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Effect of the DnaK chaperone on the conformational quality of JCV VP1 virus-like particles produced in *Escherichia coli*

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Abstract

Protein nanoparticles such as virus-like particles (VLPs) can be obtained by recombinant protein production of viral capsid proteins and spontaneous self-assembling in cell factories. Contrarily to infective viral particles, VLPs lack infective viral genome while retaining important viral properties like cellular tropism and intracellular delivery of internalized molecules. These properties make VLPs promising and fully biocompatible nano-vehicles for drug delivery. VLPs of *human JC virus (hJCV*) VP1 capsid protein produced in *Escherichia coli* elicit variable hemagglutination properties when incubated at different NaCl concentrations and pH conditions, being optimal at 200 mM NaCl and at pH rage between 5.8 and 7.5. In addition, the presence or absence of chaperone DnaK in *E. coli* cells influence the solubility of recombinant VP1 and the conformational quality of this protein in the VLPs.

The hemagglutination ability of *hJCV* VP1 VLPs contained in *E. coli* cell extracts can be modulated by buffer composition in the hemagglutination assay. It has been also determined that the production of recombinant *hJCV* VP1 in *E. coli* is favored by the absence of chaperone DnaK as observed by Western Blot analysis in different *E. coli* genetic backgrounds, indicating a proteolysis targeting role for DnaK. However, solubility is highly compromised in a DnaK$^-$ *E. coli* strain suggesting an important role of this chaperone in reduction of protein aggregates. Finally, hemagglutination efficiency of recombinant VP1 is directly related to the presence of DnaK in the producing cells.

Keywords: *hJCV* VP1 VLps, Chaperone, Hemagglutination, Protein Nanoparticle.

Introduction

The Human JC polyomavirus (*hJCV*) belongs to the family *Polyomaviridae* and is widespread throughout the human population. It causes progressive multifocal leukoencephalopathy (PML) in immunocompromised individuals[1,2] and it has been also associated to cancer.[3] The capsid of the viruses of this family is composed of three structural proteins, VP1, VP2 and VP3. VP1 is the major capsid protein forming the outer shell of the virus shell through the spatial assembly of 72 VP1 pentamers. VP2 and VP3 are involved in the interaction between the core of the viral particle and the capsid and are essential in the virus life cycle.[4-7] However, capsids formed exclusively of VP1 (*hJCV* VP1 virus-like particles-VLPs) can be obtained in heterologous expression systems including *Escherichia coli*, yeasts, mammalian cells and insect cell-baculovirus expression systems.[8-14] An interesting feature of VLPs is their ability to self-assemble, which can be controlled experimentally allowing the internalization of dyes, nucleic acids, drugs or proteins in vitro.[10,15-17] In addition, VLPs can be functionalized with cell ligands allowing the specific delivery of the cargo to target cells.[18,19] Interestingly, many VLPs have been proved to elicit sustained immune response in vaccination regimes.[20] However, the administration of *hJCV* VP1 VLPs without adjuvant is not able to stimulate any immune response,[9] making this type of VLPs suitable as nanovehicles for biotechnological and nanomedical applications.

Expression of viral proteins of eukaryotic hosts in prokaryotic expression systems copes with several difficulties. On the one hand, the prokaryotic expression system lacks many of the eukaryotic posttranslational modifications such as glycosylation or the formation of disulfide bonds in the oxidizing cytosol. In addition, the finely tuned chaperone-protease pathways of the protein quality control system are believed to be limiting in expressing cells. In that sense, it has been demonstrated that overexpression or depletion of chaperones has a significant effect over recombinant protein yield and conformational quality, being these parameters not necessarily coincident. Assembly of polyomavirus VLPs both in vivo and in vitro seems to be favored by Hsp70 family chaperones in both eukaryotic and prokaryotic systems and is inhibited by the presence of Hsp60 family chaperones.[21,22] In this work, we have studied the effect of the bacterial DnaK chaperone on the production, solubility and hemagglutination activity of *hJCV* VP1 VLPs, and the influence of this chaperone on the supramolecular organization of polyomavirus VLPs in vivo. DnaK is a folding chaperone that promotes the correct protein folding in cooperation with its co-chaperone DnaJ, and assisted by the nucleotide exchange factor GrpE.[23,24] The results obtained show that, in the presence of DnaK, the total amount of recombinant *hJCV* VP1 recombinant protein is negatively affected although the solubility and biological activity is significantly improved. Then, the increase in solubility is translated into an enhanced conformational quality as the protein obtained in DnaK$^-$ genetic background shows higher titer in hemagglutination assays.

