol, 10 mM Tris-HCl, 2.5 mM EDTA, 325 mM NaCl, pH 7.8) for at least 24 h. Protein concentration measurements were made according to Bradford (1976).

For electron microscopy, samples were adsorbed on carbon-formvar coated grids and stained with 2 % phosphotungstic acid (pH 6.8); the grids were examined with a JEM 100C electron microscope (JEOL Ltd., Tokyo, Japan) at an accelerating voltage 80 kV.

Results

Design of constructs and expression

In order to establish optimal conditions for generation of GA VLPs in yeast, we aimed to compare three well-described expression systems by cloning of phage GA CP gene in three different vectors. The resulting expression units are schematically presented in Fig. 1A. In the first approach, the pESC-URA vector was selected as a template for cloning and *GAL1* promoter-directed expression. This vector was previously used for generation of phage MS2 VLPs (Legendre, Fastrez 2005). In a second approach, we focused on the pFX-derived plasmid, which represents an already established VLP producing system exploiting hybrid *GAL10-PYK1* promoter. In addition, this vector encodes for the *FDH1* gene of *Candida maltosa*, conferring resistance to formaldehyde (Sasnauskas et al. 1992), which is very convenient for quick selection of transformants on rich media. In a third approach, the strong *AOX1* promoter-directed expression provided by the *P. pastoris* expression vector pPIC3.5K was undertaken.

All three constructs pESC-GA, pFX-GA, and pPIC-GA were transformed in their corresponding yeast host (YPH499, AH22 and GS115, respectively). While both *S. cerevisiae* vectors exist in the cells as episomes, pPIC3.5K does not contain a yeast replication origin and needs to be integrated into the host genome via homologous recombination. Due to

Table 2. Summary of generation of phage GA VLPs in yeast. Replication in at least two independent experiments

Strain/plasmid	Amount of cells after cultivation (g L^{-1})	Immunologic anti-GA titers	GA VLPs (mg from 1 g of cells)
YPH499/pESC-GA	15 - 20	1:4 - 1:8	0.6 - 0.8
AH22/pFX-GA	40 - 50	1:16	1.5 - 2
GS115/pPIC-GA	40 - 50	1:32	2.5 - 3

the presence of *Kan* gene in the expression unit, we have selected clones with multiple expression units integrated in the yeast chromosome, which accordingly exhibited increased resistance to Geneticin in *P. pastoris*.

The selected clones were cultivated in appropriate conditions ensuring maximal expression level in each particular case. Optical densities of yeast cells notably varied between strains due to the content of cultivation media, resulting in different amounts of cells obtained at the end of cultivation (Table 2). Total synthesis of GA CP was monitored by CBB-stained PAAG (Fig. 1B) showing well-detectable accumulation of ~13.6 kDa protein in strains AH22 and GS115, while in strain YPH499 the production was significantly lower. Nevertheless, presence of the specific product in the latter case was verified by Western blot with GA-specific antibodies (data not shown). Therefore synthesis of GA CP was confirmed in all three cases and we proceeded to analyze solubility and self-assembly of the target protein.

Purification and characterization of GA VLPs

As the first step of purification, the cells were disrupted by French press and the soluble protein fraction was analyzed by Ouchterlony double radial immunodiffusion with anti-GA antibodies. The obtained titres (Table 2) correlated well with the absolute GA CP amounts (Fig. 1B), suggesting that the majority of the target protein was in the soluble protein fraction and also providing indirect evidence of the presence of VLPs in cell lysates, since unassembled CP usually accumulates as unsoluble aggregates in cells (our unpublished observations).

For further purification, a concentrated mixture of soluble proteins was subjected to size-exclusion chromatography on Sepharose CL4B beads. As expected, the majority of the target protein was eluted between 36 to 42 ml, which corresponds to the calculated volume where VLPs may appear. The respective part of the elution profile is presented in Fig. 2A. Importantly, the larger part of contaminants was removed during the chromatography, indicating the effectiveness of the particular method.

To verify the presence of VLPs, the same fractions were also subjected to native agarose gel electrophoresis (Fig. 2B). The gel was stained with ethidium bromide demonstrating a strong nucleic acid signal that was correlated with the amount of GA CP (Fig. 2A). Taken together, these data strongly suggest formation of VLP nucleoprotein complexes migrating towards anode in native agarose gel. In part, this might be explained by presence of a large amount of negatively charged nucleic acid non-specifically packed inside the VLPs.

Based on the information presented above, the peak VLP-containing fractions were pooled and concentrated. Overall amounts of the obtained VLPs are presented in Table 2. These data correlated well with both the total synthesis level of GA CP (Fig. 1B) and

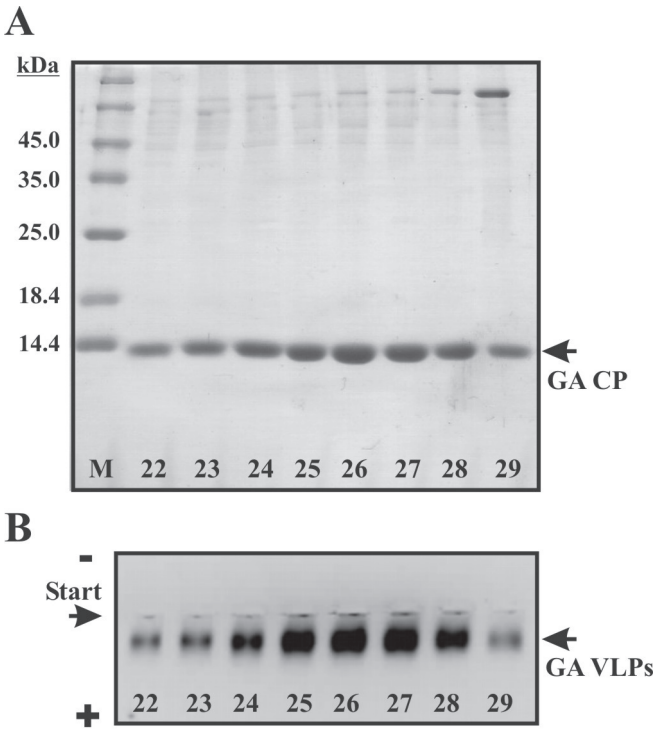

Fig. 2. Purification and detection of recombinant GA VLPs. A, CBB-stained PAAG that demonstrates protein content in *P. pastoris*-derived peak column fractions. The numbers below correspond to appropriate fractions. Both S. cerevisiae strains gave similar profiles but with accordingly lower protein amount (data not shown). M, protein molecular weight marker. The same samples were analyzed in ethidium bromide stained native agarose gel (B). Target proteins are marked by arrows. Anode and cathode are designated as "+" and "-", respectively.

immunological anti-GA titers obtained in supernatant (Table 2). Visually, the overall purity of the VLP samples in PAAG can be estimated as 80 to 90 %, which is rather high after only a single purification step (Figure 3A). Not surprisingly, the highest purity was associated with the highest expression level, obtained for *P. pastoris*. Finally, the samples were subjected to electron microscopy, which confirmed the formation of icosahedral phage GA-like particles in all three cases (Fig. 3B).

Taken together, an efficient GA VLP generation system was established in both yeast *S. cerevisiae* and *P. pastoris*. The highest yield of VLPs was found in the case of *P. pastoris*. Such recombinant wild-type GA VLPs may be further used for exposition of foreign peptides on their surface via chemical coupling and/or packaging of immunostimulatory DNA sequences internally.

Discussion

Highly immunogenic VLPs generated by heterologous expression of viral structural genes have become a powerful tool for vaccine development. In addition to being effective

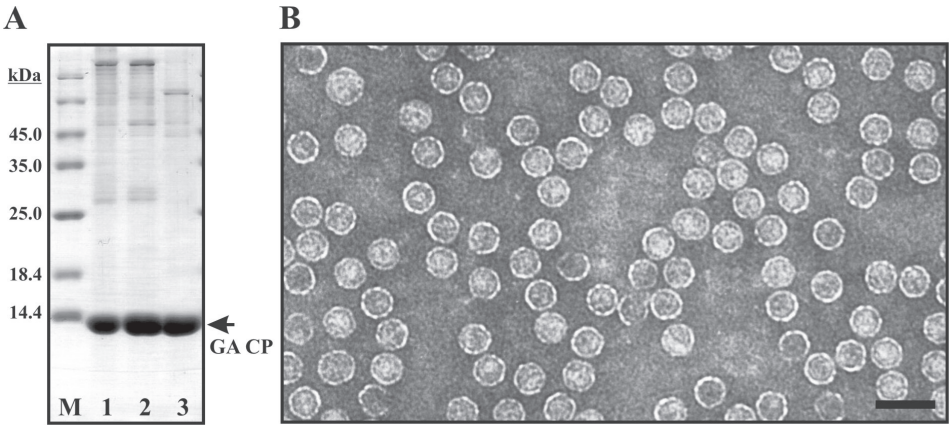

Fig. 3. Characterization of purified GA VLPs. A, CBB-stained PAAG of the final product after size-exclusion chromatography. M, protein molecular weight marker. Lanes 1, 2 and 3 demonstrate the purity of VLPs obtained from strains YPH499, AH22, and GS115, respectively. B, direct evidence of VLP formation by electron microscopy. Only VLPs purified from yeast *P. pastoris* are presented. Scale bar: 50 nm.

vaccines against the corresponding virus from which they were derived, VLPs can also be used to present foreign epitopes to the immune system. This approach might be combined with the packaging of selected genes and drugs inside VLPs (for recent review articles see Georgens et al. 2005; Xu et al. 2006; Jennings, Bachmann 2008).

Icosahedral capsids of the simple RNA phages have attracted attention of scientists as promising carriers of foreign epitopes and nucleic acids. A special interest has been devoted to group III phage Qb VLPs. Bacterially expressed Qb VLPs have been explored for chemical coupling of desired peptides to surface-exposed lysine residues. To increase their immunogenicity, such chimeric VLPs can be further engineered by loading them with short synthetic DNA sequences (Storni et al. 2004; Schwarz et al. 2005). Several Qb phage-derived therapeutic vaccine candidates have already entered phase I to III clinical trials (Kündig et al. 2006; Maurer, Bachmann 2007; Tissot et al. 2008).

However, for vaccine development, *E. coli*-derived VLPs need to be purified from contamination of bacterial endotoxins, which is costly and time-consuming. Alternatively, VLPs could be produced in "endotoxin-free" organisms, such as yeast, which has been regarded as generally safe for human use. Up to now, a large number of structural genes from mammalian viruses have been expressed in yeast resulting in formation of VLPs (Valenzuela et al. 1982; Kniskern et al. 1986; Sasnauskas et al. 1999; Samuel et al. 2002; Slibinskas et al. 2004; Juozapaitis et al. 2007). The yeast expression system has been used successfully to produce licensed prophylactic vaccines against human hepatitis B virus (McAleer et al. 1984) and human papillomavirus (Bryan 2007).

An important drawback of repetitive vaccination with chimeric VLPs might be their limited effectiveness due to the presence of neutralizing antibodies against capsid protein induced after the first application (Da Silva et al. 2001). Therefore, technologies to generate a broad spectrum of carrier VLPs need to be developed. It should be also noted that humans normally do not possess pre-existing antibodies to RNA phages and the immune response will therefore not be impaired. Taking into account these considerations, we extended our

investigations to optimize generation of phage GA-derived VLPs in yeast. The phage GA CP gene was therefore cloned and expressed in three different vectors.

Despite the rather small production of GA CP observed under GAL1 promoter in pESC-URA, this vector contains another GAL10 promoter located in the opposite orientation. This might be advantageous for co-expression of two genes for protein-protein or protein-nucleic acid interaction studies in *S. cerevisiae*. Significantly higher expression of target protein was observed in case of pFX-GA. This is in line with previous data about pFX-directed high-level expression and VLP formation of polyomavirus VP1 and mumps virus nucleoprotein in *S. cerevisiae* (Sasnauskas et al. 1999; Samuel et al. 2002). Finally, methylotrophic yeast, *P. pastoris*, was superior in production of GA CP and yield of recombinant VLPs, therefore confirming its selection as a host microorganism for high-level expression of recombinant genes for both basic laboratory research and industrial manufacture (for a review see Macauley-Patrick et al. 2005).

Recently, we demonstrated the assembly of phage Qb VLPs in *S. cerevisiae* and *P. pastoris* using pFX- and pPIC3.5K-derived expression vectors, respectively (Freivalds et al. 2006). The results obtained were quite similar to those described in this article in that selection of *P. pastoris* clones with multiple expression units integrated in the yeast chromosome resulted in increased expression and outcome of recombinant VLPs while those with single insertion demonstrated rather low synthesis of the target protein. However, not always more integration events leads to higher production, as shown for synthesis of the measles virus nucleoprotein (Slibinskas et al. 2004). Thus, wide screening and selection of individual *P. pastoris* clones is needed for obtaining maximum production in each particular case.

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References

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.

Bradley D.E. 1966. The structure and infective process of a *Pseudomonas aeruginosa* bacteriophage containing ribonucleic acid. *J. Gen. Microbiol.* 45: 83–96.

Bryan J.T. 2007. Developing an HPV vaccine to prevent cervical cancer and genital warts. *Vaccine* 25: 3001–3006.

Coffi H. 1995. *Lysotypie des Acinetobacter.* Thèse de Maîtrise dès Sciences, Laval University, Québec, Canada.

Da Silva D.M., Pastrana D.V., Schiller J.T., Kast W.M. 2001. Effect of preexisting neutralizing antibodies on the anti-tumor immune response induced by chimeric human papillomavirus virus-like particle vaccines. *Virology* 290: 350–360.

Freivalds J., Dislers A., Ose V., Skrastina D., Cielens I., Pumpens P., Sasnauskas K., Kazaks A. 2006. Assembly of bacteriophage Q_ virus-like particles in yeast *Saccharomyces cerevisiae* and *Pichia pastoris*. *J. Biotechnol.* 123: 297–303.

Furuse K. 1987. Distribution of the coliphages in the environment: general considerations. In: Goya S.M, Gerba C.P., Bitton G. (eds) *Phage Ecology*. Wiley Interscience, New York, pp. 87–124.

Georgens C., Weyermann J., Zimmer A. 2005. Recombinant virus like particles as drug delivery system. *Curr. Pharm. Biotechnol*. 6: 49–55.

Gietz D., St Jean A., Woods R.A., Schiestl R.H. 1992. Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.* 20: 1425.

Golmohammadi R., Fridborg K., Bundule M., Valegård K., Liljas L. 1996. The crystal structure of bacteriophage Qb at 3.5 Å resolution. *Structure* 4: 543–554.

Heal K.G., Hill H.R., Stockley P.G., Hollingdale M.R., Taylor-Robinson A.W. 1999. Expression and immunogenicity of a liver stage malaria epitope presented as a foreign peptide on the surface of RNA-free MS2 bacteriophage capsids. *Vaccine* 18: 251–258.

Inokuchi Y., Takahashi R., Hirose T., Inayama S., Jacobson A.B., Hirashima A. 1986. The complete nucleotide sequence of the group II RNA coliphage GA. *J. Biochem*. 99: 1169–1180.

Jegerlehner A., Tissot A., Lechner F., Sebbel P., Erdmann I., Kündig T., Bächi T., Storni T., Jennings G., Pumpens P., Renner W.A., Bachmann M.F. 2002. A molecular assembly system that renders antigens of choice highly repetitive for induction of protective B cell responses. *Vaccine* 20: 3104–3112.

Jennings G.T., Bachmann M.F. 2008. The coming of age of virus-like particle vaccines. *Biol. Chem*. [in Press].

Juozapaitis M., Serva A., Kucinskaite I., Zvirbliene A., Slibinskas R., Staniulis J., Sasnauskas K., Shiell B.J., Bowden T.R., Michalski W.P. 2007. Generation of menangle virus nucleocapsid-like particles in yeast *Saccharomyces cerevisiae*. *J. Biotechnol.* 130: 441–447.

Kniskern 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.

Kozlovska T.M., Cielens I., Dreilina D., Dislers A., Baumanis V., Ose, V., Pumpens, P. 1993. Recombinant RNA phage Qb capsid particles synthesized and self-assembled in *Escherichia coli*. *Gene* 137: 133–137.

Kündig T.M., Senti G., Schnetzler G., Wolf C., Prinz Vavricka B.M., Fulurija A., Hennecke F., Sladko K., Jennings G.T., Bachmann M.F. 2006. Der p 1 peptide on virus-like particles is safe and highly immunogenic in healthy adults. *J. Allergy Clin. Immunol.* 117: 1470–1476.

Legendre D., Fastrez J. 2005. Production in Saccharomyces cerevisiae of MS2 virus-like particles packaging functional heterologous mRNAs. *J. Biotechnol.* 117: 183–194.

Liljas L., Fridborg K., Valegård K., Bundule M., Pumpens P. 1994. Crystal structure of bacteriophage fr capsids at 3.5 Å resolution. *J. Mol. Biol.* 244: 279–290.

Loeb T., Zinder N.D. 1961. A bacteriophage containing RNA. *Proc. Natl. Acad. Sci. USA* 47: 282–289.

Macauley-Patrick S., Fazenda M.L., McNeil B., Harvey L.M. 2005. Heterologous protein production using the Pichia pastoris expression system. *Yeast* 22: 249–270.

Mastico R.A., Talbot S.J., Stockley P.G. 1993. Multiple presentation of foreign peptides on the surface of an RNA-free spherical bacteriophage capsid. *J. Gen. Virol.* 74: 541–548.

Maurer P., Bachmann M.F. 2007. Vaccination against nicotine: an emerging therapy for tobacco dependence. *Expert Opin. Investig. Drugs* 16: 1775–1783.

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: 178–180.

Pushko P., Kozlovskaya T., Sominskaya I., Brede A., Stankevica E., Ose V., Pumpens P., Grens, E. 1993. Analysis of RNA phage fr coat protein assembly by insertion, deletion and substitution mutagenesis. *Protein Eng.* 6: 883–891.

Sambrook J., Fritsch E.F., Maniatis T. 1989. *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, N.Y.

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.

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.

Schmidt J.M. 1966. Observations on the adsorption of Caulobacter bacteriophages containing ribonucleic acid. *J. Gen. Microbiol.* 45: 347–53.

Schwarz K., Meijerink E., Speiser D.E., Tissot A.C., Cielens I., Renhof R., Dishlers A., Pumpens P., Bachmann, M.F. 2005. Efficient homologous prime-boost strategies for T cell vaccination based on virus-like particles. *Eur. J. Immunol.* 35: 816–821.

Slibinskas R., Samuel D., Gedvilaite A., Staniulis J., Sasnauskas K. 2004. Synthesis of the measles virus nucleoprotein in yeast *Pichia pastoris* and *Saccharomyces cerevisiae*. *J. Biotechnol.* 107: 115–124.

