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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%

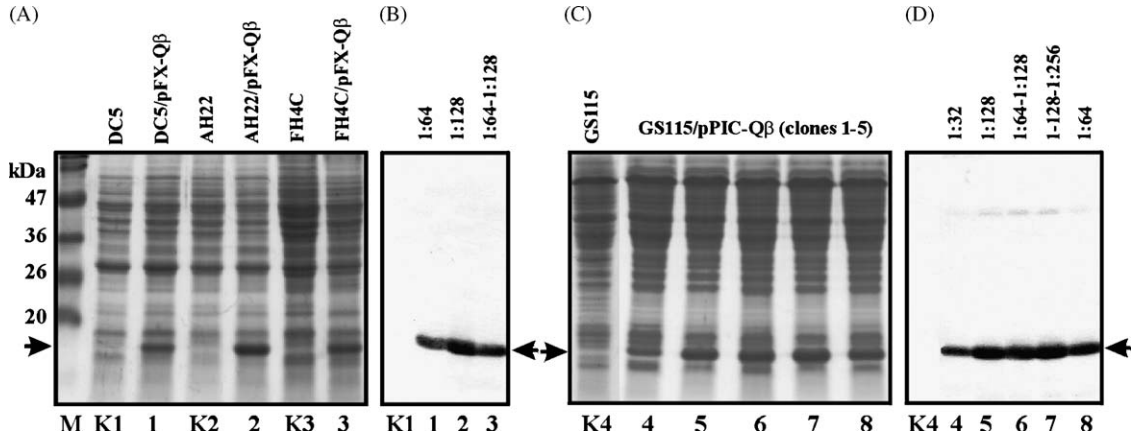

Fig. 1. Expression of Qβ CP in *S. cerevisiae* and *P. pastoris*, estimated by Coomassie-stained 15% SDS-PAGE (A and C) and Western blotting with rabbit polyclonal anti-Qβ antibody (B and D). K1, K2, K3 in (A) represent non-transformed *S. cerevisiae* DC5, *S. cerevisiae* AH22, and *S. cerevisiae* FH4C 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%

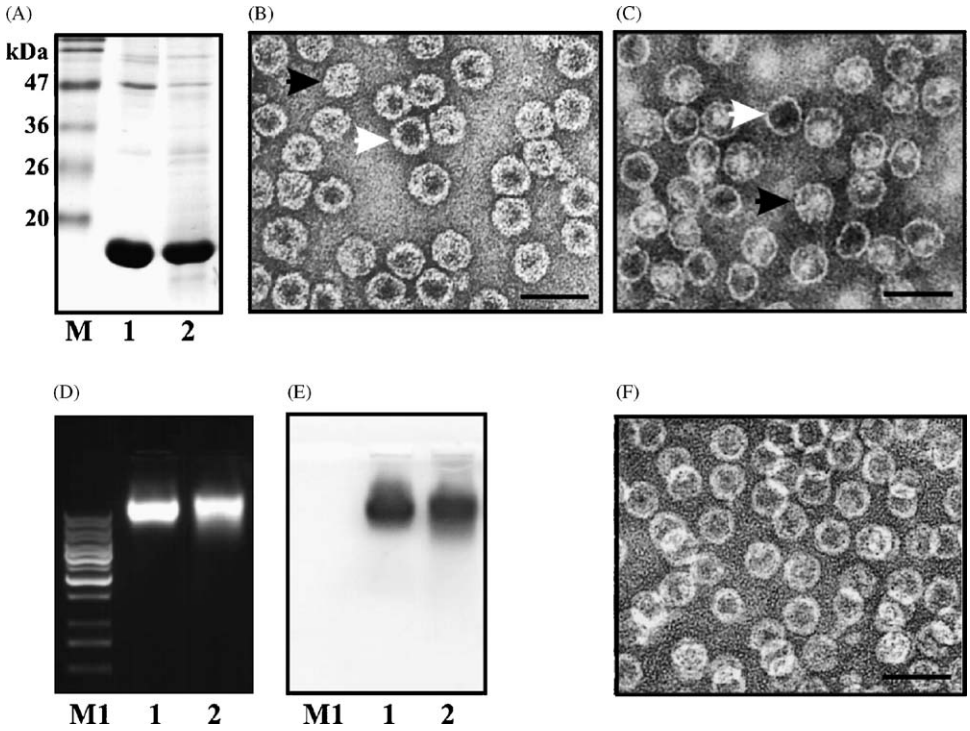

Fig. 2. Characterization of yeast-derived Qβ VLPs. Particles after purification from *S. cerevisiae* (lane 1) and *P. pastoris* (lane 2) were analyzed in Coomassie-stained 15% SDS-PAGE (A) by electron microscopy (B and C) and in native 1% agarose gel (D and E) stained with ethidium bromide (D) and subsequently 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

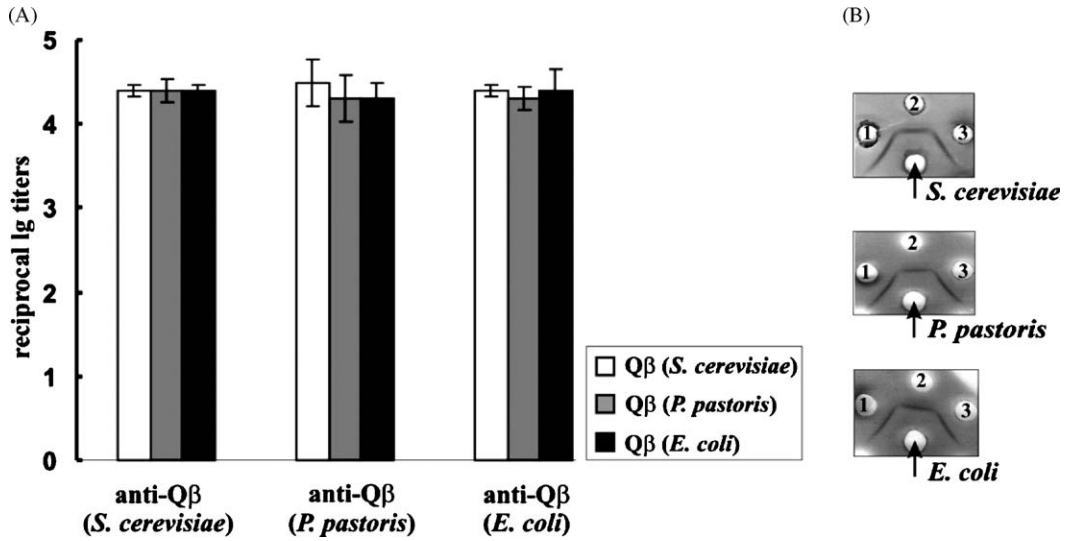

Fig. 3. Antigenicity and immunogenicity of Qβ VLPs. Reactivity of murine anti-Qβ sera with the recombinant Qβ VLPs from different hosts in direct ELISA (A) and in immunodiffusion according to Ouchterlony (B). ELISA plates were coated with particles derived from *S. cerevisiae* (open bars), *P. pastoris* (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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