Material and methods

hJCV VP1 gene cloning and E. coli strains

The *human JCV* VP1 gene (*Jvgp4*, NC_001699.1) was codon optimized for *E. coli* expression and provided by Geneart (Regensburg, Germany). The artificial gene (KF488587) was cloned into pTrc99A expression vector (Amersham Pharmacia Biotech, catalog no. 275007-01) by using *Nc*oI-*Bam*HI (Roche) restriction sites and transformed in *E. coli* DH5α strain (Invitrogen). Expression of *JCV* VP1 gene was under transcriptional control of the Isopropyl-beta-D-thiogalactopyranoside (IPTG) inducible promoter Ptrc.

Plasmid pTrc99a-VP1 was transformed by heat shock pulse into three different *E. coli* expression strains: the pseudo wild type MC4100 [F^- *araD139* Δ(*argF-lac*) *U169 rspL150 relA1 flbB5301 deoC1 ptsF25 rbsR,* $Strep^R$],[25] its $DnaK^-$ derivative JGT20 [MC4100 dnaK thr::tn10, $Step^R$, Tc^R, CGSC#: 6152][25] and MC4100 bearing pBB535[26] that harbours IPTG-inducible, $P_{A1/lac\text{-}O1}$-controlled *DnaK-J* chaperone genes.

hJCV VP1 production and purification

The transformed *E. coli* cells were cultured overnight in LB medium at 37ºC. The starter culture was then diluted at 1/20 with LB to a final volume of 500 ml and the mixture cultured again at 37ºC until the optical density at 550 nm reached 0.5. The gene expression was induced by the addition of IPTG to final concentration of 0.5 mM. Cells were incubated at 30ºC for 4 hours, and 65 ml of the culture were then separated and cells harvested (15,000g for 15 min at 4ºC), washed with phosphate buffered saline (PBS) and harvested again. Pellets were

suspended in 6.5 ml of PBS with EDTA-free protease inhibitor cocktail Complete (Roche, catalog no. 11873580001). Cells were disrupted by two 10 min-sonication cycles at 40 %-50 % amplitude with a Labsonic U sonicator, 8-mm probe (B. Braun Biotech International, Melsungen, Germany). Soluble and insoluble fractions were separated by centrifugation.

Protein detection was performed by Western blotting in protein samples resolved in 10 % SDS-PAGE transferred onto a nitrocellulose membrane. After blocking with 5 % milk O/N, the nitrocellulose membrane was incubated with mouse monoclonal to *Human Polyomavirus JCV* capsid protein VP1 primary antibody (Abcam, catalog no. ab34756) at a dilution of 1:1,500 in PBS. For detection, a 1:2000 diluted goat anti-mouse IgG (H+L)-HRP conjugate antibody (Bio-Rad) was used. Recombinant *hJCV Polyomavirus* Major Capsid VP1 Protein (Abcam, catalog no. ab74569) was used as protein standard in protein quantification experiments; for that, protein samples were loaded containing increasing amounts of recombinant protein: 15 ng, 30 ng, 60 ng, 90 ng, 120 ng and 240 ng. The protein amount ($\mu g\ ml^{-1}$ culture was normalized according to optical density at 550 nm).

Hemagglutination assays.