Storni T., Ruedl C., Schwarz K., Schwendener R.A., Renner W.A., Bachmann M.F. 2004. Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic side effects. *J. Immunol.* 172: 1777–1785.

Tars K., Bundule M., Fridborg K., Liljas L. 1997. The crystal structure of bacteriophage GA and a comparison of bacteriophages belonging to the major groups of *Escherichia coli* leviviruses. *J. Mol. Biol.* 271: 759–773.

Tars K., Fridborg K., Bundule M., Liljas L. 2000. The three-dimensional structure of bacteriophage PP7 from Pseudomonas aeruginosa at 3.7–A resolution. *Virology* 272: 331–337.

Tissot A.C., Maurer P., Nussberger J., Sabat R., Pfister T., Ignatenko S., Volk H.D., Stocker H., Müller P., Jennings G.T., Wagner F., Bachmann M.F. 2008. Effect of immunisation against angiotensin II with CYT006-AngQb on ambulatory blood pressure: a double-blind, randomised, placebo-controlled phase IIa study. *Lancet* 371: 821–827.

Valegård K., Liljas L., Fridborg K., Unge T. 1990. The three-dimensional structure of the bacterial virus MS2. *Nature* 345: 36–41.

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.

van Duin J. 1999. Single-stranded RNA phages. In: Webster R.G., Granoff A. (eds) *Encyclopedia of Virology,* Academic Press, London, pp. 1663–1668.

Voronkova T., Grosch A., Kazaks A., Ose V., Skrastina D., Sasnauskas K., Jandrig B., Arnold W., Scherneck S., Pumpens P., Ulrich R. 2002. The highly flexible and immunodominant C-terminal region of hamster polyomavirus VP1 exposed on chimeric bacteriophage fr capsids induced a strong VP1-specific antibody response in mice. *Viral Immunol.* 15: 627–643.

Weber, H. 1999. Qb replicase. In: Creighton T.E. (ed) *Encyclopedia of Molecular Biology*. John Wiley, New York, pp. 2085–2087.

Xu Y.F., Zhang Y.Q., Xu X.M., Song G.X. 2006. Papillomavirus virus-like particles as vehicles for the delivery of epitopes or genes. *Arch. Virol.* 151: 2133–2148.

Bakteriofāga GA vīrusam līdzīgo daļiņu ekspresija raugos *Saccharomyces cerevisiae* un *Pichia pastoris* un to attīrīšana

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Kopsavilkums

Rekombinētās vīrusiem līdzīgās daļiņas (VLD), kas ģenerētas RNS baketeriofāga apvalka proteīna gēnu heterologās ekspresijas ceļā, ir daudzsološi svešu epitopu un nukleīnskābju pārnesēji jaunu vakcīnu un gēnu terapijas līdzekļu attīstībai. Šajā darbā mēs pētījām iespēju ražot no bakteriofāga GA apvalka proteīna izveidotās VLD raugos *Saccharomyces cerevisiae* un *Pichia pastoris*. Augšanas apstākļu optimizēšanai pārbaudīja trīs dažādas ekspresijas sistēmas: GAL1 un GAl10 promotoru vadīto ekspresiju *S. cerevisiae* un AOX1 promotora vadīto ekspresiju *P. pastoris*. Visās sistēmās novēroja GA apvalka proteīna sintēzi un VLD veidošanos. GA VLD attīrīja ar vienas pakāpes izmēra izslēgšanas hromatogrāfiju līdz 80 - 90 % homogenitātei. Attīrīto VLD gala iznākums bija 0.6 līdz 2.0 mg uz 1 g šūnu *S. cerevisiae* gadījumā, bet ekspresija *P. pastoris* deva līdz pat 3 mg VLD uz identisku šūnu daudzumu. Iegūtās rekombinantās VLD var tālāk izmantot svešu epitopu eksponēšanai uz to virsmas ar ķīmiskas piesaistīšanas starpniecību vai imunostimulējošu DNS sekvenču ievietošanai tajās.

III

Specificity of packaging mRNAs in bacteriophage GA virus-like particles in yeast *Saccharomyces cerevisiae*

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Specificity of packaging mRNAs in bacteriophage GA virus-like particles in yeast *Saccharomyces cerevisiae*

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Abstract

Heterologous expression of RNA bacteriophage coat protein genes leads to formation of virus-like particles that encapsulate intracellular RNA. Here we packaged specific mRNA molecules into bacteriophage GA capsids *in vivo*. For specificity we employed a GA operator – a 21-nucleotide-long RNA sequence that folds into a stem-loop structure and can specifically bind to the coat protein of the phage. Several plasmids were constructed for expression in *Saccharomyces cerevisiae*, which directed synthesis of both the GA coat protein and the mRNA to be packaged. As models for packaging, three different mRNAs (GA coat protein, ENA-78 and GFP) were used, each constructed in two versions that differed by the presence of the GA operator within their sequence. RNA content analysis of the produced capsids in gel electrophoresis revealed the existence of RNAs of predicted lengths. The presence of the packaged mRNAs in the capsids was further verified by reverse transcription PCR. However, the operator had rather small effect on the specificity of capsid contents, since the mRNA of GA coat protein was also invariably detected inside the capsids.

Key words: bacteriophage GA, coat protein, mRNA, packaging, virus-like particles.

Introduction

RNA bacteriophages belonging to the Leviviridae family are small viruses that infect several Gram-negative bacteria genera. RNA phages infecting *Escherichia coli* have been divided into four groups denoted I to IV (Fiers 1979). Groups I and II are related to each other and belong to the Levivirus genus; phages from groups III and IV are also closely similar and assigned to the Allolevivirus genus. The best-known representatives are phages MS2 from group I, GA from group II, Qβ from group III and SP from group IV.

The genomic RNA of leviviruses is an approximately 3500-nucleotides-long single-stranded RNA molecule that codes for only four proteins: coat protein, lysis protein, maturation protein and a subunit of the viral replicase – an RNA-dependent RNA polymerase (for a review, see van Duin 1988). The viral genome is enclosed in a $T = 3$ quasi-equivalent icosahedral capsid with diameter about 28 nm, which consists of 180 coat protein molecules. Each virion also contains a single copy of the maturation protein, which is essential for the attachment of the phage to bacterial F-pili and successful infection.

Besides its structural role, the coat protein also functions as a specific RNA binding protein that recognizes a stem-loop structure at the very beginning of the replicase gene (Bernardi, Spahr 1973). The stem-loop is often designated a translational operator, as its binding to the coat protein effectively represses the synthesis of replicase. The operator is

also believed to be a packaging signal that initiates the assembly of the capsid and thus ensures recognition and selective encapsidation of the phage RNA (Hohn 1969a; Beckett, Uhlenbeck 1988). Although the coat protein can assemble into phage-like particles *in vitro* when mixed with the genomic RNA of the phage or unspecific RNAs of different lengths (Hohn 1969b), the presence of the operator lowers the concentration requirements for capsid assembly (Beckett et al. 1988).

The coat protein genes from numerous RNA phages have been reverse-transcribed, cloned and expressed in *Escherichia coli*, leading to assembly of virus-like particles (VLPs) without the need for any other viral components (Kastelein et al. 1983; Kozlovskaya et al. 1986; Peabody 1990; Kozlovska et al. 1993; Lim et al. 1994). Such recombinant VLPs encapsulate intracellular RNA (Pickett, Peabody 1993), but are morphologically and immunologically indistinguishable from native phages.

There has also been some interest to produce VLPs *in vivo* with a specific nucleic acid content. For this, a coexpression system that directs simultaneous production of both the coat protein and the RNA to be packaged is generally utilized. For specificity, the target RNA contains the translational operator of the replicase gene. In this way, recombinant MS2 capsids containing *LacZ* RNA were produced in *E. coli* (Pickett, Peabody 1993). A similar system was recently employed also in yeast *Saccharomyces cerevisiae* where the mRNA of human growth hormone was packaged into MS2 VLPs (Legendre, Fastrez 2005), demonstrating the potential of RNA phage capsids as carriers and possible delivery vehicles for therapeutic mRNAs. Others have made use of the remarkable stability of RNA phage capsids and engineered VLPs as containers for ribonuclease-protected RNA molecules of choice. As potential controls and standards for RT-PCR detection of RNA viruses, MS2 capsids have been produced that contain RNAs with a consensus sequence from HIV-1 *gag* gene (Pasloske et al. 1998) or, similarly, a longer RNA with sequences combined from hepatitis C virus, SARS coronavirus and avian influenza virus genomes (Wei et al. 2008).

Up to now, all efforts to encapsulate heterologous RNAs into RNA phage capsids *in vivo* have been directed towards phage MS2, a type species of group I. Since the details of molecular interactions between the coat protein and RNA operator vary among different RNA phages, the results obtained with one phage cannot be extrapolated to others. We investigated whether specific RNAs can be encapsulated *in vivo* using the coat protein from phage GA, a characteristic representative of group II.

Materials and methods

Construction of plasmids

The construction of pESC-GA has been described elsewhere (Freivalds et al. 2008). pESC-GAop was constructed by amplifying a fragment from pGACPop with primers GA_fwd and GAop_rev3 (for nucleotide sequences of primers, see Table 1), which was cloned into *Bam*HI/*Hind*III-digested pESC-URA vector (Stratagene). Note: pGACPop is an *E. coli* expression plasmid that contains the GA operator sequence downstream the GA coat protein gene, with nucleotide sequence corresponding to primers GAop_rev1, GAop_rev2 and GAop_rev3. Vectors for the two-gene coexpression plasmids were prepared by digesting pESC-GA with *Not*I and *Bgl*II (pESC-GAv1) or with *Eco*RI and *Not*I (pESC-GAv2). A short double-stranded DNA fragment containing GA operator sequence and

Table 1. Oligonucleotides used for the construction of plasmids. Restriction sites for cloning are underlined and the initiation and termination codons of genes are shown in bold

Oligonucleotide	Nucleotide sequence (5' to 3')
ENA_fwd	TCGAATTC**ATG**GCTGGTCCTGCCGC
ENA_rev	ATGCGGCCGC**TTA**GTTTTCCTTGTTTCCA
GA_fwd	CAGGATCC**ATG**GCAACTTTACGCAGTTTCGT
GA_rev	TGAAGC**TTA**CGCGTAGAAGCCACTCTG
GAop_rev1	ACATAGGTTTTCCTTATGTTTTGC**TTA**CGCGTAGAAGCCACTCTG
GAop_rev2	CATGATCAATTGACCTCCTTATCGGAACATAGGTTTTCCTTATGTT
GAop_rev3	AGAAGCTTCATGATCAATTGACCTCC
GAop1	GGCCGCAAAACATAAGGAAAACCTATGTTCCA
GAop2	GATCTGGAACATAGGTTTTCCTTATGTTTTGC
GFP_fwd	TCGAATTCC**ATG**GTGAGCAAGGGCGAGGA
GFP_rev	GAGCGGCCGCAAGC**TTA**CTTGTACAGCTCGTCCAT

*Not*I and *Bgl*II sticky ends was obtained by hybridizing GAop1 and GAop2 and ligated into pESC-GAv1. The resulting plasmid was digested with *Eco*RI and *Not*I, thus producing pESC-GAv3. A DNA fragment containing the coding sequence of ENA-78 flanked by *Eco*RI and *Not*I restriction sites was amplified from plasmid pTRC-ENA (I. Cielēns, unpublished data) using primers ENA_fwd and ENA_rev and cloned into pESC-GAv2 and pESC-GAv3, resulting in plasmids pESC-GA-ENA and pESC-GA-ENAop, respectively. In a similar manner, the sequence coding for GFP was amplified using primers GFP_fwd and GFP_rev from plasmid pA62 (kindly provided by A. Strods), which contains the gene for CXCR4-eGFP fusion protein (unpublished data). The resulting fragment was cloned into pESC-GAv2 and pESC-GAv3, thus producing plasmids pESC-GA-GFP and pESC-GA-GFPop, respectively.

Production and purification of GA VLPs

Yeast transformants were obtained, cultivated and the recombinant GA capsids purified essentially as described (Freivalds et al. 2008), with an additional last purification step of ion-exchange chromatography. After gel-filtration, the fractions containing GA VLPs in TEN buffer (20 mM Tris-HCl, pH 7.8, 5 mM EDTA, 150 mM NaCl) were loaded on a DEAE-Sephadex A50 column (V = 3.5 mL, h = 5 cm) and washed with four column volumes of TEN buffer. GA capsids were eluted in the void volume while nucleic acid contaminants remained bound to the column and were subsequently eluted with TEN buffer supplemented with 1 M NaCl. Fractions containing GA VLPs were pooled and stored frozen at –20 °C until use.

RNA extraction

An equal volume of Tris-HCl buffered phenol (pH 6.7) was added to a preparation of purified capsids, vortexed for 30 s and centrifuged at 10 000 g for 5 min. The aqueous phase was collected and repeatedly phenol-extracted until no protein band could be observed at the phase interface. The aqueous phase was then washed three times with diethyl ether and the RNA concentrated by ethanol precipitation. Finally, the RNA was dissolved in a small

volume of sterile water and aliquots stored frozen at –20 °C until use.

RNA electrophoresis

RNA samples were thawed on ice. After adding of 2X RNA loading dye (Fermentas) samples were heated at 70 °C for 10 min, cooled 2 min on ice and immediately loaded on a denaturing urea-polyacrylamide gel (8 M urea, 4 % polyacrylamide, 1X TBE). After electrophoresis, the gel was stained with ethidium bromide and RNA detected by fluorescence in UV light.

Reverse transcription PCR

Synthesis of the first strand cDNA was conducted by the RevertAid kit (Fermentas) according to manufacturer's protocol and using 1.2 μg of the extracted RNA as template and either 20 pmol of sequence-specific primer (GA_rev for all RNA samples and ENA_rev for RNA extracted from GA-ENA and GA-ENAop capsids) or 90 pmol of oligo$(dT)_{18}$ for RNA preparations from GA-GFP and GA-GFPop. The reason for using oligo$(dT)_{18}$ instead of GFP_rev was the apparent formation of a stable secondary structure of the latter, resulting in no detectable reaction products at 37 °C (data not shown). After reverse transcription, 2 μL of the mixture was used as a template for second strand cDNA synthesis, using primers GA_fwd / GA_rev, ENA_fwd / ENA_rev and GFP_fwd / GFP_rev for the amplification of GA coat protein, ENA-78 and green fluorescent protein cDNAs, respectively.

Results

Construction of the in vivo *packaging system*

To attempt to produce GA VLPs *in vivo* with a specified RNA content, we chose the expression system in yeast *Saccharomyces cerevisiae*. Although the levels of heterologous protein expression in yeast are generally lower compared to those attainable in *E. coli*, the *S. cerevisiae* system is beneficial in a number of aspects. Yeast provides a source of eukaryotic, 5'-capped and 3'-poly(A)-tailed mRNAs, which is attractive considering our further goals to test GA VLPs as RNA packaging and delivery tools to mammalian cells. Also, the absence of bacterial endotoxins in yeast preparations simplifies the capsid purification procedures for this purpose. As Legendre and Fastrez (2005) demonstrated with MS2 coat protein that such system is functional in *S. cerevisiae*, we used a similar approach for the coat protein of phage GA.

To construct the *in vivo* packaging system, we used the commercial pESC-URA plasmid, which contains divergent galactose-inducible promoters GAL1/GAL10. We have previously described the construction of pESC-GA, a pESC-URA-derived plasmid that contains the wild-type GA coat protein gene under the control of GAL1 and directs production of GA VLPs in *S. cerevisiae* cells (Freivalds et al. 2008). In order to determine the effect of GA operator on the RNA content of the VLPs, we constructed in an analogous way a plasmid pESC-GAop, which contains the GA operator sequence downstream the coat protein gene.

We proceeded to modify pESC-GA to allow the encapsidation of heterologous mRNAs of choice into GA VLPs. The system was designed in a way that any gene of interest can be inserted under the GAL10 promoter in two vectors using the same cloning sites, in which one vector (pESC-GAv3) contains the GA operator just downstream the inserted

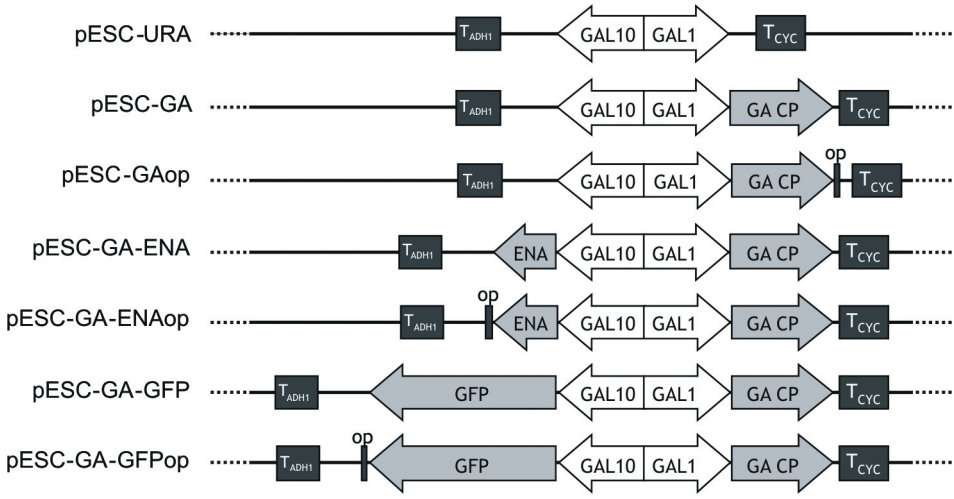

Fig. 1. Constructed plasmids for expression in S.cerevisiae. A schematic representation of the developed constructions. Genes coding for the GA coat protein (GA CP), epithelial neutrophil-activating peptide 78 (ENA) and green fluorescent protein (GFP) are indicated as well as promoters (GAL1 and GAL10), transcription terminators (T_{ADH1} and T_{CYC}) and GA operator (op).

gene, while the other (pESC-GAv2) does not and serves as a control to examine the encapsidation specificity that the operator provides. We used in the above-mentioned way two different genes to test the system: one coding for the epithelial neutrophil-activating peptide 78 (ENA-78), resulting in plasmids pESC-GA-ENA and pESC-GA-ENAop, and the other for enhanced green fluorescent protein (eGFP) with plasmids pESC-GA-GFP and pESC-GA-GFPop, respectively (Fig. 1).

Purification of GA VLPs

The purification of recombinant GA capsids from *S. cerevisiae* cells followed directly the procedures described by Freivalds et al. (2008). The fractions containing GA VLPs were remarkably pure from protein contaminants after the last step. However, electrophoretic analysis in an ethidium bromide-stained agarose gel revealed that the preparation also contains a substantial amount of free RNA that was not incorporated into capsids (data not shown). In order to adequately analyze the RNA content of the particles, removal of all other nucleic acids from the sample is of key importance. The RNA contaminants were effectively separated by introducing an additional purification step of ion-exchange chromatography on a DEAE-Sephadex A50 column. This ensured that the RNA subsequently extracted from the preparation originated only from the interior space of GA VLPs.

For convenience, the capsids produced from pESC-GA, pESC-GAop, pESC-GA-ENA, pESC-GA-ENAop, pESC-GA-GFP and pESC-GA-GFPop will further be denoted as GA, GAop, GA-ENA, GA-ENAop, GA-GFP and GA-GFPop, respectively.

Analysis of capsid RNA contents

The RNA was phenol-extracted from purified GA capsids and subjected to electrophoresis

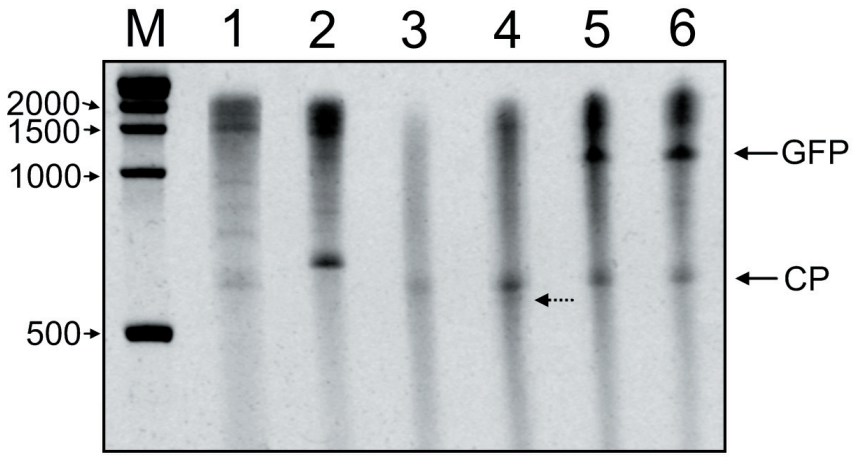

Fig. 2. Analysis of capsid RNA contents in a denaturing polyacrylamide gel. 1 μg of RNA extracted from GA (lane 1), GAop (lane 2), GA-ENA (lane 3), GA-ENAop (lane 4), GA-GFP (lane 5) or GA-GFPop (lane 6) capsids were loaded on a 4% urea-polyacrylamide gel. The positions of the GA coat protein (CP) and green fluorescent protein (GFP) mRNAs are indicated by arrows; the dotted arrow shows the supposed location of the mRNA of ENA-78. M, RNA ladder.

in a denaturing polyacrylamide gel. The RNA preparations in all cases were not homogeneous (Fig. 2). A distinct band corresponding to RNA somewhat longer than 500 nucleotides could be observed in all cases except in the preparation from GAop (lane 2), where a slightly longer RNA species was detected. Since in pESC-GAop the additional sequence with GA operator adds approximately 50 nucleotides to the 3'-untranslated region of the RNA transcript (see Table 2), this strongly suggests that the respective bands in the gel correspond to the mRNA of GA coat protein. In the case of GA-GFP and GA-GFPop (lanes 5 and 6), an additional prominent band of about 1000 nucleotides was detected, which apparently was formed by the mRNA of GFP. In the RNA preparation from GA-ENAop, a faint band could be observed just below that of the coat protein mRNA (lane 4). Further analysis in a more concentrated gel confirmed the existence of an RNA species migrating slightly faster than the mRNA of GA coat protein in preparations from GA-ENAop and also from GA-ENA, but not in those from other VLPs (data not shown). Although the sequences coding for ENA-78 and GA coat protein differ by approximately 150 nucleotides, the difference fell to about 50 nucleotides in mRNA due to unequal lengths of the untranslated regions of transcripts from GAL1 and GAL10 (Table 2). This explains the close migration and poor separation of both mRNAs and suggests that the faster-migrating band corresponds to the mRNA of ENA-78.

In order to prove unambiguously that the produced GA VLPs have packaged the expected mRNAs, the extracted RNA was analyzed by reverse transcription PCR (RT-PCR). The results (Fig. 3) showed that the mRNA of ENA-78 is present in both GA-ENA and GA-ENAop capsids (lanes 4 and 6). The mRNA of the green fluorescent protein was correspondingly present in GA-GFP and GA-GFPop capsids (lanes 8 and 10). The RT-PCR confirmed that the mRNA of the GA coat protein is present not only in GAop capsids (lane 2), but also in all other GA VLPs, regardless of the presence or absence of the operator within other mRNAs in the cell (lanes 1, 2, 3, 5, 7 and 9).

Table 2. Calculated lengths of the mRNAs packaged into the capsids. The mRNA transcription start points from GAL1 and GAL10 were determined after Johnston and Davis (1984) and the polyadenylation sites (which therefore mark the end of the 3' untranslated region) of CYC1 and ADH1 after Heidmann et al. (1992). The calculations do not include the 3' poly(A) tail, the length of which was not known but which generally spans 50-90 adenine residues in *S. cerevisiae* (Brown, Sachs 1998)

mRNA	Length of the sequence (nucleotides)			
	5'-untranslated	Protein-coding	3'-untranslated	Total
GA	66	393	126	585
GAop	66	393	178	637
ENA	12	240	280	532
ENAop	12	240	270	522
GFP	13	720	284	1017
GFPop	13	720	274	1007

Discussion

We demonstrated that it is possible to produce recombinant GA capsids *in vivo* that contain heterologous RNAs of choice. We developed a system in *S. cerevisiae* that provides a simple way to encapsulate any desired RNA sequence in GA VLPs and succeeded in packaging different model mRNAs into the particles. However, the GA operator failed to provide high encapsidation specificity of the target RNAs. This was clearly demonstrated by the incorporation of GA coat protein in mRNAs in the VLPs even in situations when other mRNAs containing the GA operator were present in the cell at the time of capsid assembly.

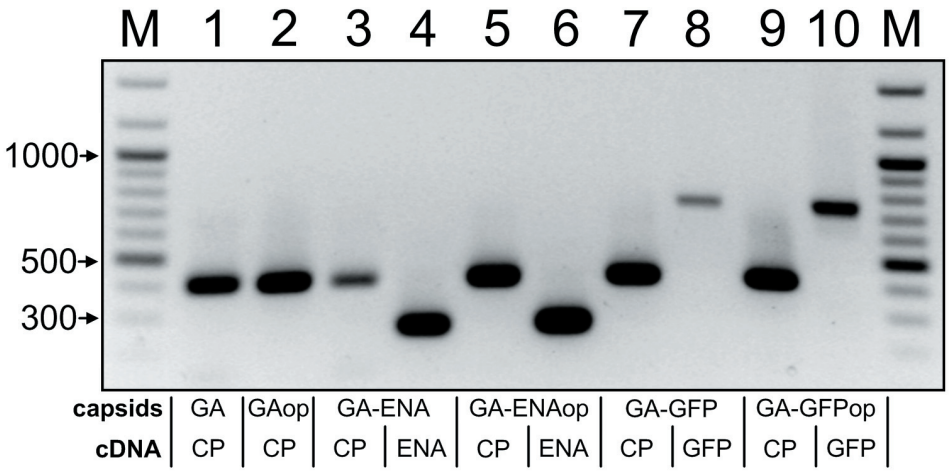

Fig. 3. Analysis of capsid RNA contents by RT-PCR. RNA was extracted from each type of the produced capsids and assayed for the presence of particular mRNAs, as indicated below. CP, cDNA of the GA coat protein; ENA, cDNA of the epithelial neutrophil-activating peptide 78; GFP, cDNA of the green fluorescent protein; M, DNA ladder.

The RNA binding properties of MS2 and GA coat proteins are slightly different. The crystal structure of the MS2 coat protein and RNA operator complex indicated that the specificity of the interaction is governed by three bases in the operator, which make direct contacts with the protein (Valegård et al. 1994). In GA this number is reduced to two, and the requirements for specific nucleotides at certain positions are also higher for MS2 than GA. MS2 coat protein only weakly binds to the GA operator, whereas GA coat protein has a similar affinity for both GA and MS2 operators (Gott et al. 1991). Consequently, GA coat protein can bind equally well to a larger pool of different RNA sequences, and the lower specificity facilitates the competition of non-operator sequences for association with the coat protein and subsequent encapsidation into the particles.

The ratio of coat protein and RNA in the cell is also important for the specificity of packaging. In a phage-infected cell, the coat protein concentration is initially low and increases gradually (Nathans et al. 1969). The high affinity of the RNA operator for the coat protein ensures specific packaging of the viral genome before the coat protein concentration reaches levels at which unspecific binding to intracellular RNA can occur (Beckett et al. 1988). The *in vivo* packaging experiments with *LacZ* mRNA showed that the specificity is indeed considerably higher at high *LacZ* and low coat protein concentrations (Pickett, Peabody 1993). The divergent GAL1/GAL10 promoters used in our system direct the production of similar levels of mRNA (Hadfield et al. 1993). In the case of MS2, this was apparently sufficient to provide a rather high specificity of packaging (Legendre, Fastrez 2005). However, to achieve high packaging specificity with GA, a higher proportion of RNA over coat protein is probably required.

For the ultimate goal to develop GA VLPs as nucleic acid delivery vehicles to eukaryotic cells, further modifications of the capsid are clearly required, which would allow to address the particles to particular types of cells. This problem might be potentially solved by chemically coupling a cell-specific peptide ligand to the surface of the capsid (Storni et al. 2004). Alternatively, chimeric RNA phage capsids can be produced by genetically fusing a foreign amino acid sequence to the coat protein (Mastico et al. 1993; Heal et al. 1999; Voronkova et al. 2002). The *in vivo* packaging system could then be used with modified coat proteins to produce chimeric capsids that contain therapeutic mRNAs or other kinds of RNA-based drugs, like ribozymes and antisense RNAs. Although the relatively low specificity of the GA coat protein-operator interaction renders the *in vivo* RNA packaging system in GA VLPs less advantageous compared to that of MS2, optimizations like adjustment of coat protein and RNA levels in the cell, the use of GA coat protein mutants that bind the operator stronger, similarly to those that are known for MS2 (Lim, Peabody 1994), and possibly other measures might significantly enhance the RNA encapsidation specificity into GA capsids in the future. Eventually, VLPs loaded with therapeutic RNAs and equipped with ligands on their surface may become powerful tools for cell-specific delivery of nucleic acid-based drugs.

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References

Beckett D., Uhlenbeck, O.C. 1988. Ribonucleoprotein complexes of R17 coat protein and a translational operator analog. *J. Mol. Biol.* 204: 927–938.

Beckett D., Wu H.N., Uhlenbeck O.C. 1988. Roles of operator and non-operator RNA sequences in bacteriophage R17 capsid assembly. *J. Mol. Biol.* 204: 939–947.

Bernardi A., Spahr, P.F. 1973. Nucleotide sequence at the binding site for coat protein on RNA of bacteriophage R17. *Proc. Natl. Acad. Sci. USA* 69: 3033–3037.

Brown C.E., Sachs A.B. 1998. PolyA tail length control in *Saccharomyces cerevisiae* occurs by message-specific deadenylation. *Mol. Cell Biol.* 18: 6548–6559.

Fiers W. 1979. Structure and function of RNA bacteriophages. In: Fraenkel-Conrat H., Wagner R.R. (eds) *Comprehensive Virology*, vol. 13. Plenum Publishing Corporation, New York, pp. 69–204.

Freivalds J., Rūmnieks J., Ose V., Renhofa R., Kazāks A. 2008. High-level expression and purification of bacteriophage GA virus-like particles from yeast *Saccharomyces cerevisiae* and *Pichia pastoris*. *Acta Univ. Latv.* 745: 75–85.

Gott J.M., Wilhelm L.J., Uhlenbeck O.C. 1991. RNA binding properties of the coat protein from bacteriophage GA. *Nucleic Acids Res.* 19: 6499–6503.

Hadfield C., Raina K.K., Shashi-Menon K., Mount R.C. 1993. The expression and performance of cloned genes in yeasts. *Mycol. Res.* 97: 897–944.

Heal K.G., Hill H.R., Stockley P.G., Hollingdale M.R., Taylor-Robinson A.W. 1999. Expression and immunogenicity of a liver stage malaria epitope presented as a foreign peptide on the surface of RNA-free MS2 bacteriophage capsids. *Vaccine* 18: 251–258.

Heidmann S., Obermaier B., Vogel K., Domdey H. 1992. Identification of pre-mRNA polyadenylation sites in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 12: 4215–4229.

Hohn T. 1969a. Studies on a possible precursor in the self-assembly of bacteriophage fr. *Eur. J. Biochem.* 8: 552–556.

Hohn T. 1969b. Role of RNA in the assembly of bacteriophage fr. *J. Mol. Biol.* 43: 191–200.

Johnston M., Davis R.W. 1984. Sequences that regulate the divergent GAL1-GAL10 promoter in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 4: 1440–1448.

Kastelein R.A., Berkhout B., Overbeek G.P., van Duin J. 1983. Effect of the sequences upstream from the ribosome-binding site on the yield of protein from the cloned gene for phage MS2 coat protein. *Gene* 23: 245–254.

Kozlovska T.M., Cielens I., Dreilina D., Dislers A., Baumanis V., Ose V., Pumpens, P. 1993. Recombinant RNA phage Qβ capsid particles synthesized and self-assembled in *Escherichia coli*. *Gene* 137: 133–137.

Kozlovskaya T.M., Pumpen P.P., Dreilina D.E., Tsimanis A.J., Ose V.P., Tsibinogin V.P., Gren E.J. 1986. Formation of capsid-like structures as a result of expression of the cloned gene of the envelope protein of the RNA-containing bacteriophage fr. *Dokl. Akad. Nauk. SSSR* 28: 452–455. (in Russian)

Legendre D., Fastrez J. 2005. Production in *Saccharomyces cerevisiae* of MS2 virus-like particles packaging functional heterologous mRNAs. *J. Biotechnol.* 117: 183–194.

Lim F., Peabody D.S. 1994. Mutations that increase the affinity of a translational repressor for RNA. *Nucleic Acids Res.* 22: 3748–3752.

Lim F., Spingola M., Peabody D. S. 1994. Altering the RNA binding specificity of a translational repressor. *J. Biol. Chem.* 269: 9006–9010.

Mastico R.A., Talbot S.J., Stockley P.G. 1993. Multiple presentation of foreign peptides on the surface of an RNA-free spherical bacteriophage capsid. *J. Gen. Virol.* 74: 541–548.

Nathans D., Oeschger M.P., Polmar S.K., Eggen K. 1969. Regulation of protein synthesis directed by coliphage MS2 RNA. I. Phage protein and RNA synthesis in cells infected with suppressible mutants. *J. Mol. Biol.* 39: 279–292.

Pasloske B.L., Walkerpeach C.R., Obermoeller R.D., Winkler M., DuBois D.B. 1998. Armored RNA

technology for production of ribonuclease-resistant viral RNA controls and standards. *J. Clin. Microbiol.* 36: 3590–3594.
Peabody D.S. 1990. Translational repression by bacteriophage MS2 coat protein expressed from a plasmid. A system for genetic analysis of a protein-RNA interaction. *J. Biol. Chem.* 265: 5684–5689.
Pickett G.G., Peabody D.S. 1993. Encapsidation of heterologous RNAs by bacteriophage MS2 coat protein. *Nucleic Acids Res.* 21: 4621–4626.
Storni T., Ruedl C., Schwarz K., Schwendener R.A., Renner W.A., Bachmann M.F. 2004. Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic side effects. *J. Immunol.* 172: 1777–1785.
Valegård K., Murray J.B., Stockley P.G., Stonehouse N. J., Liljas L. 1994. Crystal structure of an RNA bacteriophage coat protein-operator complex. *Nature* 371: 623–626.
van Duin J. 1988. Single-stranded RNA bacteriophages. In: Calendar R. (ed) *The Bacteriophages*, vol. 1. Plenum Press, New York, pp. 117–167.
Voronkova T., Grosch A., Kazaks A., Ose V., Skrastina D., Sasnauskas K., Jandrig B., Arnold W., Scherneck S., Pumpens P., Ulrich R. 2002. Chimeric bacteriophage fr virus-like particles harboring the immunodominant C-terminal region of hamster polyomavirus VP1 induce a strong VP1-specific antibody response in rabbits and mice. *Viral Immunol.* 15: 627–643
Wei Y., Yang C., Wei B., Huang J., Wang L., Meng S., Zhang R., Li J. 2008. Ribonuclease-resistant virus-like particles containing long chimeric RNA sequences produced by a two-plasmid coexpression system. *J. Clin. Microbiol.* 46: 1734–1740.

mRNS iepakošanas specifiskums bakteriofāga GA vīrusveidīgajās daļiņās raugā *Saccharomyces cerevisiae*

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Kopsavilkums

RNS bakteriofāgu apvalka proteīna gēnu heterologa ekspresija izraisa vīrusveidīgo daļiņu veidošanos, kas ietver iekššūnas RNS. Šajā darbā mēs iepakojām specifiskas mRNS molekulas bakteriofāga GA kapsīdās *in vivo*. Specifiskuma nodrošināšanai izmantojām GA operatoru – 21 nukleotīdu garu RNS sekvenci, kas salokās kāta-cilpas struktūrā un var specifiski piesaistīties fāga apvalka proteīnam. Ekspresijai *Saccharomyces cerevisiae* konstruēja vairākas plazmīdas, kas nodrošināja gan GA apvalka proteīna, gan iepakojamās mRNS sintēzi. Par iepakošanas modeļiem izmantoja trīs dažādas mRNS (GA apvalka proteīna, ENA-78 un GFP), katru no tām konstruējot divos variantos, kas atšķīrās ar GA operatora klātbūtni to sekvencēs. Iegūto kapsīdu RNS satura analīze ar gēla elektroforēzi parādīja, ka ir radušās paredzētā garuma RNS. Iepakoto mRNS klātbūtni kapsīdās apstiprināja apgrieztās transkripcijas PCR. Tomēr operatoram bija samērā niecīga ietekme uz kapsīdu satura specifiskumu, jo kapsīdās vienmēr konstatēja arī GA apvalka proteīna mRNS.

IV

Yeast-expressed bacteriophage-like particles for packaging of nanomaterials

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RESEARCH

Yeast-Expressed Bacteriophage-Like Particles for the Packaging of Nanomaterials

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Abstract Virus-like particles (VLPs) generated by heterologous expression of viral structural genes have become powerful tools in vaccine development. Recently, we and others have reported on the assembly of VLPs of the RNA bacteriophages MS2, Qβ, and GA in yeast. Here, we investigate the formation of VLPs of five additional phages in the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris*, namely, the coliphages SP and fr, *Acinetobacter* phage AP205, *Pseudomonas* phage PP7, and *Caulobacter* phage φCb5. In all cases except SP, particle formation was detected, although VLP outcome varied from 0.2 to 8 mg from 1 g of wet cells. We have found that phage φCb5 VLPs easily dissociate into coat protein dimers when applied to strong anion exchangers. Upon salt removal and the addition of nucleic acid or its mimics and calcium ions, the dimers re-assemble into VLPs with high efficiency. A variety of compounds, including RNA, DNA, and gold nanoparticles can be packaged inside φCb5 VLPs. The ease with which phage φCb5 coat protein dimers can be purified in high quantities and re-assembled into VLPs makes them attractive for downstream applications including the internal packaging of nanomaterials and the chemical coupling of peptides of interest on the surface.