Detection of red blood cell (RBC) hemagglutination was performed by mixing 0.1 μg of *hJCV* VP1 (obtained from *E. coli* lysates and adjusted to a final volume of 50 μl with PBS) with 50 μl of 0.5% chicken RBCs, kindly provided by Gerard Eduard Martin Valls from CReSA (Universitat Autònoma de Barcelona), in a serial two fold dilution in 96 well plates. Samples were incubated for

approximately 48 h at 4ºC and then observed. Different buffers were used in hemagglutination assays: Tris 20 mM, 0/40/110/150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH7.5 and also Tris 20 mM, 200 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 5.8/7.5/9. The hemagglutination titer for each sample has been calculated as the median value from three different plates taking into account that 50 μl (containing 0.1 μg of protein) is the initial volume. Briefly, the individual titer for each point in the data set corresponds to the highest dilution factor that produced a positive reading. This value is then corrected to ml by a factor of 20 giving HA Units/ml.

Transmission electron microscopy (TEM)

E. coli MC4100/pBB535 soluble cell fraction was loaded on 40 % sucrose cushion and centrifuged at 100,000 g at 4°C for 4h (Beckman SW27 swinging bucket rotor). Resulting pellet was dissolved in Tris 20 mM, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH7.5 buffer. Sample was applied on carbon coated grids and negatively stained with uranyl acetate 2 % [w/v] aqueous solution. Photographs were taken with JEM-1400 transmission electron microscope at 250000 X nominal magnification.

Statistical analysis.

Significance of differences between means of recombinant protein production in the insoluble, soluble and total cell fractions were evaluated by a Student's t-test. Each experiment was performed in triplicate in intra and inter experiments. Briefly, each data point presented in the graphs corresponds to the results

obtained in three independent experiments and each experiment was performed in triplicate. Therefore, nine different measures have been analized for each data point.

Results and discussion

In vitro stabilization of VP1 VLPs produced in wild type E. coli

Production of recombinant *human JCV* VP1 has been described in several expression systems including *E. coli*, yeast, insect cells-baculovirus and mammalian cells. In all cases, the presence of the VLPs has been demonstrated by using the hemagglutination assay or by transmission electron microscopy.[8,9,12,14] In this study we wanted to determine the effect of the incubation buffer on the efficiency of VP1 VLPs assembly in cell extracts. For that reason, chicken red blood cells were incubated with VP1 VLP containing samples produced in an *E. coli* wild type genetic background under different buffer conditions (Fig. 1a). It has been described the importance of the presence of Ca^{2+} and oxidizing conditions in the stability of the SV40 and *hJCV* VLPs. In fact, the formation of the VLPs depends on the interaction of Ca^{2+} within the GH loop and probably on the presence of disulfide bonds between CD loops of neighboring capsomeres.[8] Therefore, a buffer containing Ca^{2+} and Mg^{2+} was selected and different monovalent ion concentration and pH conditions were tested regarding the stability of *hJCV* VP1 VLPs. The results show that the presence of NaCl is a favoring factor, being optimal at 200 mM. At higher concentrations (1 M), a clear interference with the hemagglutination assay was detected (Fig. 1b). The dependence in monovalent ions on the assembly of VP1 VLPs has been also demonstrated for SV40 VP1 in insect cell nuclear extracts and it seems to be related to the presence of cellular factors.[27] The influence of pH was also tested showing better hemagglutination performance at pH ranging

from 5.8 to 7.5 in the 200 mM NaCl containing buffer (Fig. 1c). VP1 VLPs stability was compromised at higher pH values in the same buffer. Consequently, the hemagglutination buffer used in subsequent experiments contained 200 mM NaCl and it was adjusted to pH=7.5.