Keywords RNA bacteriophages · Virus-like particles · Yeast · Packaging · Reassembly · Nanomaterials

Janis Freivalds and Svetlana Kotelovica contributed equally to this study.

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Introduction

Currently, highly immunogenic VLPs generated by the heterologous expression of viral structural genes have become powerful tools for vaccine development. In addition to being effective vaccines against the corresponding virus from which they are derived, VLPs can also be used to present foreign epitopes to the immune system [1, 2]. This approach could be potentially combined with the packaging of selected genes, drugs, and proteins inside VLPs [3–5]. Furthermore, targeted VLPs can be generated by including specific peptide ligands as "molecular addresses" on the surface of the particles [6].

RNA bacteriophages (phages) are small viruses with simple structural organization. Their approximately 3,500 nucleotide long-genomic RNA is encapsulated in a $T = 3$ icosahedral shell composed of 180 copies of the coat protein (CP) and one copy of the maturation protein. These phages were first isolated from *Escherichia coli* [7], but later were also found in *Caulobacter* [8], *Pseudomonas* [9], and *Acinetobacter* [10]. Recombinantly expressed CP genes leads to formation of VLPs with a diameter of 25–30 nm which are morphologically and immunologically indistinguishable from native phages [11, 12]. The icosahedral capsids of RNA phages have attracted the attention of researchers as promising carriers of foreign epitopes and nucleic acids. *E. coli*-derived phage VLPs have been explored for both genetic and chemical coupling of desired peptides to their surface. To increase their immunogenicity, such chimeric VLPs can be further engineered by loading them with short synthetic DNA sequences [13]. Several Qβ phage-derived therapeutic vaccine candidates against nicotine addiction, hypertension, and allergy have entered clinical trials [14–16]. Another set of vaccine prototypes targeting West

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Nile and influenza virus infections has been recently constructed using the phage AP205 VLP platform [17, 18].

During vaccine development, *E. coli*-derived VLPs need to be purified from bacterial endotoxin contamination, which is a costly and time consuming process. Alternatively, VLPs can be produced in "endotoxin-free" organisms such as yeast, which have been regarded as generally safe for human use. We and others have demonstrated the potential of yeast cells as hosts for producing properly assembled VLPs of the phages MS2, Qβ, and GA [19–21]. Here, we continue our investigations with five additional phages, namely, coliphages SP and fr, *Acinetobacter* phage AP205, *Pseudomonas* phage PP7, and *Caulobacter* phage φCb5. Notably, CP genes from all abovementioned phages have been successfully expressed in *E. coli* resulting in formation of VLPs ([12, 17, 22, 23] and our unpublished data). In addition to the generation of VLPs, we aimed to improve the purity of our final phage VLP preparations to near homogeneity. We have also shown that phage φCb5 VLPs can be easily re-assembled in vitro and thus are particularly suitable for the packaging of foreign materials.

Materials and Methods

Construction of the Expression Vectors and Selection of Clones

Construction of the expression vectors was performed as described previously [20]. The genes were PCR-amplified and ligated either into the *Bam*HI/*Eco*105I-treated vector pPIC3.5 K (Invitrogen) under control of the *AOX1* promoter or into the *Xba*I/*Bgl*II-treated vector pFX-Qβ [20] under control of the hybrid *GAL10*/*PYK1* promoter [24]. The amplification primers are listed in Table 1. After sequencing, the resulting plasmids were transformed into *S. cerevisiae* strain AH22 *MATa leu2 his4* according to a standard lithium acetate/PEG transformation procedure [25]. The clones were selected on yeast expression dextrose (YEPD) agarized medium supplemented with 2.5 mM formaldehyde. Alternatively, after linearization with *Ecl*136II in the *AOX1* promoter region, expression plasmids were used for transformation of the *P. pastoris* GS115 *his4* strain by electroporation (Bio Rad, Gene Pulser). Mut^+His^+ transformants were isolated on agarized minimal medium (1.34 % yeast nitrogen base, 2 % glucose, 4×10^{-5} % biotin, 2 % agar). For the selection of clones with multiple integration units, transformants were pooled and plated onto YEPD agarized medium containing increased concentrations of the antibiotic G418 (1.8–2.5 mg/mL).

Expression Conditions

To prepare a start culture, an individual colony was picked and placed in 5 mL of liquid YEPD medium and incubated at 30 °C overnight without shaking. Further cultivation was performed in 2 L Erlenmeyer flasks with 200 mL of medium on an *Infors* shaker.

For expression in *S. cerevisiae*, the start culture was diluted in YEPD medium containing 3 % (w/v) galactose and 2.5 mM formaldehyde until OD_{590} ~0.05. Flasks were incubated at 30 °C on a shaker at 200 rpm. Every 24 h, solid galactose was added to a 2 % final concentration. The cells were cultivated for 72 h total.

The expression in *P. pastoris* was performed according to the manufacturer's protocol (www.invitrogen.com) with the following minor modifications: the start culture was diluted until OD_{590} ~0.05 in buffered complex glycerol (BMGY) medium and incubated at 30 °C on a shaker at 250 rpm. After 24 h at OD_{590} 6–8, expression was induced by the addition of 1 % methanol. On each subsequent day, methanol was added to a final concentration of 1 %. The cells were harvested 72 h after induction. The final OD_{590} of expression cultures typically reached 12–14.

Purification of VLPs

Six grams of frozen yeast cells was resuspended in 24 mL of lysis buffer (20 mM Tris HCl, pH 7.2, 0.1 mM PMSF) and disrupted with a French press (three cycles, 20,000 psi). The soluble proteins were separated by centrifugation for 15 min at 15,500×*g* following fractionation by size-exclusion chromatography (SEC) on a Sepharose 4 Fast Flow column ($h = 80$ cm, $V = 500$ mL) equilibrated in column buffer (20 mM Tris HCl, pH 8.0). The preparation of lysate for SEC was phage-dependent. For φCb5 VLPs, the soluble fraction was directly loaded onto the column without clarification. For phages fr and PP7, the supernatant was cleared by incubation for 0.5 h at +50 °C and subsequent centrifugation for 25 min at 15,500×*g*. Phage AP205 VLPs were precipitated with 40 % ammonium sulfate (w/v) for 1 h at +4 °C, centrifuged for 25 min at 15,500×*g* and dissolved in a minimal amount of column buffer. The flow rate during chromatography was 3 mL/h, and the size of the fractions collected was 10 mL. The peak fractions were pooled, and VLPs were concentrated using a 60 % ammonium sulfate (w/v) precipitation overnight at +4 °C (except phage φCb5 VLPs), centrifuged for 25 min at 15,500×*g* and dissolved in a minimal amount (1 mL) of column buffer. φCb5 VLPs were concentrated with Amicon filters (Millipore; MWCO 100 kDa). VLPs were then passed through an anion exchange HiPrep 16/10 DEAE FF column connected to an ÄKTA chromatography system at 5 mL/min. For final polishing, particles were subjected to

Table 1 Oligonucleotide primers used for the amplification of CP genes from phage AP205, fr, PP7, SP, and φCb5 genomes (GenBank accession Nos. AF334111, X15031, X80191, X07489, and HM066936, respectively)

Name	Sequence (5′-3′)
AP_Xba_Fw^{Sc}	TTTCTAGAACAA**TG**GCAAATAAGCCAATG
AP_Bgl_Rv^{Sc}	TTAGATCT**TTA**AGCAGTAGTATCAGAC
AP_Bgl_Fw^{Pp}	TTAGATCTACC**ATG**GCAAATAAGCCAATG
AP_Sna_Rv^{Pp}	TTTACGTA**TTA**AGCAGTAGTATCAGACG
fr_Xba_Fw^{Sc}	TTTCTAGAACAA**TG**GCTTCGAACTTTGAAGAGTTC
fr_Bgl_Rv^{Sc}	TTAGATC**TTA**GTAGATTCCCGAGTTGG
fr_Bam_Fw^{Pp}	TTGGATCCACC**ATG**GCTTCGAACTTTGAAGAG
fr_Sna_Rv^{Pp}	TTTACGTA**TTA**GTAGATTCCCGAGTTGG
PP7_Xba_Fw^{Sc}	TTTCTAGAACAA**TG**TCCAAAACCATCGTTC
PP7_Bam_Rv^{Sc}	TTGGATCC**TTA**ACGGCCCAGCGGCACAAG
PP7_Bam_Fw^{Pp}	TTGGATCCACC**ATG**TCCAAAACCATCGTTCTTTC
PP7_Sna_Rv^{Pp}	TTTACGTA**TTA**ACGGCCCAGCGGCACAAG
SP_Xba_Fw^{Sc}	TTTCTAGAACAA**TG**GCAAAATTAAATCAGGTAAC
SP_Bgl_Rv^{Sc}	TTAGATC**TTA**GTAGGCTGGGTTCAGATTGTC
SP_Bgl_Fw^{Pp}	TTAGATCTACC**ATG**GCAAAATTAAATCAGG
SP_Sna_Rv^{Pp}	TTTACGTA**TTA**GTAGGCTGGGTTCAGATTGTC
Cb_Xba_Fw^{Sc}	TTTCTAGAACAA**TG**GCTCTCGGCGACACTC
Cb_Bgl_Rv^{Sc}	TTAGATC**TTA**CCTTCACGATTCCCAGC
Cb_Bam_Fw^{Pp}	TTGGATCCACC**ATG**GCTCTCGGCGACACTC
Cb_Sna_Fw^{Pp}	TTTACGTA**TTA**CCTTCACGATTCCCAGC

Restriction sites used for cloning are underlined. Initiation and termination codons are shown in bold (^{Sc}) Primers were used for cloning in *S. cerevisiae* expression vector; (^{Pp}) primers were used for cloning in *P. pastoris* expression vector

an ÄKTA-connected HiLoad 16/600 Superdex 200 column (Amersham Biosciences) in column buffer at 1 mL/min.

Analytical Methods

Preparation of the yeast cell extracts for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Kushnirov [26] with minor modifications. Briefly, two optical units of yeast cells (at $A = 590$ nm) was resuspended in 100 μL of water. Then, 100 μL of 0.2 M NaOH was added, and the mixture was incubated for 5 min at room temperature. The cells were pelleted and resuspended in 200 μL of Laemmli's sample buffer, boiled for 6–8 min and pelleted again. Protein samples were analyzed by SDS-PAGE according to standard protocols with a 4 % stacking and 15 % separating polyacrylamide gel (PAAG). To visualize protein bands, the gels were stained with Coomassie Brilliant Blue G-250.

Protein samples were subjected to native 1 % agarose gel electrophoresis in TAE buffer (pH 8.4) for approximately 0.5 h at 5 V/cm. Nucleic acids in the agarose gels were visualized by ethidium bromide staining. Protein concentration was determined by the formula OD(A_{260})/8, which has been used previously to determine the concentrations of native phages. The purity of the protein samples was estimated by densitometric analysis of the Coomassie-stained PAAG.

For electron microscopy, the protein samples were adsorbed on carbon–Formvar-coated copper grids and negatively stained with 1 % uranyl acetate aqueous solution. The grids were examined with a JEM-1230 electron microscope (JEOL Ltd., Tokyo, Japan) at 100 kV.

Disassembly of φCb5 VLPs

Phage φCb5 VLPs were applied to an anion exchange HiPrep 16/10 Q XL column in column buffer and eluted by a linear salt gradient (0–1 M NaCl) at 5 mL/min. Dimer-containing fractions were pooled, diluted two times with column buffer, and applied to a MonoQ TM column for final purification. Dimers were eluted by a linear salt gradient (0–1 M NaCl in column buffer, peak fractions contained approximately 0.25 M NaCl) at 1 mL/min and were stored at +4 °C until further use for VLP reconstruction. Both chromatography steps were performed using the ÄKTA chromatography system.

Reconstruction and Packaging of φCb5 VLPs

For VLP reconstruction, 1 volume of φCb5 dimers (3.2 mg/mL) in 0.3 M NaCl was mixed with 1 volume of 5 mM $CaCl_2$, 7 volumes of 20 mM Tris–HCl (pH 8.0) and 1 volume of nucleic acid or its mimic solution, as explained below, and further dialyzed at 4 °C for 1 h against 20 mM Tris–HCl, pH

8.0. *E. coli* total tRNA was dissolved to a volume of 1 mg/mL in 20 mM Tris–HCl pH 8.0. CpG DNA oligonucleotide G10 (5′-GGG GGG GGG GGA CGA TCG TCG GGG GGG GGG-3′; [27]) was dissolved to 2 mg/mL in water. Diphtheria toxin (DT) mRNA was prepared as described below and dissolved in DEPC-treated water to 10 mg/mL. 23-(9-Mercaptononyl)-3,6,9,12,15,18,21-heptaoxatricosanoic acid (MNHA) was dissolved in water to 10 mg/mL.

In Vitro Transcription of DT mRNA

The DT catalytic domain gene corresponding to amino acids 26-215 (UniProt: Q6NK15) was synthetized commercially in GenScript and cloned between the *Nde*I and *Pst*I sites of the Internal Ribosome Entry Site-containing vector pT7CFE1-CHis (Thermo Scientific). In vitro transcription was carried out using the TranscriptAid T7 High Yield Transcription Kit according to the manufacturer's instructions.

For VLP reconstruction, 2 volumes of φCb5 dimers (3.2 mg/mL) in 0.3 M NaCl were mixed with 1 volume of DT mRNA and 7 volumes of 20 mM Tris–HCl (pH 8.0) and dialyzed at 4 °C for 1 h against 20 mM Tris–HCl pH 8.0.

Packaging of Gold Nanoparticles in φCb5 VLPs

To coat the gold nanoparticles with MNHA, 100 volumes of 10 nm colloidal gold (Sigma-Aldrich) was mixed with 1.6 volumes of MNHA (10 mg/mL) and incubated at room temperature for 20 h.

To package the gold nanoparticles, 100 volumes of φCb5 dimers (0.5 mg/mL in 20 mM Tris–HCl, pH 8.0, 0.3 M NaCl) and 12 volumes of MNHA-coated gold particles were mixed and dialyzed overnight against 20 mM Tris–HCl, pH 8.0, at 4 °C.

Results and Discussion

Expression of Phage CP Genes in Yeast

It has been shown that repetitive vaccination with chimeric VLPs might be ineffective due to the presence of neutralizing antibodies against the capsid protein induced after the first application [28]. Additionally, different VLPs display different properties regarding their stability, tolerance to foreign amino acid insertions and chemical modifications, ease of in vitro reconstruction, and ability to pack foreign materials during assembly. Therefore, technologies to generate a broad spectrum of carrier VLPs need to be developed. Here, we investigated CP genes from the phages fr, SP, AP205, PP7, and φCb5 for the generation of their respective VLPs in yeast. Consequently, expression levels were compared in *S. cerevisiae* and *P. pastoris* using episomal and integrative vectors, respectively. The synthesis of phage CPs in all cases was detectable by SDS-PAGE, however, the amount of target protein varied significantly (Fig. 1a, b). Generally, increased synthesis of phage CPs was observed in *P. pastoris* clones containing multiple integration units. Especially, efficient *P. pastoris* producer strains were selected for phage PP7 and φCb5 CPs. However, for phages fr and SP, slightly better CP production was observed in *S. cerevisiae*. Our previous experience with the expression of viral structural proteins in *P. pastoris* has led us to the conclusion that many multicopy clones need to be analyzed to select the best producer strains [20, 21, 29]. This finding is supported by novel data, as superproducer clones were successfully obtained for phage φCb5 and PP7 VLPs (Fig. 1c, d). Dot-blot hybridization with a *his4* specific probe revealed that these clones contained an increased amount of integrated expression units (data not shown). The majority of target protein appeared in the soluble fraction, and the total

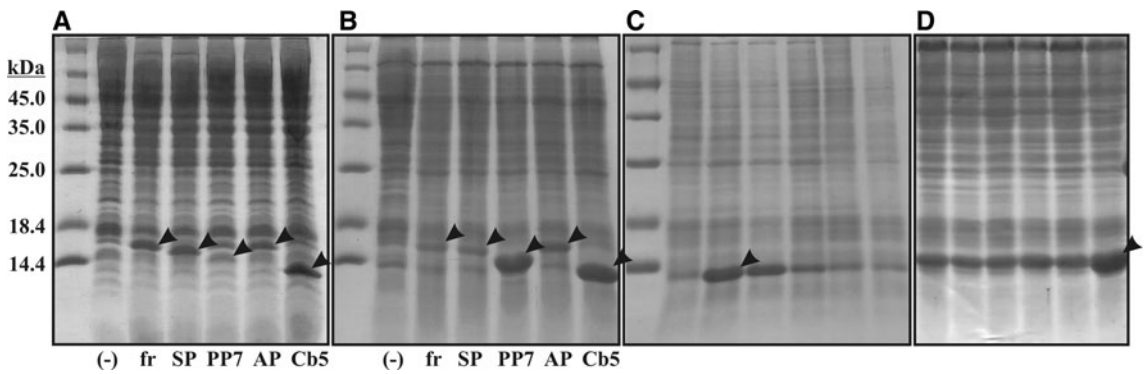

Fig. 1 Coomassie-stained SDS-PAAG demonstrating the synthesis of phage CPs in yeast *S. cerevisiae* (**a**) and *P. pastoris* (**b**). Calculated molecular masses (including the first methionine) of the CPs are as follows: fr, 13.9 kDa; AP205, 14.0 kDa; SP, 14.1 kDa; PP7, 14.0 kDa; and φCb5, 13.6 kDa. **c**, **d** show the selection of superproducer clones for phage φCb5 and PP7 CPs, respectively. Target proteins and selected producer clones are marked by *arrowheads*

amount synthesized correlated well with the amount of target protein in the soluble fraction with the sole exception of phage SP CP, which was completely insoluble when expressed in *S. cerevisiae* and partially soluble in *P. pastoris* (see Table 2).