hJCV VP1 expression in diverse E. coli DnaK genetic backgrounds

The production of recombinant proteins in heterologous expression systems provokes a stress situation in the cell factory leading, in most cases, to the accumulation of the protein of interest in insoluble structures known as inclusion bodies (IBs). The formation of IBs in expression experiments is enhanced by the limitation of the chaperone/protease system and therefore, the increase in the amount of chaperones has been used as a strategy to overcome this limitation with the final aim to increase the amount of recombinant protein in the cellular soluble fraction.[28,29] In summary, the results obtained so far by using several chaperone cocktails suggest that, overall; solubility takes priority over conformational quality. Consequently, the more recombinant protein is present in the soluble fraction the less biological activity is retained.[30-32] However, most of the studies performed so far are related to proteins that are not able to form protein complexes and only few of them describe the influence of chaperones in the production of protein complexes as VLPs.[21] Therefore, in order to gather information related to the influence of chaperones on the production of protein complexes, we have studied the effect of the presence of DnaK, a bacterial chaperone of the Hsp70 family on the production of JCV VP1. We transformed

wild type *E. coli* MC4100, *E coli* MC4100 DnaK$^-$ (JGT20) and *E. coli* MC4100/pBB535 with an expressing vector coding the VP1 *hJCV* gene and induced its expression under standard conditions. The total amount of recombinant protein was significantly higher in Dnak$^-$ *E. coli* strain, and the presence of DnaK in MC4100 had a profound negative effect on protein yield (Fig. 2a). In addition, the fourfold increase in DnaK concentration in MC4100/pBB535 *E. coli* strain[33] did not promote further reduction in protein production. This result might indicate a limited access of overexpressed DnaK to available DnaJ and GrpE cellular levels. However, when soluble and insoluble cellular fractions were individually analyzed, wild type MC4100 and DnaK overexpressing MC4100/pBB535 *E. coli* cells presented higher soluble/insoluble protein ratio and contained significantly more soluble protein than *E coli* MC4100 DnaK$^-$ (JGT20) (Fig. 2b). Under the tested experimental conditions, expression of the chaperone DnaK enhances the solubility of VP1 *hJCV* in the cell although it also induces a reduction in protein yield.

Hemagglutination assays of recombinant hJCV VP1 VLPs produced in E. coli.

The effect in biological activity of *hJCV* VP1 VLPs produced in the presence of different amounts of DnaK was determined by analyzing their ability to hemagglutinate red blood cells (Fig. 3a). In those experiments, the same amount of recombinant *hJCV* VP1 was used and VP1 concentration in cell extracts was calculated by Western blot using standard VP1. The used incubation buffer was

the optimized reaction buffer selected in the stabilization experiments described above. In those experiments, an *E. coli* cell extract lacking VP1 has been used as negative control. The hemagglutination assay showed that in all three *E. coli* genetic backgrounds, VP1 forms VLPs that can hemagglutinate RBC. In addition, the presence of 42-45 nm particles obtained after ultracentrifugaction of *E. coli* MC4100/pBB535 cell extracts confirmed the formation of VLPs under experimental conditions (Fig. 3b). However, the presence of DnaK has a positive effect on the ability of VP1 VLPs to hemagglutinate RBC indicating a direct effect of DnaK on the correct assembly of these protein supramolecular assemblies. In addition, it can be seen that when comparing hemagglutination activity on DnaK containing lysates the overexpression of DnaK had no effect (Fig. 3c).

Conclusions

Stability of VLPs depends on several factors as oxidative environment and divalent ion concentration among others. It has been demonstrated that pH and monovalent cations are also relevant for the correct assembly of *hJCV* VP1 as they might collaborate in van der Waals interactions.[34]

Cellular protein quality control is accomplished by the coordinated action of chaperones and proteases acting on nascent polypeptides for protein folding and holding, sending folding reluctant intermediates to the proteolysis pathway or accumulating them in dynamic protein based structures (IBs) for further processing through either folding or proteolysis. The general outcome of this process is improvement on protein solubility, defined as the portion of protein