Purification and Characterization of VLPs

Several methods were compared for lysate clarification. Recently, we successfully applied heat treatment for the purification of hepatitis B core particles [29]. Here, the same strategy was explored, however, with partial success. By optimizing the thermal conditions, we observed that heating for 30 min at 50 °C was optimal for the clarification of phage fr and PP7 lysates (Fig. 2a). For phages φCb5 and AP205, a large amount of target protein was lost during heating, apparently due to denaturation and/or association with the insoluble cell fraction (data not shown). Thus, ammonium sulfate precipitation was applied as an alternative purification strategy. This approach worked well for AP205 VLPs but not for φCb5 VLPs, which confirms previous observations about the salt sensitivity of native φCb5 as well as that of the corresponding VLPs [12, 30]. Nevertheless, high-level synthesis allowed for the direct loading of φCb5 supernatant onto a SEC column and successful recovery of the VLPs. For phage SP, after the direct loading of *P. pastoris* supernatant onto a SEC column, aggregated structures were detected by electron microscopy but not VLPs (data not shown). This observation was rather unexpected because for phage Qβ also belonging to the same *Allolevivirus* genus of the *Leviviridae* family and displaying 80 % amino acid identity to SP, we previously obtained high-level synthesis of correctly assembled VLPs [20]. In addition, efficient formation of SP VLPs has been observed in *E. coli* (our unpublished data). This only indicates that viral structural genes with high similarity may demonstrate completely different expression pattern and assembly properties in heterologous hosts.

After the first SEC step, the VLP-containing fractions were identified by native agarose gel electrophoresis and SDS-PAGE; however, the purity was unsatisfactory (data not shown). For further purification, peak fractions were concentrated and applied to a weak DEAE anion exchanger as described previously [29]. The VLPs rapidly passed through the column, while most of the contaminating proteins were captured by the DEAE matrix (data not shown). Finally, the VLPs were applied to a second SEC column for polishing. VLP preparations were analyzed by native agarose gel electrophoresis, where they migrated as distinct bands between the 1 and 2 kb dsDNA markers (Fig. 2b). The quality of the purified VLPs was confirmed by electron microscopy (Fig. 2c).

Table 2 Summary of phage VLP synthesis, solubility, and outcomes in yeast

Strain	Expr[a]	Solub[b]	VLP yield[c]
fr^{Sc}	++	+++	0.4–0.6
fr^{Pp}	+	+++	0.2–0.3
SP^{Sc}	++	–	ND
SP^{Pp}	+	+	ND
$PP7^{Sc}$	+	+++	0.6–0.8
$PP7^{Pp}$	+++	+++	6–8
$AP205^{Sc}$	+	+++	0.4–0.6
$AP205^{Pp}$	++	+++	1–1.5
$φCb5^{Sc}$	++	+++	0.8–1
$φCb5^{Pp}$	+++	+++	5–7

(^{Sc}) are *S. cerevisiae* strains; (^{Pp}) are *P. pastoris* strains

ND not detected

[a] The total synthesis level of target proteins estimated by Coomassie-stained PAAG was as follows: (+++) indicates high-level expression exceeding 10 % of total cell proteins, (++) indicates well-detectable synthesis reaching 5–10 % of total cell proteins, and (+) indicates detectable synthesis below 5 % of total cell proteins

[b] The solubility of target proteins estimated by Coomassie-stained PAAG was as follows: (+++) indicates more than 50 % of CP in the soluble fraction, (+) indicates below 50 % of CP in the soluble fraction, and (–) indicates no CP in the soluble fraction

[c] VLP yield was calculated in mg per 1 g of wet cells

Disassembly of φCb5 VLPs

To obtain φCb5 CP dimers, the appropriate VLPs were applied to strong anion exchangers. In our first approach, partially purified VLP fractions from the SEC column (Fig. 3a) were captured by the AEC Q XL column. After elution with a linear salt gradient, the product still contained minor contaminant protein bands (Fig. 3b) and was therefore applied to a second AEC MonoQ column (Fig. 3c). In our second approach, φCb5 VLPs were purified as described in the previous section and then applied directly to an AEC MonoQ column. Both methods worked equally well, although the first approach was faster and more convenient. By elution with increasing salt concentration both from AEC Q XL and AEC Mono Q columns, the VLPs were disrupted to dimers as confirmed by analytical SEC (Fig. 3d). Our assumption is that for VLPs to disassemble it was necessary to both adsorb them on Q anion exchanger and elute by high salt. Although, φCb5 VLPs have reduced thermostability in presence of high ionic strength, salt treatment does not disassemble the capsids at room temperature [12]. After the second AEC column, the dimers were eluted in ~250 mM NaCl. Such a salt concentration prevents dimers from re-assembly into VLPs and is optimal for the long-time storage of dimers at +4 °C until their use for reassembly/packaging experiments.

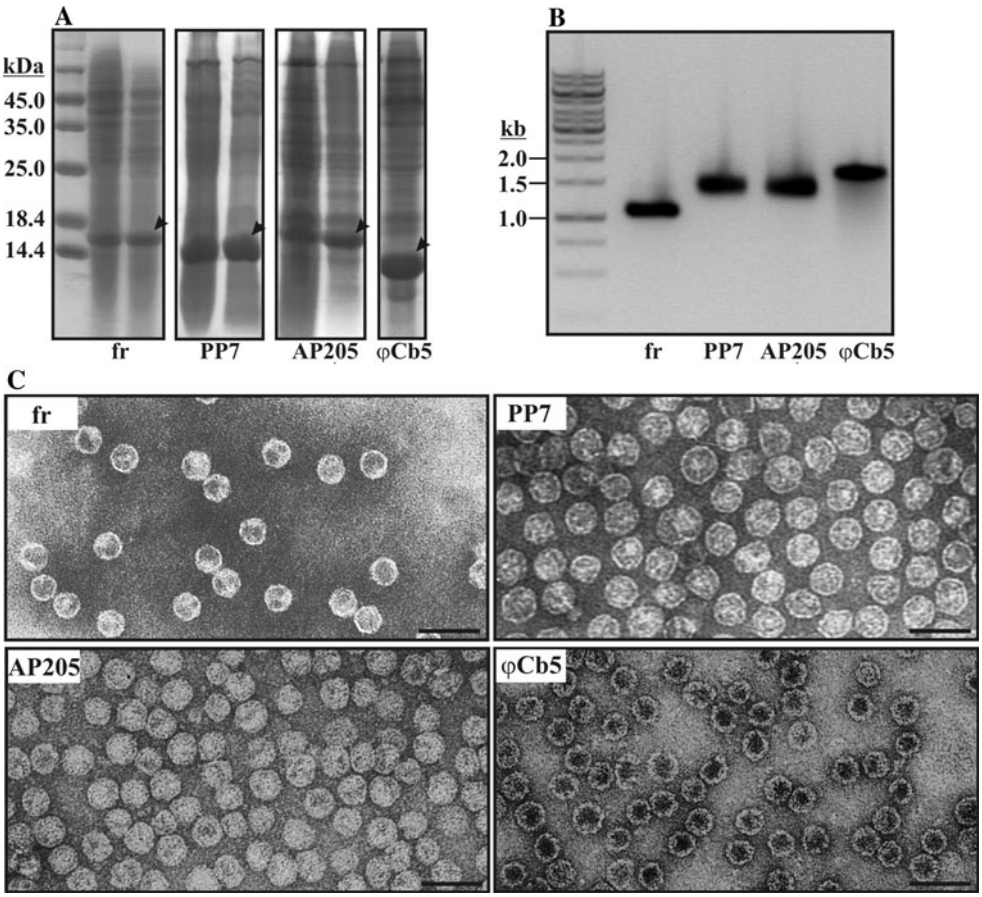

Fig. 2 Purification and characterization of phage VLPs. **a** Preparation of lysates for the first SEC. *Arrowheads* show the material loaded onto the column. **b** Ethidium bromide stained native agarose gel electrophoresis of purified VLPs. **c** Electron microscopy of the final product after the second SEC. *Scale bar* 50 nm

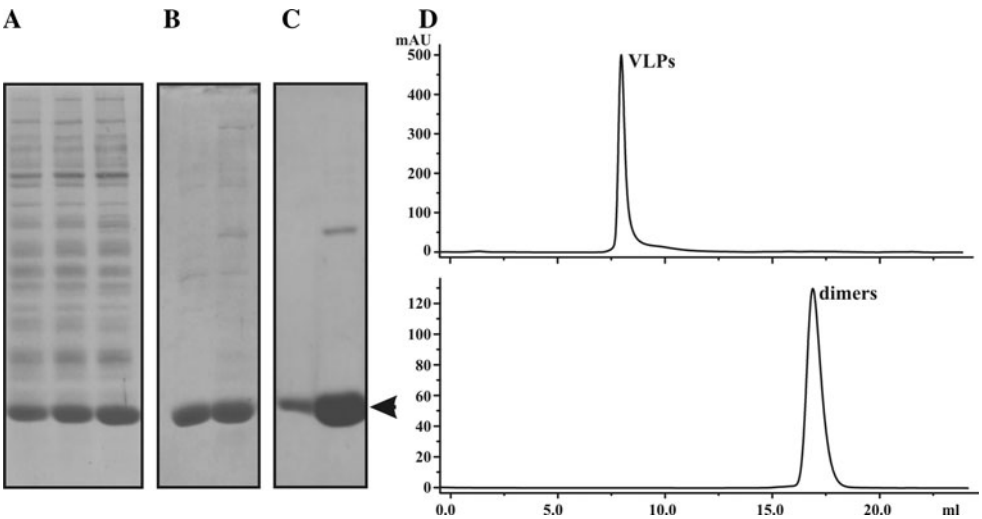

Fig. 3 Purification and detection of phage φCb5 CP dimers. **a**–**c** Peak protein fractions from the first SEC (**a**), first AEC (**b**) and second AEC (**c**) columns. Target protein is marked by *arrowhead*. **d** Monitoring of phage φCb5 VLPs and CP dimers on a Superdex 200 10/300 GL column

In Vitro Reconstruction of φCb5 VLPs and Packaging of Various Compounds

Lately, VLPs have gained attention as suitable nanocontainers for the packaging of various compounds for immunization and other therapeutic or diagnostic purposes. One important condition for material packaging is the ability to routinely disassemble and assemble the corresponding VLPs. Disassembly/assembly protocols have been worked out for several RNA phages ([31] and references therein), but the procedures involve the use of denaturing conditions, and the yields are generally low. From our previous structural studies, it is known that, unlike the VLPs of other phages, φCb5 VLPs are stabilized

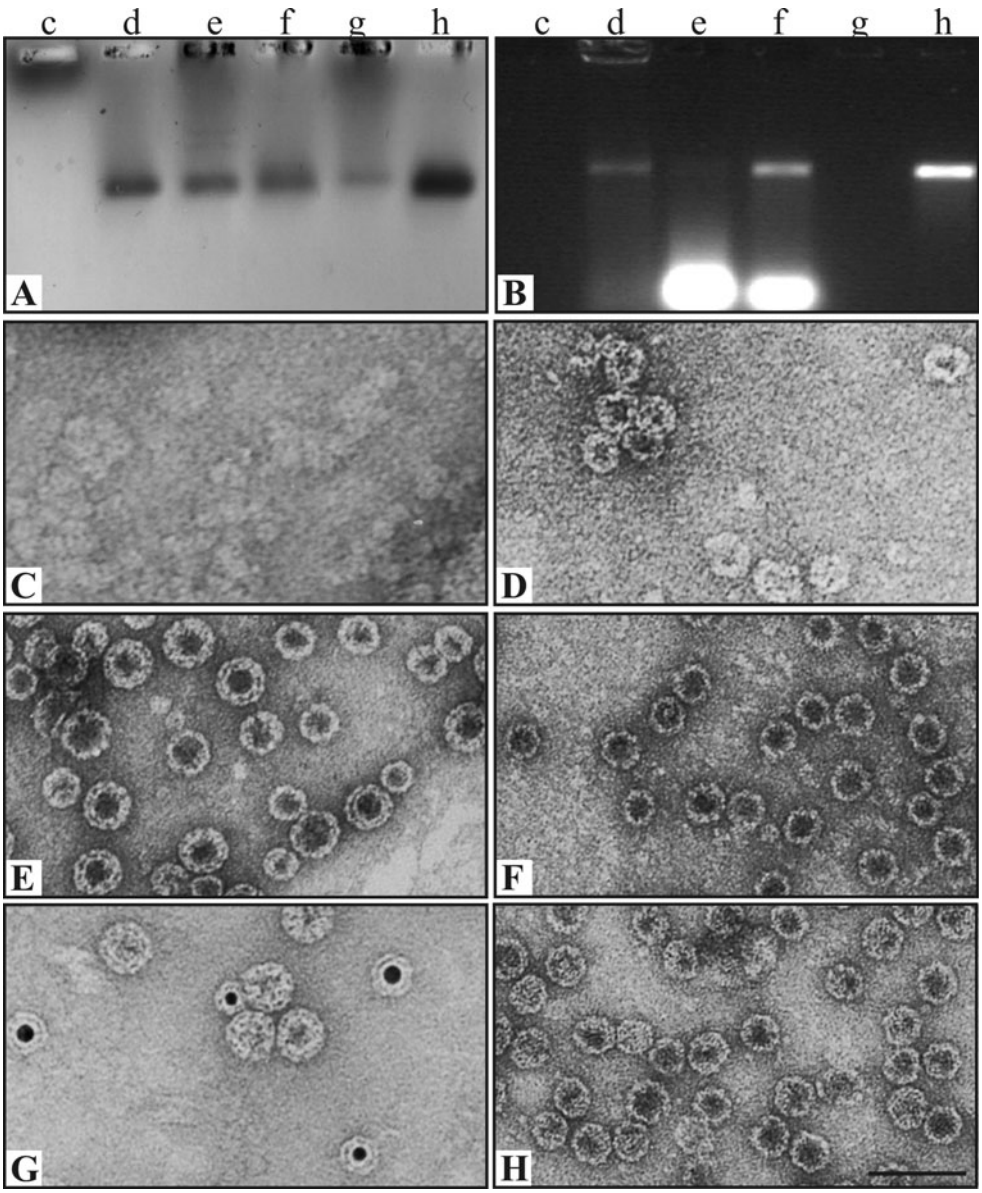

Fig. 4 Reassembly and packaging of φCb5 VLPs. **a**, **b** Native agarose gel electrophoresis of φCb5 CP assemblies visualized by Coomassie (**a**) and ethidium bromide (**b**) staining. *Lanes c–h* correspond to electron microscopy pictures (**c**–**h**), respectively. **c**–**h** Electron microscopy of φCb5 CP assemblies: φCb5 CP dimers (**c**), reassembly with: CpG oligonucleotide (**d**), DT mRNA (**e**), *E. coli* tRNA (**f**), and MNHA-coated gold nanoparticles (**g**), initial φCb5 VLPs (**h**). *Scale bar* 50 nm

by metal ions and can be consequently destabilized by the addition of chelating agents or increasing the ionic strength. However, under physiologically relevant conditions, φCb5 VLPs are as stable as the VLPs of similar phages [12]. As described above, φCb5 VLPs disassembled during purification on an ion exchange column, but the resulting CP dimers were soluble, stable and apparently not denatured. Furthermore, we tested whether the dimers could be reassembled back into particles by simply lowering the ionic strength and adding metal ions along with nucleic acid or its mimic. As seen from Fig. 4, φCb5 coat protein does assemble into VLPs in the presence of various nucleic acids such as *E. coli* total tRNA, CpG oligonucleotide, and the mRNA of the catalytic domain of DT. Packaging of bacterial RNA or CpG oligonucleotide is beneficial for increasing the immune response if the corresponding VLPs with exposed foreign epitopes are to be used for vaccination purposes. DT is one of the most potent known venoms, and it is believed that a single molecule can kill a eukaryotic cell via the inactivation of ribosomes. The DT catalytic domain is responsible for ribosome inactivation, while the other domains are necessary for cellular binding and translocation. Functional DT mRNA could be targeted to specific eukaryotic cells, if it is packaged in VLPs with exposed polypeptides, able to recognize certain cellular receptors. In our experiments, some DT mRNA-packaged VLPs appeared to be double-layered, whereas others seemed to possess $T = 1$ symmetry, as judged by their smaller particle size. In contrast, the packaging of *E. coli* tRNA led to the formation of VLPs very similar to those before disassembly. The packaging of CpG DNA oligonucleotide was somewhat less efficient but

also led to the formation of native-sized VLPs. Clearly, the size of assembled particles is dependent on the exact nature of packaged material. Presumably, the genome of φCb5 phage is tailored for efficient assembly of $T = 3$ particles, while other nucleic acids in some cases induce formation of smaller or double-layered VLPs.

Although, the VLPs of many viruses seem to require RNA or DNA for efficient assembly, the nucleic acid often does not have to be virus-specific and can even be replaced by other compounds with acidic functional groups that presumably mimic the negative charge on phosphate groups in nucleic acid. In a study by Chen et al. [32], VLPs were reassembled in vitro in the presence of gold nanoparticles coated with PEG-like compounds with attached carboxyl groups. Here, we attempted to use a similar compound, MNHA, for the re-assembly of φCb5 VLPs in vitro. As shown in Fig. 4, MNHA-coated gold nanoparticles can indeed be packaged in φCb5 VLPs, and on average one of three VLPs contains a gold nanoparticle. In our experiments, MNHA alone was also able to trigger particle formation (data not shown), so the "empty" VLPs in gold nanoparticle packaging experiment presumably also contain free MNHA. Interestingly, all gold nanoparticle-containing VLPs were smaller, corresponding to a $T = 1$ size, and none were double-layered. Possibly, the small size of MNHA-coated nanoparticles (smaller than $T = 1$ VLPs) constrains the size of packaged VLPs. In principle, MNHA could also be easily attached to a variety of other compounds, such as toxic or therapeutic proteins, thereby enabling these compounds to be packaged inside VLPs.

In conclusion, our yeast system is suitable for the production of various RNA phage VLPs. Especially, high synthesis levels can be obtained for phages PP7 and φCb5 VLPs. For the latter, an efficient disassembly and reassembly system allows for the packaging of nanomaterials within the VLPs which could be used for therapeutic purposes.

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References

1. Jennings, G. T., & Bachmann, M. F. (2008). The coming of age of virus-like particle vaccines. *Biological Chemistry, 389*, 521–536.
2. Buonaguro, L., Tagliamonte, M., Tornesello, M. L., & Buonaguro, F. M. (2011). Developments in virus-like particle-based vaccines for infectious diseases and cancer. *Expert Review of Vaccines, 10*, 1569–1583.
3. Xu, Y. F., Zhang, Y. Q., Xu, X. M., & Song, G. X. (2006). Papillomavirus virus-like particles as vehicles for the delivery of epitopes or genes. *Archives of Virology, 151*, 2133–2148.
4. Kaczmarczyk, S. J., Sitaraman, K., Young, H. A., Hughe, S. H., & Chatterjee, D. K. (2011). Protein delivery using engineered virus-like particles. *Proceedings of the National Academy of Sciences of the United States of America, 108*, 16998–17003.
5. Ma, Y., Nolte, R. J., & Cornelissen, J. J. (2012). Virus-based nanocarriers for drug delivery. *Advanced Drug Delivery Reviews, 64*, 811–825.
6. Yildiz, I., Shukla, S., & Steinmetz, N. F. (2011). Applications of viral nanoparticles in medicine. *Current Opinion in Biotechnology, 22*, 901–908.
7. Loeb, T., & Zinder, N. D. (1961). A bacteriophage containing RNA. *Proceedings of the National Academy of Sciences of the United States of America, 47*, 282–289.
8. Schmidt, J. M. (1966). Observations on the adsorption of *Caulobacter* bacteriophages containing ribonucleic acid. *Journal of General Microbiology, 45*, 347–353.
9. Bradley, D. E. (1966). The structure and infective process of a *Pseudomonas aeruginosa* bacteriophage containing ribonucleic acid. *Journal of General Microbiology, 45*, 83–96.
10. Coffi, H. (1995). Lysotypie des *Acinetobacter*. Thèse de Maîtrise dès Sciences, Québec: Laval University, p. 82.
11. Kozlovska, T. M., Cielens, I., Dreilinņa, D., Dislers, A., Baumanis, V., Ose, V., et al. (1993). Recombinant RNA phage Q beta capsid particles synthesized and self-assembled in *Escherichia coli*. *Gene, 137*, 133–137.
12. Plevka, P., Kazaks, A., Voronkova, T., Kotelovica, S., Dishlers, A., Liljas, L., et al. (2009). The structure of bacteriophage phiCb5 reveals a role of the RNA genome and metal ions in particle stability and assembly. *Journal of Molecular Biology, 391*, 635–647.
13. Storni, T., Ruedl, C., Schwarz, K., Schwendener, R. A., Renner, W. A., & Bachmann, M. F. (2004). Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic side effects. *Journal of Immunology, 172*, 1777–1785.
14. Cornuz, J., Zwahlen, S., Jungi, W. F., Osterwalder, J., Klingler, K., van Melle, G., et al. (2008). A vaccine against nicotine for smoking cessation: A randomized controlled trial. *PLoS ONE, 3*, e2547.
15. Tissot, A. C., Maurer, P., Nussberger, J., Sabat, R., Pfister, T., Ignatenko, S., et al. (2008). Effect of immunisation against angiotensin II with CYT006-AngQb on ambulatory blood pressure: a double-blind, randomised, placebo-controlled phase IIa study. *Lancet, 371*, 821–827.
16. Beeh, K. M., Kanniess, F., Wagner, F., Schilder, C., Naudts, I., Hammann-Haenni, A., et al. (2013). The novel TLR-9 agonist QbG10 shows clinical efficacy in persistent allergic asthma. *Journal of Allergy and Clinical Immunology, 131*, 866–874.
17. Spohn, G., Jennings, G. T., Martina, B. E., Keller, I., Beck, M., Pumpens, P., et al. (2010). A VLP-based vaccine targeting domain III of the West Nile virus E protein protects from lethal infection in mice. *Virology Journal, 7*, 146.
18. Tissot, A. C., Renhofa, R., Schmitz, N., Cielens, I., Meijerink, E., Ose, V., et al. (2010). Versatile virus-like particle carrier for epitope based vaccines. *PLoS ONE, 5*, e9809.
19. Legendre, D., & Fastrez, J. (2005). Production in *Saccharomyces cerevisiae* of MS2 virus-like particles packaging functional heterologous mRNAs. *Journal of Biotechnology, 117*, 183–194.
20. Freivalds, J., Dislers, A., Ose, V., Skrastina, D., Cielens, I., Pumpens, P., et al. (2006). Assembly of bacteriophage Qβ virus-like particles in yeast *Saccharomyces cerevisiae* and *Pichia pastoris*. *Journal of Biotechnology, 123*, 297–303.
21. Freivalds, J., Rūmnieks, J., Ose, V., Renhofa, R., & Kazāks, A. (2008). High-level expression and purification of bacteriophage GA virus-like particles from yeast *Saccharomyces cerevisiae* and *Pichia pastoris*. *Acta Universitatis Latviensis ser Biology, 745*, 75–85.

22. Kozlovskaia, T. M., Pumpen, P. P., Dreilinia, D. E., Tsimanis, A. I., & Ose, V. P. (1986). Formation of capsid-like structures as a result of expression of the cloned gene of the envelope protein of the RNA-containing bacteriophage fr. *Doklady Akademii Nauk SSSR, 287*, 452–455. In Russian.
23. Caldeira, J. C., & Peabody, D. S. (2007). Stability and assembly in vitro of bacteriophage PP7 virus-like particles. *Journal of Nanobiotechnology, 5*, 10.
24. Samuel, D., Sasnauskas, K., Jin, L., Beard, S., Zvirbliene, A., Gedvilaite, A., et al. (2002). High-level expression of recombinant mumps nucleoprotein in *Saccharomyces cerevisiae* and its evaluation in mumps IgM serology. *Journal of Medical Virology, 66*, 123–130.
25. Gietz, D., St Jean, A., Woods, R. A., & Schiestl, R. H. (1992). Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Research, 20*, 1425.
26. Kushnirov, V. V. (2000). Rapid and reliable protein extraction from yeast. *Yeast, 16*, 857–860.
27. Kuramoto, E., Yano, O., Kimura, Y., Baba, M., Makino, T., Yamamoto, S., et al. (1992). Oligonucleotide sequences required for natural killer cell activation. *Japanese Journal of Cancer Research, 83*, 1128–1131.
28. Da Silva, D. M., Pastrana, D. V., Schiller, J. T., & Kast, W. M. (2001). Effect of pre-existing neutralizing antibodies on the anti-tumor immune response induced by chimeric human papillomavirus virus-like particle vaccines. *Virology, 290*, 350–360.
29. Freivalds, J., Dislers, A., Ose, V., Pumpens, P., Tars, K., & Kazaks, A. (2011). Highly efficient production of phosphorylated hepatitis B core particles in yeast *Pichia pastoris*. *Protein Expression and Purification, 75*, 218–224.
30. Bendis, I., & Shapiro, L. (1970). Properties of *Caulobacter* ribonucleic acid bacteriophage phi Cb5. *Journal of Virology, 6*, 847–854.
31. Rumnieks, J., Ose, V., Tars, K., Dislers, A., Strods, A., Cielens, I., et al. (2009). Assembly of mixed rod-like and spherical particles from group I and II RNA bacteriophage coat proteins. *Virology, 391*, 187–194.
32. Chen, C., Daniel, M. C., Quinkert, Z. T., De, M., Stein, B., Bowman, V. D., et al. (2006). Nanoparticle-templated assembly of viral protein cages. *Nano Letters, 6*, 611–615.

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Highly efficient production of phosphorylated hepatitis B core particles in yeast *Pichia pastoris*

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ABSTRACT

Virus-like particles (VLPs) of the recombinant hepatitis B virus (HBV) core protein (HBc) are routinely used in HBV diagnostics worldwide and are of potential interest as carriers of foreign peptides (e.g., immunological epitopes and targeting addresses, and/or as vessels for packaged diagnostic and therapeutic nanomaterials). Despite numerous reports exploiting different expression systems, a rapid and comprehensive large-scale methodology for purification of HBc VLPs from yeast is still lacking. Here, we present a convenient protocol for highly efficient production and rapid purification of endotoxin-free *ayw* subtype HBc VLPs from the methylotrophic yeast *Pichia pastoris*. The HBc gene expression cassette along with the geneticin resistance gene was transferred to the *P. pastoris* genome via homologous recombination. A producer clone was selected among 2000 transformants for the optimal synthesis of the target protein. Fermentation conditions were established ensuring biomass accumulation of 163 g/L. A simple combination of pH/heat and salt treatment followed by a single anion-exchange chromatography step resulted in a more than 90% pure preparation of HBc VLPs, with a yield of about 3.0 mg per 1 g of wet cells. Purification is performed within a day and may be easily scaled up if necessary. The quality of HBc VLPs was verified by electron microscopy. Mass spectrometry analysis and direct polyacrylamide gel staining revealed phosphorylation of HBc at at least two sites. To our knowledge, this is the first report of HBc phosphorylation in yeast.

Introduction

Hepatitis B virus (HBV)[1] from the *Hepadnaviridae* family is a major cause of human liver disease, resulting in approximately 620,000 deaths worldwide each year [1]. About 4.5 million new HBV infections occur each year, of which a quarter progress to liver disease [2]. The virus is composed of an outer envelope containing surface proteins integrated in a lipoprotein shell and an inner nucleocapsid assembled from the HBV core protein (HBc) that encloses the viral genomic DNA and the viral polymerase [3]. Virtually all HBV infected individuals can produce high titers of an anti-HBc antibody, which is one of the most specific serological markers of past (anti-HBc IgG) or current (anti-HBc IgM) HBV infections [4].

Heterologously expressed HBc spontaneously assembles into the virus-like particles (VLPs) that are routinely used for HBV diagnostics worldwide [5]. Because HBc is known to induce strong B-cell, T-cell, and cytotoxic T-cell responses in hepatitis B patients [6] in both a T-cell dependent and T-cell independent manner [7], it might be regarded as a component of a novel prophylactic and/or therapeutic HBV vaccine [8]. The ability of HBc carrier to provide inserted epitopes with T-cell help [9] and preferential priming of Th1 cells, without any requirement for adjuvants [10], stimulates the development of a broad range of vaccine prototypes on the basis of HBc VLPs [8]. Recently, HBc has attracted special interest in medicinal nanotechnology as a putative packager of organic and inorganic compounds, including stimulatory oligonucleotides, low molecular weight drugs, and magnetic particles.

Yeast systems have been used extensively for expression of a large number of structural genes from many mammalian viruses, which results in a formation of naturally folded VLPs [11–16]. This has led to a generation of licensed prophylactic vaccines against human HBV and papillomaviruses [8,17]. One of the most prominent yeast expression systems is based on the methylotrophic *Pichia pastoris* strain, which is used successfully to produce more than 500 proteins both for basic laboratory research and industrial manufacturing [18,19]. Although expression of the *HBc* gene in yeast cells including *P. pastoris* have been described by several authors [20–24], the majority of published HBc VLP purification methods remain either too complicated and time-consuming or

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[1] *Abbreviations used:* AEC, anion-exchange chromatography; CBB, Coomassie Brilliant Blue; HBV, hepatitis B virus; HBc, HBV core protein; PAAG, polyacrylamide gel; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; VLP(s), virus-like particle(s).

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non-effective due to the high costs and/or low output of the target protein.

Naturally folded HBc, either isolated from human tissues or recombinantly expressed in mammalian cells, appears as a phosphoprotein [25,26], being phosphorylated on serine residues at the carboxy-terminal part of the molecule [27]. In contrast, phosphorylation has not been detected for *Escherichia coli*- and yeast-derived HBc [28]. It has been demonstrated that HBc phosphorylation plays an important role in HBV replication and capsid localization [29,30]. In the present work, we establish the efficient expression of the *HBc* gene in *P. pastoris* and develop a rapid purification method for HBc VLPs. In contrast to previous reports, HBc is found to be phosphorylated at at least two sites within the molecule. We suggest such yeast-derived HBc VLPs are of value for diagnostic purposes and vaccine development as well as for HBV replication studies.

Materials and methods

Construction of an expression vector and selection of clones

The *HBc* gene from plasmid pHB320 containing the full HBV genome, genotype D, subtype *ayw* (GenBank Accession No. X02496; [31]), was PCR-amplified and ligated into the *Bam*HI/*Eco*105I-treated vector pPIC3.5K (Invitrogen), under control of the *AOX1* promoter. After sequencing, the resulting pPIC-HBc plasmid was linearized with *Ecl*136II in *AOX1* promoter region and used for transformation of the *P. pastoris* GS115 *his4* strain by electroporation. Mut^+His^+ transformants were isolated on agarized minimal medium (1.34% yeast nitrogen base, 2% glucose, 4×10^{-5}% biotin, 2% agar). For selection of clones with multiple integration units, transformants were replica-plated onto yeast extract peptone dextrose (YEPD) agarized medium containing increased concentrations of the G418 antibiotic (1.8–2.5 mg/mL).

Southern blotting

To estimate the approximate expression cassette copy number in *P. pastoris*, chromosomal DNA from selected transformants was digested with *Bgl*II and separated by agarose gel electrophoresis. After transferring to a nitrocellulose membrane, DNA was hybridized with a biotin-labeled *HIS4*-specific probe (~600-bp *Kpn*I restriction fragment from the pPIC3.5K) using the Biotin DecaLabel™ DNA Labeling Kit. The reaction was further processed and developed by the Biotin Chromogenic Detection Kit. All the enzymes, protein and DNA molecular weight (MW) markers as well as kits used in our study were purchased from Fermentas (Vilnius, Lithuania) unless otherwise indicated.

Cultivation in flasks

Induction of *HBc* gene expression in *P. pastoris* was achieved according to the protocol of the manufacturer with the following minor modifications: selected clones were incubated at 30 °C on a shaker in 0.5-L Erlenmeyer flasks containing 100 mL of buffered complex glycerol medium (BMGY) for 20–24 h until OD_{590} 6–8; cells were then harvested by low-speed centrifugation and resuspended in the same volume of buffered complex methanol medium (BMMY) containing 0.5% methanol. Each subsequent day, 100% methanol was added to a final concentration of 0.5%, and cells were harvested 3 days after induction.

The level of HBc production was estimated by disrupting 20 optical units of yeast cells by 425–600-μ glass beads (Sigma) in 200 μL of 20 mM Tris–HCl, pH 8.0, eight times for 0.5 min. Debris was separated by low-speed centrifugation, and the supernatant was serially diluted for an immunodiffusion assay [32] using a polyclonal rabbit anti-HBc antibody (obtained after immunization with *E. coli*-derived HBc VLPs).

Large-scale cultivation

A volume of 500 mL of seed material was used to inoculate 4.5 L of BMGY in a fermentor. Seed material was prepared in two steps: stock culture from storage at −80 °C was plated on agarized minimal medium, and a single colony was inoculated in 5 ml of YEPD and cultured at 30 °C for 48 h. A volume of 0.1 mL of this primary seed material was transferred to each of five 0.5-L flasks containing 100 mL of BMGY and 10 mg/L chloramphenicol, and flasks were incubated at 250 rpm at 30 °C for 20–24 h to a final OD_{590} 6.0–8.0.

Fermentation conditions: A 10-L fermentor (Bioflo 410, New Brunswick Scientific) was filled with 4.5 L BMGY (with glycerol concentration 40 g/L) and 0.5 L seed material in BMGY. Non-enriched air was used throughout the fermentation. The dissolved oxygen was set at 20%, the aeration rate on the first day was up to 1 vol/min, and the stirring speed was up to 1200 rpm, while the incubation temperature was 30 °C, and the pH was controlled with 25% (v/v) NH_4OH to keep the pH above 5.0. After the glycerol exhaustion at 18 h, 20 mL of 50% glycerol and 10 mL of 100% methanol was added. One hour later, the methanol supply was set at a rate of 3.0 mL/L/h, and the air supply was increased to 5 vol/min. Cultivation continued for 92 h with methanol feeding adjusted to a consumption rate of 6.8 g/L/h. Antifoam M30 (Serva) was used to prevent extensive foam formation. Cells were harvested at 3000*g* for 10 min at 4 °C. After washing once with dH_2O, the cell pellet was stored at −80 °C until use.

Purification of HBc VLPs

A 4-g portion of the frozen yeast cells was resuspended in 16 mL of lysis buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.1 mM PMSF) and disrupted with a French press (3 cycles, 20,000 psi). The soluble fraction was separated by centrifugation for 15 min at 15,500*g* and the pH was adjusted to 8.0 with 0.5 M NaOH. The supernatant was incubated for 1 h at +65 °C and subsequently centrifuged for 25 min at 15,500*g*. Solid ammonium sulfate was then added to the supernatant to 40% saturation, which was incubated for 0.5 h at 4 °C and centrifuged again for 25 min at 15,500*g*. The sediment was dissolved in a minimal amount (1 mL) of phosphate buffer containing 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.1% Triton X-100, pH 7.4, and loaded onto a pre-packed anion-exchange HiPrep 16/10 DEAE Fast Flow column (20-mL bed volume) connected to an ÄKTA chromatography system (Amersham Biosciences). The column was preequilibrated with phosphate-buffered saline, pH 7.4 (Sigma), and run at 5 mL/min. Column-bound proteins were eluted by a linear gradient with phosphate buffer containing 1 M NaCl.

E. coli-derived HBc VLPs used as a control were purified essentially as described previously [33].

Analytical methods

Protein samples were monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), with a 4% stacking and 15% separating polyacrylamide gel (PAAG), according to standard protocols. To visualize protein bands, the gels were stained with Coomassie Brilliant Blue (CBB) G-250. Alternatively, separated proteins were transferred onto nitrocellulose membranes and detected by immunoblotting with the monoclonal anti-HBc antibody 13C9 [34] and the anti-mouse IgG peroxidase conjugate (Sigma).

Protein samples were subjected to native 1% agarose gel electrophoresis in TAE buffer (pH 8.4) for about 0.5 h at 5 V/cm. Nucleic

acids in agarose gels were visualized by ethidium bromide staining. For determination of protein concentration, a Bradford assay [35] was used, and the purity of HBc samples was estimated by densitometric analysis of the CBB-stained PAAG.

The endotoxin level in the protein samples was determined by a Limulus amoebocyte lysate (LAL) Pyrogent® Plus test kit according to the manufacturer's protocol (BioWhittaker, Walkersville, MD).

To detect phosphorylated proteins, PAAG was stained directly with the ProQ® Diamond phosphoprotein gel stain (Invitrogen) according to the manufacturer's instructions. Full-length HBc molecules as well as their C-terminal domains resulting from proteinase K cleavage were analyzed. For the proteinase K reaction, VLPs were treated for 10 min at 95 °C in buffer containing 1% SDS and 2% β-mercaptoethanol followed by the addition of proteinase K and incubation for 5 min at 50 °C.

For electron microscopy, the protein samples were adsorbed on carbon–formvar-coated copper grids and negatively stained with 1% uranyl acetate aqueous solution. The grids were examined with a JEM-1230 electron microscope (JEOL Ltd., Tokyo, Japan) at 100 kV.

For whole protein analysis in mass spectrometry, 2 μL of purified VLPs at 1 mg/mL concentration in 20 mM Tris–HCl, pH 8.0, was mixed with 2 μl 10% trifluoroacetic acid and 2 μl DHAP. For tryptic digestion of proteins, the CBB-stained band was excised from SDS–PAAG, incubated for 2 × 1 h in 0.2 M ammonium bicarbonate and 50% acetonitrile at 30 °C and incubated for 2 × 20 min in 100% acetonitrile at room temperature. A volume of 0.1 mg/mL trypsin (Sigma, proteomics grade) in 1 mM HCl was mixed 1:1 with 50 mM ammonium bicarbonate in 10% acetonitrile. Gel pieces were covered with the trypsin solution so that the gel absorbed approximately two thirds of the solution's volume. The gel was further incubated for 3 h at 30 °C. A volume of 2 μL of buffer covering the gel pieces was mixed with 2 μL 10% trifluoroacetic acid and 2 μl DHAP solution (15 mg/mL DHAP in 75% ethanol, 2.5 mM diammonium hydrogen citrate). All samples were analyzed on a Bruker Daltonics Autoflex MALDI-TOF mass spectrometer.