present in the soluble fraction of the cell. In this work, it has been demonstrated the positive effect on solubility observed in the presence of DnaK chaperone in recombinant *hJCV* VP1 production. In addition, saturation in solubility seems to be reached at DnaK concentration obtained in wild type *E. coli* genetic background since fourfold higher concentration of DnaK in MC4100/pBB535 is not translated in an increase VP1 solubility (Fig. 3b). In addition, the presence of DnaK seems to target *hJCV* VP1 to proteolysis pathway since production of VP1 is dramatically reduced in MC4100 and MC4100/pBB535 *E. coli* strains. Production of VP1 of related polyomavirus simian virus 40 (SV40) in *E. coli* is also dependent on the presence of chaperones although in this case, the presence of DnaK produces an increase in the yield of SV40 VP1.[21] In this study, the different induction conditions used might account for the dissimilar results (2 h at 30ºC instead of 4 h at 30ºC). On the other hand, in eukaryotic expression systems, heat shock cognate protein 70 (hsc70), one of the Hsp70 family member, is involved in the in vivo assembly of polyomavirus VP1 VLPs in the nuclei while in prokaryotes DnaK, one of the Hsp70 family prokaryotic counterparts, recognizes the C-terminus of VP1 and enhances VP1 assembly.[17,21] Therefore, the positive effect on hemagglutination efficiency in DnaK producing *E. coli* strains is in accordance with direct interaction of DnaK with recombinant *hJCV* VP1. This result contrasts with the production of recombinant proteins that do not form supramolecular structures. In this scenario, it has been demonstrated that biological activity is not necessarily favored when gaining solubility. In fact, a wide spectrum of soluble species can be found in the

soluble fraction of induced cells.[21,35] This study reveals the positive impact of DnaK on solubility and conformational competence of multiprotein complexes formed by *hJCV* VP1 which give priority to protein yield that is negatively affected in a DnaK-positive genetic background.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure 1. Hemagglutination assays of 0.1 μg of *hJCV* VP1 VLPs obtained in *E. coli* soluble cell fraction under different NaCl concentrations (A). Hemagglutination assays of *hJCV* VP1 VLPs obtained in the *E. coli* soluble cell fraction under different pH conditions in 200 mM NaCl in Tris buffer (B). Control wells show the hemagglutination activity of a negative *E. coli* cell extract under different buffering conditions. Effect of NaCl concentration on red blood cell precipitation in the absence of *E. coli* cell extract (C).