Results and discussion

Selection and cultivation of the HBc producer strain

The yeast expression system was chosen due to its relatively simple fermentation design and high final cell densities. In addition, data from other groups indicate that *P. pastoris*-derived HBc preparations are superior to *E. coli*-derived HBc VLPs in anti-HBc antibody diagnostic assays [23,24]. Additionally, yeast products do not harbor pathogens, viruses, or pyrogens.

It has been shown previously that the expression level in *P. pastoris* VLP producers can be substantially increased by extensive screening for clones with multicopy insertions of expression cassettes [15,23,36,37]. For this, about 2000 $Mut^{+}His^{+}$ transformants were screened for their resistance level to increased doses of the G418 antibiotic as a selection marker. The majority of the clones were resistant to G418 at concentrations below 1.8 mg/mL, while 1–2% of clones were able to grow at concentrations up to 2.5 mg/mL, indicating a possible multiple-insertion event. Clones with increased resistance generally exhibited higher though variable synthesis of the target protein (Fig. 1a) which correlated well with their anti-HBc titers in immunodiffusion: highly resistant clones reacted at dilutions up to 1:128, while control clones with single-copy inserts reacted at a dilution 1:8 (data not shown). In this way, clone E1, with the highest production level confirmed by SDS–PAGE (Fig. 1a, lane 4), was selected.

To verify multiple-target gene insertion events in clones with increased resistance to G418, samples of chromosomal DNA from individual clones were subjected to Southern blot analysis (Fig. 1b). This analysis revealed the presence of a 2.7-kb fragment corresponding to the chromosomal *HIS4* gene and a larger, approximately 6.0-kb fragment corresponding to the expression cassette integrated at the *AOX1* locus. As expected, a putative single-copy transformant exhibited two bands of similar intensity (Fig. 1b, lane 2), whereas in clones with an increased resistance, the 6.0-kb band dominated over the 2.7-kb band, suggesting a multiple-insertion pattern (Fig. 1b, lanes 3–7). Notably, clones with apparently even more copies than E1 (e.g., clone E) still produced remarkably less HBc (compare Fig. 1a and b, lanes 3 and 4). Although the exact insert copy number was not detected, this finding indicates a certain optimal level of target gene dosage. This is consistent with data for expression of other viral structural genes in *P. pastoris* (e.g., maximal synthesis of measles virus nucleoprotein was detected in a transformant with 10 copies of the target gene, whereas a further increase in the gene copy number led to reduced expression [15]). Our data clearly show that the expression potential of the *P. pastoris* expression system could sometimes be underevaluated by limited screening of clones.

Clone E1 was cultivated in a fermentor as described in the Materials and methods section, with an HBc expression level of 2–3% of the total cell proteins at the end of cultivation. Although this was about three times lower than the HBc level obtained with the *E. coli* expression system [38,39], it was high enough to establish an efficient and short purification protocol ensuring about 90% purity of the target protein (discussed below). For our fermentation, we did not use oxygen-enriched air, which has often been used in *P. pastoris* fermentation systems [23,40]. The final biomass yield under these conditions was 163 g/L (an average from two independent cultivations); with oxygen enrichment, it was possible to obtain twice the amount of biomass, but with a three times lower HBc synthesis level (data not shown). Although more fermentation experiments are needed to make definite conclusions about the effect of oxygen, we propose that slower biomass accumulation is beneficial for HBc synthesis.

Purification of HBc VLPs

To make purification cost-effective and fast, we aimed to avoid many commonly used protein purification steps such as centrifugation in a sucrose gradient, affinity and size-exclusion chromatography, filter-concentration, and dialysis. Disrupting yeast cells with the French press is highly reproducible and easy to scale-up. The majority of the HBc was found in the soluble fraction (Fig. 2a, lane 1), though a strong degradation pattern was detectable by a Western blot (Fig. 2b, lane 1). A heat treatment has been used by several groups as a purification step applicable for both bacterial and yeast-derived HBc preparations [22,41,42]. In addition, Naito et al. [41] demonstrated that contaminating proteins were removed from bacterial lysates more efficiently at pH 6.0 rather than at neutral pH. Under our conditions, heat treatment of yeast lysates at pH 6.2 (this was the pH of the non-adjusted crude cell lysate) precipitated the majority of HBc, while a rise in the pH significantly increased the solubility of HBc, with pH 8.0 being optimal for recovery of the target protein (Fig. 2a, and data not shown). Further experiments revealed that a heat treatment at pH 8.0 of either the supernatant or crude cell homogenate resulted in nearly identical HBc recovery (data not shown), enabling the exclusion of the initial centrifugation step from the protocol. Moreover, heat treatment led to precipitation of degraded forms of HBc (Fig. 2b, lanes 1 and 2). We suggest that most HBc degradation products are not incorporated in the capsid structure and therefore are thermally less stable than VLPs. Altogether, our data demonstrate the effect

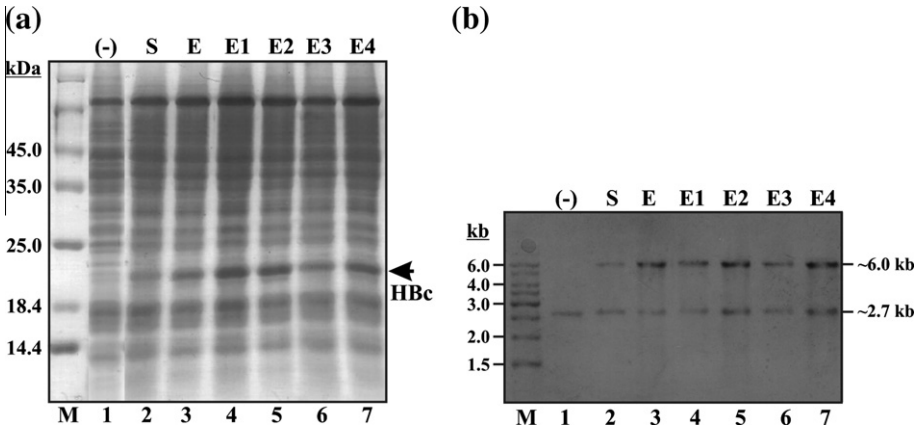

Fig. 1. Correlation between *HBc* gene expression level and the amount of integration units in individual *P. pastoris* subclones estimated by CBB-stained PAAG (a) and Southern blotting (b). Lane 1, negative control, non-transformed *P. pastoris* cells. Lanes 2–7, *P. pastoris* clones harboring single (S; lane 2) and multiple (E–E4; lanes 3–7) *HBc* gene insertions. Only a part of the multicopy clones is shown to demonstrate deviation in expression level. M, protein (a) and DNA (b) MW standards.

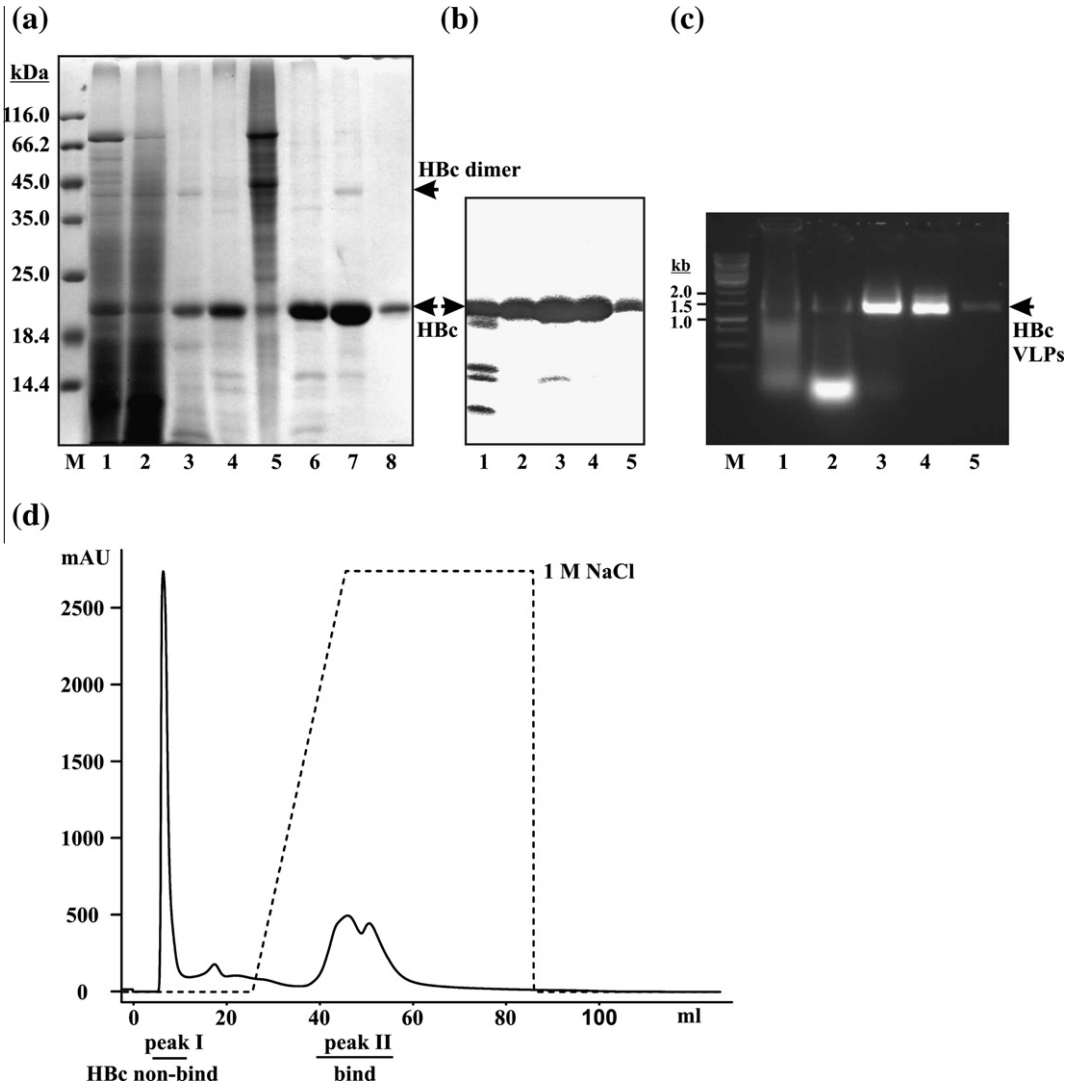

Fig. 2. Main purification steps for HBc VLPs. (a) CBB-stained PAAG illustrating HBc purity in protein samples. Lanes 1 and 2, soluble and unsoluble fractions, respectively. Lanes 3 and 4, soluble fraction after heat treatment at pH 6.2 and 8.0, respectively. Lane 5, heat-precipitated proteins from supernatant at pH 8.0. Lane 6, ammonium sulfate precipitate. Lanes 7 and 8, proteins from AEC peaks I and II, respectively. (b) and (c) Western blotting and native agarose gel electrophoresis, showing HBc degradation pattern and presence of nucleic acids, respectively. Lanes 1 and 2, non-treated and heat-treated cell supernatants, respectively. Lane 3, dissolved ammonium sulfate precipitate. Lanes 4 and 5, proteins from AEC peaks I and II, with column flow-through and bound material, respectively (d). M, protein (a) and DNA (c) MW standards.

of the pH during the heat treatment procedure, although it is clear that the optimal pH value should be determined for each individual expression event.

Ammonium sulfate precipitation has been routinely used in HBc VLP concentration and purification protocols [22,33,43]. Under our conditions, a 0.5-h incubation was enough to precipitate more than 90% of the HBc from solution, with further successful and complete solubilization of the precipitate (data not shown). This procedure also removed most of the non-specific nucleic acids from the HBc preparation (Fig. 2c). Thus, a simple combination of pH/heat treatment followed by salt precipitation effectively enriched the concentration of the HBc in solution (Fig. 2a, lane 6).

Size-exclusion chromatography and/or sucrose gradient ultracentrifugation are often recommended as final steps in HBc purification [23,24,44]. These methods, however, strongly limit sample volume and increase costs. We looked to ion exchangers as a relatively cheap, robust material able to withstand harsh cleaning-in-place conditions, typically with sodium hydroxide. Other researchers have reported recovery of *E. coli*-derived HBc VLPs from weak anion exchange matrices such as DEAE Sephacel or Streamline DEAE [43,45]. We subjected the enriched HBc solution to DEAE Sepharose with similar characteristics and scalability possibilities. According to the anion-exchange chromatography (AEC) profile, proteins were separated into two dominant peaks representing column-bound and non-bound material (Fig. 2d). SDS–PAGE revealed that both peaks contain target protein, though with strong predominance of HBc in peak I (Fig. 2a, lanes 7 and 8). Thus, under conditions described, the majority of the HBc material did not bind to the matrix and was eluted as a sharp peak within the column void volume (between 5 and 8 mL). However, about 10% of the HBc was retained on the column and was eluted only at increased salt concentrations.

Characterization of HBc VLPs

As detected by SDS–PAGE, purified HBc migrated in PAAG according to its calculated MW, namely, 21.1 kDa, and showed no visible degradation in Western blotting (Fig. 2a, lane 7 and b, lane 4). Despite the presence of some minor bands of contaminants visible in overloaded PAAGs, the final purity of the HBc might be roughly estimated as at least 90%. The output of HBc VLPs reached 3 mg from 1 g of wet cells or about 700 mg from 1 L of initial *P. pastoris* fermentation culture. For comparison, reported yields by others who used *P. pastoris*-driven *HBc* gene expression were 69 and 64 mg/L, respectively [23,24]. Our greater output might be a consequence of a more efficient producer strain and/or an optimized purification protocol. In addition, one can speculate that different HBV genotypes (*adw* or *ayw* used in these or our studies, respectively) can influence the yield due to different behaviors of the target protein during purification.

The presence of nucleic acids in HBc VLPs was detected by agarose gel electrophoresis, where particles migrated along with the 1.5-kb dsDNA band (Fig. 2c). These VLP-associated nucleic acids can be eliminated by RNase (but not DNase) treatment (data not shown), confirming that heterologously expressed HBc VLPs predominantly encapsidate host-derived RNA [23,46]. To obtain empty RNA-free HBc VLPs for *in vivo* applications, Broos et al. [43] used combined Mono Q and Heparin column chromatography, though the yield was rather low. Alternatively, we and others have demonstrated that under low ionic strength, VLP-associated RNA can be substituted by short, defined DNA fragments [33,47]. Moreover, potential for controlled dis- and re-assembly makes VLPs especially attractive for gene and drug delivery applications [48,49]. The latter approach is also of value for the removal of non-specific nucleic acids from VLP preparations.

For further characterization of the final product, we subjected protein from both AEC peaks to electron microscopy. The non-bound fraction appeared as a heterogeneous mixture of correctly folded icosahedral $T = 3$ and $T = 4$ particles, with a predominance of the larger $T = 4$ form (Fig. 3a). These VLPs exhibited similar morphology to the authentic HBc particles derived from an HBV-infected liver as well as to recombinant VLPs obtained from bacterial cells expressing the *HBc* gene [50,51]. Interestingly, column-bound material also contained assembled VLPs, but these particles tended to aggregate possibly due to their association with remaining impurities (Fig. 3b). Thus, the final chromatography step improved not only the purity, but also the homogeneity of the VLP preparation.

The endotoxin level in the final product was less than 100 EU/mg of pure protein, which makes it attractive both for *in vitro* and *in vivo* applications. It should be noted that the low endotoxin level was achieved with relatively low synthesis of HBc VLPs compared to *E. coli* expression systems (data not shown).

The length of yeast-expressed HBc was investigated by MALDI-TOF mass spectrometry. The MW of full-length HBc theoretically is 21,116 Da (or 20,985 Da without the first methionine). Our data indicated that the MW of yeast-derived HBc is 21,324 Da (Fig. 4a). In parallel, we determined the MW of the same protein produced in *E. coli* to be 21,136 Da. As discussed in Watelet et al. [23], *E. coli*-produced HBc has its first *N*-acyl-methionine preserved, thus the theoretical MW is 21,144 Da, which is close to our observed data and well within the instrument precision for the given MW. However, the MW of the yeast-produced HBc was significantly higher than the theoretical value, and the difference could not be explained solely by instrument error. Therefore, we assumed that the increase in MW is due to some post-translational modification that does not occur in *E. coli*. Additionally, the protein peak in the mass spectrometer was significantly wider in the case of the yeast-expressed protein, suggesting that the material might

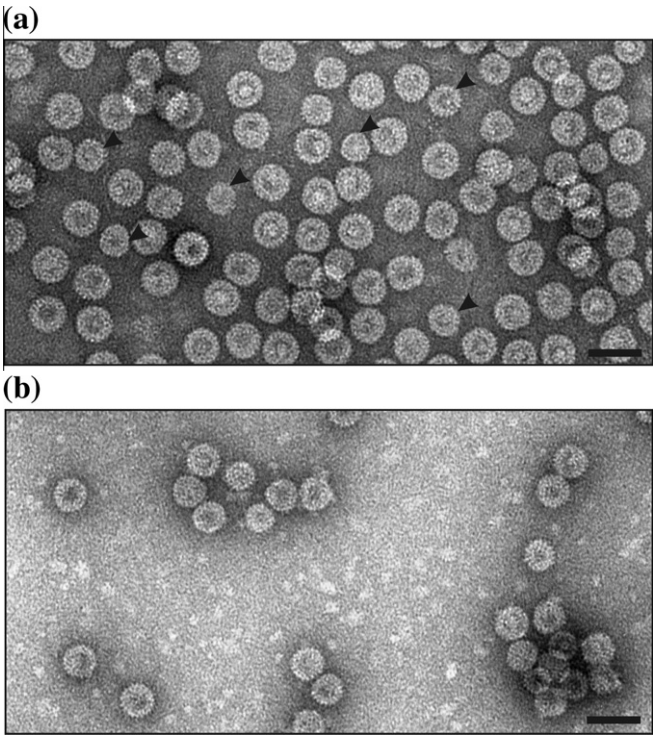

Fig. 3. Electron microscopy of purified HBc VLPs from AEC eluted in column void volume (a), and at an increased salt concentration (b). Some of the smaller $T = 3$ quasi-symmetry particles are marked by arrowheads. Scale bar: 50 nm.