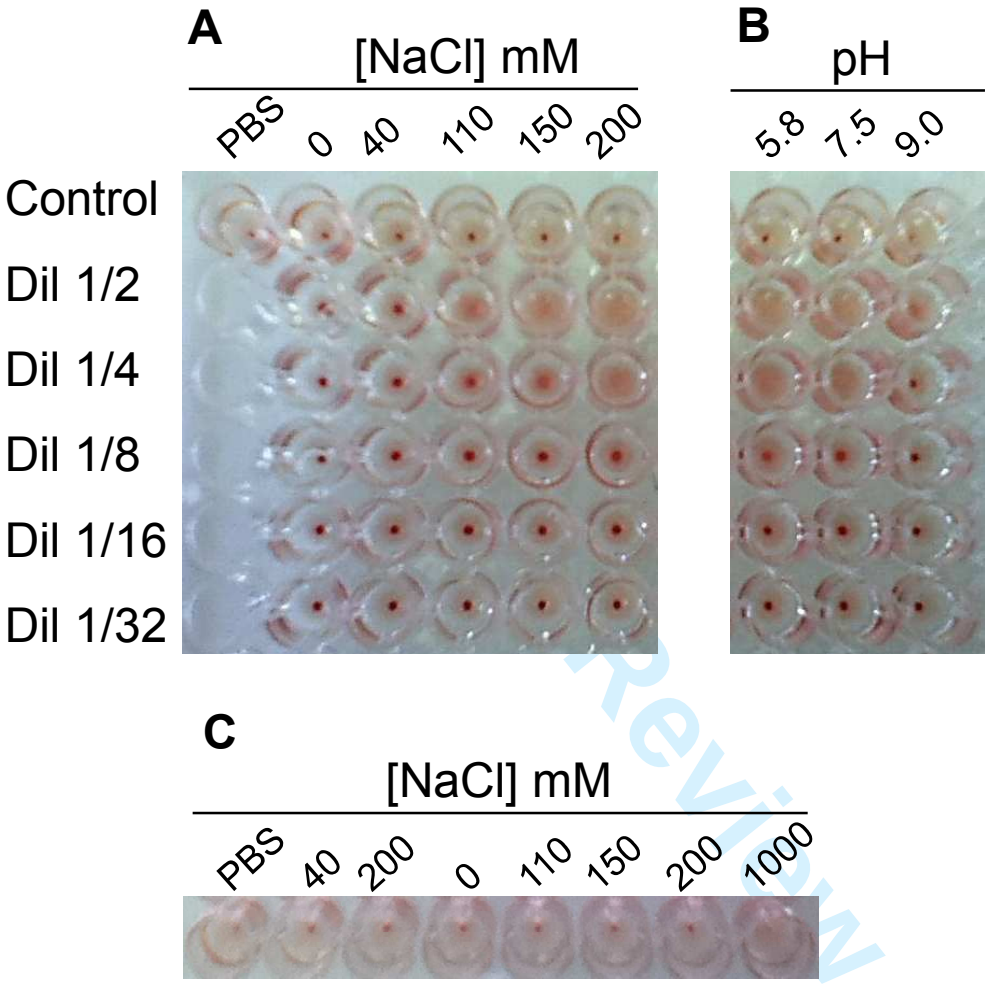
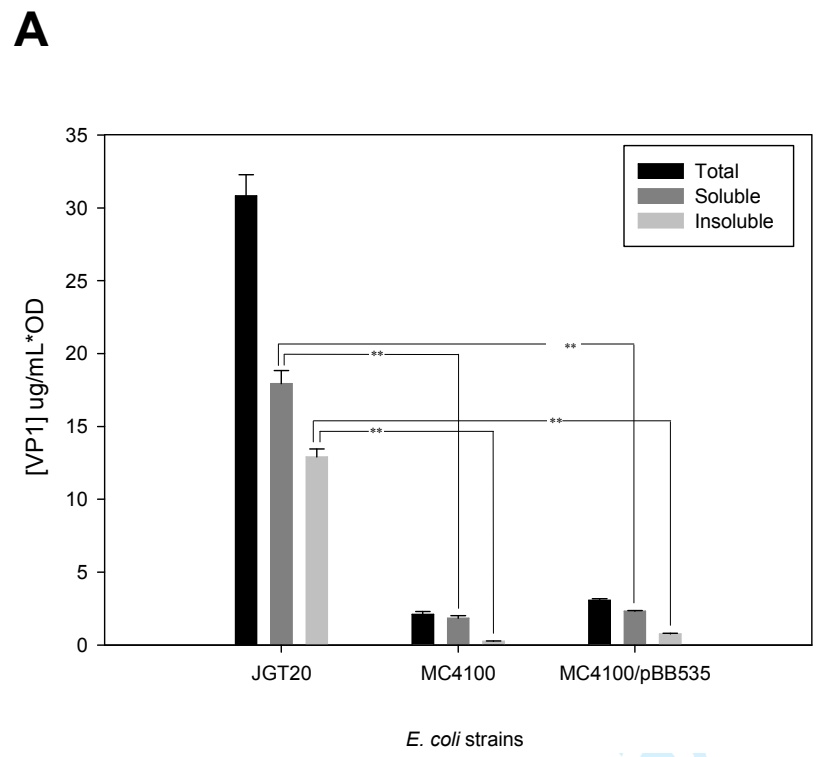


Figure 2. Protein quantification by Western blot of JCV VP1 VLPs in total, soluble and insoluble cell fractions using commercial VP1 as standard (A). Relative JCV VP1 VLPs presence in soluble cell fractions (B). Asterisks indicate significant differences when comparing with any other data group (*p<0.05; **p<0.01 and ***p<0.001). The amount of protein was normalized according to OD_{550nm}.



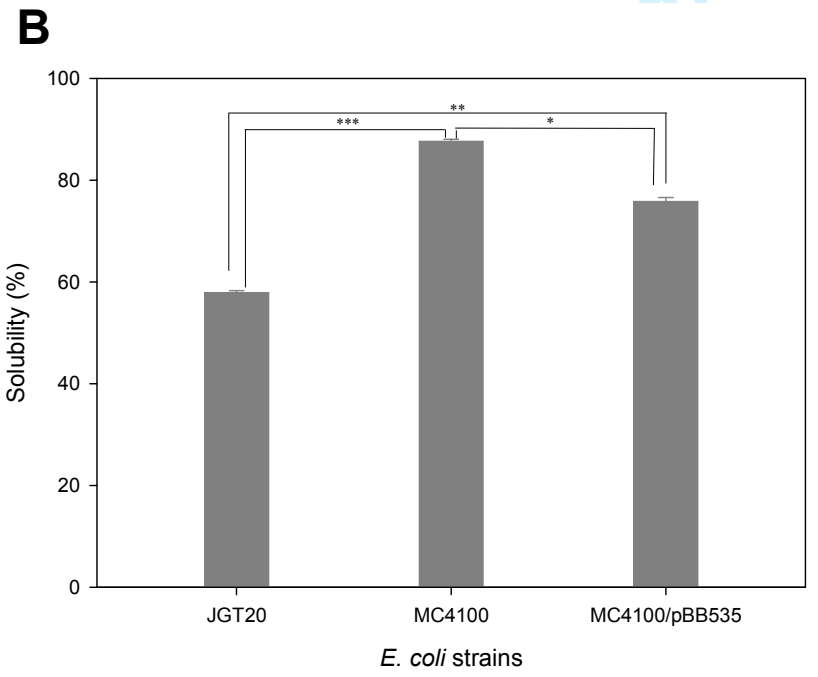
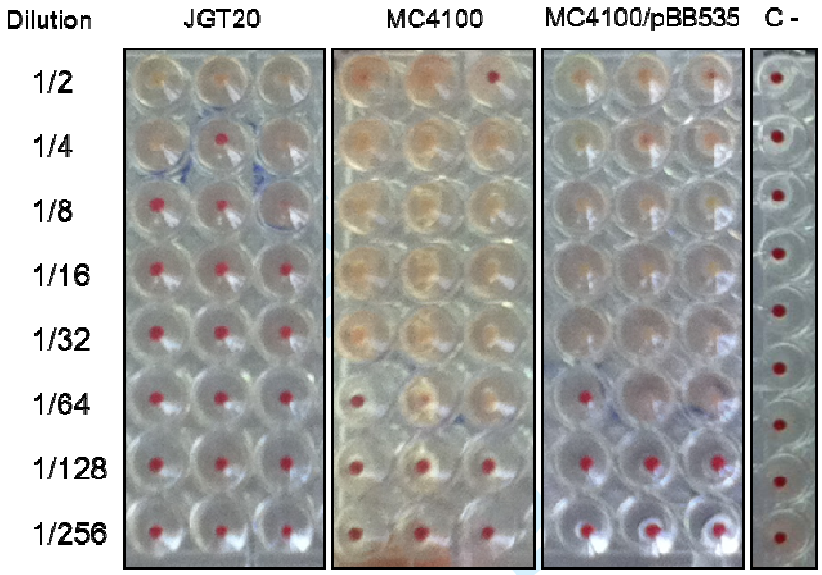


Figure 3. Hemagglutination assay of JCV VP1 containing *E. coli* lysates (A). Negative control wells (C-) correspond to MC4100 *E. coli* strain lysates lacking JCV VP1. Quantification of HA activity of 0.1 μg of *hJCV* VP1 VLPs obtained in different *E. coli* genetic backgrounds (B). Purified JCV VP1 obtained from *E. coli* MC4100/pBB535 induced cell lysates (C).

A



B

E. coli strain	HA titre (Units/mL)
JGT20	133±27
MC4100	1067±213
MC4100/pBB535	1067±213

C