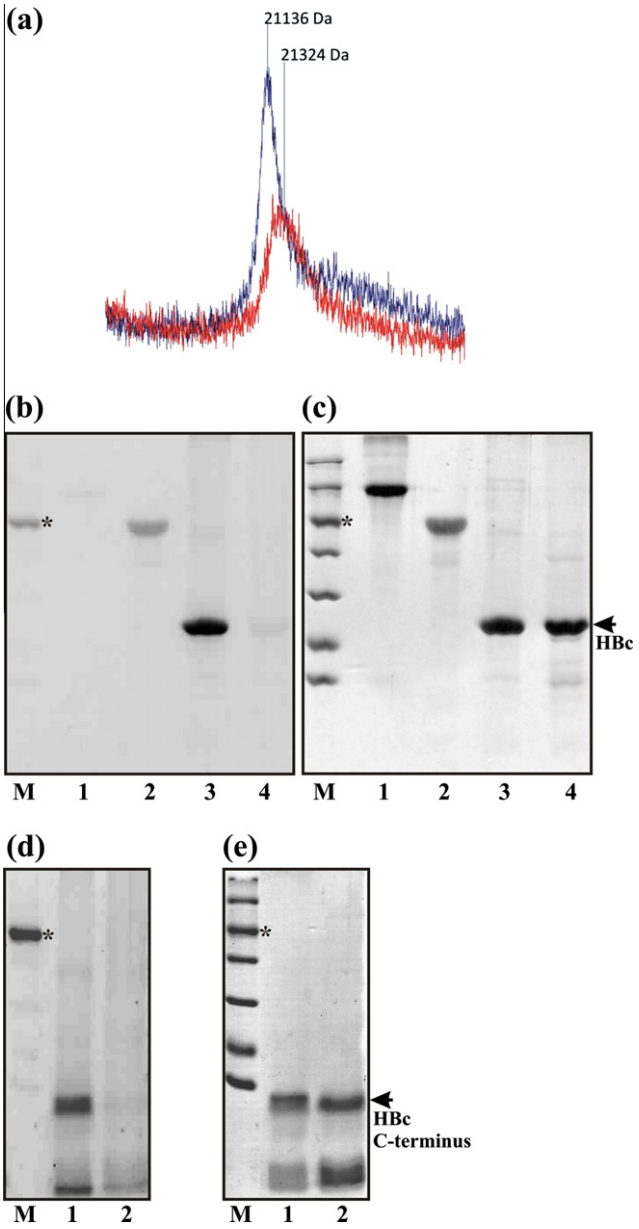

Fig. 4. Phosphorylation of *P. pastoris*-derived HBc. Comparison of full-length yeast- and *E. coli*-derived HBc molecules by mass spectrometry (a), and by denaturing SDS–PAAG stained directly with phosphoprotein stain (b), and subsequently with CBB (c). BSA and pepsin were loaded as negative and positive controls (lanes 1 and 2, respectively). Lanes 3 and 4 represent *P. pastoris*- and *E. coli*-derived HBc, respectively. Lower panel represents denaturing SDS–PAAG with proteinase K-cleaved C-terminal domains of *P. pastoris*- (lane 1) and *E. coli* (lane 2)-derived HBc stained directly with phosphoprotein stain (d), and subsequently with CBB (e). M, protein MW standards. Phosphoprotein ovalbumin is marked by an asterisk.

be somewhat heterogenous, which is a common outcome due to partial post-translational modifications (Fig. 4a).

According to Liao and Ou [27], native HBc is phosphorylated at three serine residues at the carboxy-terminal part of the molecule. In contrast, *E. coli*-derived HBc was found non-phosphorylated [28]. To investigate whether our yeast-produced protein is phosphorylated, we subjected full-length HBc molecules to SDS–PAGE and performed phosphoprotein and CBB staining (Fig. 4b and c). The results clearly indicated that the yeast-produced HBc is indeed phosphorylated. Furthermore, we attempted to localize the phosphorylation sites. First, we performed mass spectrometry of tryptic peptides obtained from in-gel digestion of both yeast- and bacteria-derived HBc. Comparing both spectra, one peptide with a MW of 1553 Da (corresponding to the sequence DLVVSYVNTNMGLK) was present only in the bacteria-derived protein, but a peptide with a MW of 1630 Da was present only in the yeast-expressed material (data not shown). Because the difference in both MWs corresponds to the MW of a phosphate group, we conclude that phosphorylation has occurred in the DLVVSYVNTNMGLK peptide, presumably on the Ser87 residue, phosphorylation of which was recently demonstrated *in vitro* [52]. No other similar differences in mass spectra could be observed. However, potential phosphorylation sites in the C-terminal part of the molecule, similar to those observed in the native virus, are surrounded by frequent arginine residues, resulting in very short tryptic peptides that are difficult to observe with mass spectrometry. Therefore, to investigate whether there are any phosphate groups added to the C-terminal part of the polypeptide, we performed a cleavage with proteinase K, which should produce a long C-terminal peptide with a MW of 4360 Da. Cleavage products were loaded onto SDS–PAAG, and staining was performed with both phosphoprotein and Coomassie stains. The results indicated that the C-terminus of yeast-derived HBc is phosphorylated, too (Fig. 4d and e). This is consistent with data from whole protein mass spectra, indicating that there might be 2–3 phosphorylation sites per monomer. In conclusion, we have determined one phosphorylation site at Ser87 and another at the C-terminal part. Although we do not have exact experimental evidence, we speculate that the phosphorylation site(s) at the C-terminus might be the same as for the native virus, at one or several of the residues Ser155, Ser162, and Ser170. Structurally, Ser87 is located in an alpha helix, forming 4-helix bundle spikes on the surface of HBc VLPs. It should be noted that a study of Watelet et al. [23] regarding *Pichia*-derived HBc did not reveal any phosphorylation sites, although phosphoprotein detection was attempted. However, HBc from the HBV *adw* subtype contains Asn87 instead of Ser87 and therefore cannot be phosphorylated. Also, different expression conditions might influence the efficiency of phosphorylation at the C-terminal domain of the HBc molecule.

Reversible natural phosphorylation of the HBc is essential for distinct steps of HBV replication, such as pregenome packaging, plus strand DNA synthesis, capsid localization, and virus maturation and secretion [53]. Since exposure of the nuclear localization signals depends on phosphorylation of the HBc, the latter emerges as a prerequisite for transport of the viral genome to the nucleus [54]. Therefore, simple and efficient production of phosphorylated HBc particles paves a way for further functional investigations of intimate HBV replication mechanisms. Although 90% purity level is not ideal, this is close to the maximum because repeated cycles of gel-filtration or AEC did not produce any improvements (data not shown). We strongly suggest that remaining impurities have internal capsid localization and could not be removed by conventional purification methods. Controlled dis- and re-assembly of obtained VLPs in combination with packaging of therapeutic substances (e.g., antibiotics, CpG oligonucleotides, and thermotherapy agents) will be a subject for further investigation.

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References

[1] S.T. Goldstein, F. Zhou, S.C. Hadler, B.P. Bell, E.E. Mast, H.S. Margolis, A mathematical model to estimate global hepatitis B disease burden and vaccination impact, Int. J. Epidemiol. 34 (2005) 1329–1339.

[2] A.R. Zanetti, P. van Damme, D. Shouval, The global impact of vaccination against hepatitis B: a historical overview, Vaccine 26 (2008) 6266–6273.

[3] M. Nassal, H. Schaller, Hepatitis B virus replication, Trends Microbiol. 1 (1993) 221–228.

[4] G. Hess, W. Arnold, The clinical relevance of the antibody to hepatitis B core antigen (anti-HBc): a review, J. Virol. Methods 2 (1980) 107–117.

[5] S. Stahl, P. MacKay, M. Magazin, S.A. Bruce, K. Murray, Hepatitis B virus core antigen: synthesis in *Escherichia coli* and application in diagnosis, Proc. Natl. Acad. Sci. USA 79 (1982) 1606–1610.

[6] F.V. Chisari, C. Ferrari, Hepatitis B virus immunopathogenesis, Annu. Rev. Immunol. 13 (1995) 29–60.

[7] D.R. Milich, A. McLachlan, The nucleocapsid of hepatitis B virus is both a T-cell-independent and a T-cell-dependent antigen, Science 234 (1986) 1398–1401.

[8] P. Pumpens, R. Ulrich, K. Sasnauskas, A. Kazaks, V. Ose, E. Grens, Construction of novel vaccines on the basis of the virus-like particles. Hepatitis B virus proteins as vaccine carriers, in: Y. Khudyakov (Ed.), Medicinal Protein Engineering, CRC Press, 2008, pp. 205–248.

[9] T. Fehr, D. Skrastina, P. Pumpens, R.M. Zinkernagel, T cell-independent type I antibody response against B cell epitopes expressed repetitively on recombinant virus particles, Proc. Natl. Acad. Sci. USA 95 (1998) 9477–9481.

[10] D.R. Milich, F. Schödel, J.L. Hughes, J.E. Jones, D.L. Peterson, The hepatitis B virus core and e antigens elicit different Th cell subsets: antigen structure can affect Th cell phenotype, J. Virol. 71 (1997) 2192–2201.

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

[12] K.J. Hofmann, J.C. Cook, J.G. Joyce, D.R. Brown, L.D. Schultz, H.A. George, M. Rosolowsky, K.H. Fife, K.U. Jansen, Sequence determination of human papillomavirus type 6a and assembly of virus-like particles in *Saccharomyces cerevisiae*, Virology 209 (1995) 506–518.

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

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

[15] R. Slibinskas, D. Samuel, A. Gedvilaite, J. Staniulis, K. Sasnauskas, Synthesis of the measles virus nucleoprotein in yeast *Pichia pastoris* and *Saccharomyces cerevisiae*, J. Biotechnol. 107 (2004) 115–124.

[16] M. Juozapaitis, A. Serva, A. Zvirbliene, R. Slibinskas, J. Staniulis, K. Sasnauskas, B.J. Shiell, L.F. Wang, W.P. Michalski, Generation of henipavirus nucleocapsid proteins in yeast *Saccharomyces cerevisiae*, Virus Res. 124 (2007) 95–102.

[17] J.T. Bryan, Developing an HPV vaccine to prevent cervical cancer and genital warts, Vaccine 25 (2007) 3001–3006.

[18] S. Macauley-Patrick, M.L. Fazenda, B. McNeil, L.M. Harvey, Heterologous protein production using the *Pichia pastoris* expression system, Yeast 22 (2005) 249–270.

[19] E. Böer, G. Steinborn, G. Kunze, G. Gellissen, Yeast expression platforms, Appl. Microbiol. Biotechnol. 77 (2007) 513–523.

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

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

[22] D. Rolland, M. Gauthier, J.M. Dugua, C. Fournier, L. Delpech, B. Watelet, O. Letourneur, M. Arnaud, M. Jolivet, Purification of recombinant HBc antigen expressed in *Escherichia coli* and *Pichia pastoris*: comparison of size-exclusion chromatography and ultracentrifugation, J. Chromatogr. B Biomed. Sci. Appl. 753 (2001) 51–65.

[23] B. Watelet, M. Quibriac, D. Rolland, G. Gervasi, M. Gauthier, M. Jolivet, O. Letourneur, Characterization and diagnostic potential of hepatitis B virus nucleocapsid expressed in *E. coli* and *P. pastoris*, J. Virol. Methods 102 (2002) 175–190.

[24] Z.X. Li, G.Q. Hong, B. Hu, M.J. Liang, J. Xu, L. Li, Suitability of yeast- and *Escherichia coli*-expressed hepatitis B virus core antigen derivatives for detection of anti-HBc antibodies in human sera, Protein Expr. Purif. 56 (2007) 293–300.

[25] C. Albin, W.S. Robinson, Protein kinase activity in hepatitis B virus, J. Virol. 34 (1980) 297–302.

[26] M.J. Roossinck, A. Siddiqui, In vivo phosphorylation and protein analysis of hepatitis B virus core antigen, J. Virol. 61 (1987) 955–961.

[27] W. Liao, J.H. Ou, Phosphorylation and nuclear localization of the hepatitis B virus core protein: significance of serine in the three repeated SPRRR motifs, J. Virol. 69 (1995) 1025–1029.

[28] A. Machida, H. Ohnuma, F. Tsuda, A. Yoshikawa, Y. Hoshi, T. Tanaka, S. Kishimoto, Y. Akahane, Y. Miyakawa, M. Mayumi, Phosphorylation in the carboxyl-terminal domain of the capsid protein of hepatitis B virus: evaluation with a monoclonal antibody, J. Virol. 65 (1991) 6024–6030.

[29] Y.T. Lan, J. Li, W. Liao, J. Ou, Roles of the three major phosphorylation sites of hepatitis B virus core protein in viral replication, Virology 259 (1999) 342–348.

[30] B. Rabe, A. Vlachou, N. Panté, A. Helenius, M. Kann, Nuclear import of hepatitis B virus capsids and release of the viral genome, Proc. Natl. Acad. Sci. USA 100 (2003) 9849–9854.

[31] V. Bichko, P. Pushko, D. Dreilina, P. Pumpen, E. Gren, Subtype *ayw* variant of hepatitis B virus. DNA primary structure analysis, FEBS Lett. 185 (1985) 208–212.

[32] O. Ouchterlony, The antigenic pattern of immunoglobulins, G. Mal. Infett. Parassitt. 18 (1966) 942–948.

[33] A. Kazaks, R. Balmaks, T. Voronkova, V. Ose, P. Pumpens, Melanoma vaccine candidates from chimeric hepatitis B core virus-like particles carrying a tumor-associated MAGE-3 epitope, Biotechnol. J. 3 (2008) 1429–1436.

[34] P. Pushko, M. Sallberg, G. Borisova, U. Ruden, V. Bichko, B. Wahren, P. Pumpens, L. Magnius, Identification of hepatitis B virus core protein regions exposed or internalized at the surface of HBcAg particles by scanning with monoclonal antibodies, Virology 202 (1994) 912–920.

[35] M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.

[36] J. Freivalds, A. Dislers, V. Ose, D. Skrastina, I. Cielens, P. Pumpens, K. Sasnauskas, A. Kazaks, Assembly of bacteriophage Qβ virus-like particles in yeast *Saccharomyces cerevisiae* and *Pichia pastoris*, J. Biotechnol. 123 (2006) 297–303.

[37] J. Freivalds, J. Rūmnieks, V. Ose, R. Renhofa, A. Kazāks, High-level expression and purification of bacteriophage GA virus-like particles from yeast *Saccharomyces cerevisiae* and *Pichia pastoris*, Acta Univ. Latviensis ser. Biol. 745 (2008) 75–85.

[38] A. Dislers, I. Sominska, A. Kazaks, I. Petrovskis, I. Zarina, Expression system for producing recombinant virus-like particles. Patent LV 13587 (B) (2008) IPC1-7: A61K39/29; C12N15/71.

[39] A. Kazaks, J. Freivalds, P. Pumpens, A. Dislers, Recombinant strain of yeast *Pichia pastoris* for obtaining nucleocapsids of human hepatitis B virus (subtype ayw). Patent LV 13740 (B) (2008) IPC1-7: C07K14/39; C12N15/09.

[40] B. Tolner, L. Smith, R.H. Begent, K.A. Chester, Production of recombinant protein in *Pichia pastoris* by fermentation, Nat. Protoc. 1 (2006) 1006–1021.

[41] M. Naito, K. Ishii, Y. Nakamura, M. Kobayashi, S. Takada, K. Koike, Simple method for efficient production of hepatitis B virus core antigen in *Escherichia coli*, Res. Virol. 148 (1997) 299–305.

[42] M.Y. Ng, W.S. Tan, N. Abdullah, T.C. Ling, B.T. Tey, Heat treatment of unclarified *Escherichia coli* homogenate improved the recovery efficiency of recombinant hepatitis B core antigen, J. Virol. Methods 137 (2006) 134–139.

[43] K. Broos, P. Vanlandschoot, M. Maras, J. Robbens, G. Leroux-Roels, Y. Guisez, Expression, purification and characterization of full-length RNA-free hepatitis B core particles, Protein Expr. Purif. 54 (2007) 30–37.

[44] S.L. Zhou, D.N. Standring, Production of hepatitis B virus nucleocapsid like core particles in *Xenopus* oocytes: assembly occurs mainly in the cytoplasm and does not require the nucleus, J. Virol. 65 (1991) 5457–5464.

[45] M.Y. Ng, W.S. Tan, N. Abdullah, T.C. Ling, B.T. Tey, Direct purification of recombinant hepatitis B core antigen from two different pre-conditioned unclarified *Escherichia coli* feedstocks via expanded bed adsorption chromatography, J. Chromatogr. A 1172 (2007) 47–56.

[46] F. Birnbaum, M. Nassal, Hepatitis B virus nucleocapsid assembly: primary structure requirements in the core protein, J. Virol. 64 (1990) 3319–3330.

[47] T. Storni, C. Ruedl, K. Schwarz, R.A. Schwendener, W.A. Renner, M.F. Bachmann, Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic side effects, J. Immunol. 172 (2004) 1777–1785.

[48] S. Mukherjee, M.V. Thorsteinsson, L.B. Johnston, P.A. DePhillips, A. Zlotnick, A quantitative description of in vitro assembly of human papillomavirus 16 virus-like particles, J. Mol. Biol. 381 (2008) 229–237.

[49] S. Mukherjee, S. Kler, A. Oppenheim, A. Zlotnick, Uncatalyzed assembly of spherical particles from SV40 VP1 pentamers and linear dsDNA incorporates both low and high cooperativity elements, Virology 397 (2010) 199–204.

[50] B.J. Cohen, J.E. Richmond, Electron microscopy of hepatitis B core antigen synthesized in *E. coli*, Nature 296 (1982) 677–679.

[51] R.A. Crowther, N.A. Kiselev, B. Böttcher, J.A. Berriman, G.P. Borisova, V. Ose, P. Pumpens, Three-dimensional structure of hepatitis B virus core particles determined by electron cryomicroscopy, Cell 77 (1994) 943–950.

[52] H.Y. Kang, S. Lee, S.G. Park, J. Yu, Y. Kim, G. Jung, Phosphorylation of hepatitis B virus Cp at Ser87 facilitates core assembly, Biochem. J. 398 (2006) 311–317.

[53] L. Wittkop, A. Schwarz, A. Cassany, S. Grün-Bernhard, M. Delaleau, B. Rabe, C. Cazenave, W. Gerlich, D. Glebe, M. Kann, Inhibition of protein kinase C phosphorylation of hepatitis B virus capsids inhibits virion formation and causes intracellular capsid accumulation, Cell. Microbiol. 12 (2010) 962–975.

[54] M. Kann, B. Sodeik, A. Vlachou, W.H. Gerlich, A. Helenius, Phosphorylation-dependent binding of hepatitis B virus core particles to the nuclear pore complex, J. Cell Biol. 145 (1999) 45–55.