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APPLIED MICROBIOLOGY AND BIOTECHNOLOGY

Production of functional inclusion bodies in endotoxin-free *Escherichia coli*

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Abstract

Escherichia coli is the workhorse for gene cloning and production of soluble recombinant proteins in both biotechnological and biomedical industries. The bacterium is also a good producer of several classes of protein-based self-assembling materials such as inclusion bodies (IBs). Apart from being a relatively pure source of protein for *in vitro* refolding, IBs are under exploration as functional, protein-releasing materials in regenerative medicine and protein replacement therapies. Endotoxin removal is a critical step for downstream applications of therapeutic proteins. The same holds true for IBs as they are often highly contaminated with cell-wall components of the host cells. Here we have investigated the production of IBs in a recently developed endotoxin-free *E. coli* strain. The characterization of IBs revealed this mutant as a very useful cell factory for the production of functional endotoxin-free IBs that are suitable for the use at biological interfaces without inducing endotoxic responses in human immune cells.

Introduction

Recombinant proteins produced in the Gram-negative bacterium *Escherichia coli* tend to be deposited as insoluble protein clusters known as inclusion bodies (IBs). These protein particles, ranging from 50 to around 500 nm in diameter, are emerging protein materials with intriguing biomedical applications (Garcia-Fruitos et al. 2011; Villaverde et al. 2012; Villaverde 2012), that can be regarded as biomimetics of functional amyloids (protein repositories for sustained release) acting in the endocrine system (Maji et al. 2009; Badtke et al. 2009; Mankar et al. 2011; Cano-Garrido et al. 2013). Bacterial IBs show high biological activity (Garcia-Fruitos et al. 2005; Gonzalez-Montalban et al. 2007; Garcia-Fruitos et al. 2012), spontaneous penetrability in mammalian cells (Vazquez et al. 2012) and also mechanical and biological stability (Diez-Gil et al. 2010; García-Fruitós et al. 2009), which has allowed to adapt them as nanostructured materials for surface decoration in tissue engineering (García-Fruitós et al. 2009; Seras-Franzoso et al. 2013b; Tatkiewicz et al. 2013; Diez-Gil et al. 2010; Seras-Franzoso et al. 2011; Seras-Franzoso et al. 2012; Seras-Franzoso J et al. 2013) and in the context of top-down and bottom-up protein replacement/delivery cell therapies (Vazquez et al. 2012; Cano-Garrido et al. 2013; Talafova et al. 2013; Liovic et al. 2012). However, these applications require the bacterial product being free of *E. coli*-derived lipopolysaccharide (LPS) that is capable of inducing an endotoxic immune response in humans and other mammals. If present, these bacterial contaminants can severely restrict the biomedical applicability of the material. So far, the efforts addressed to obtain pure IBs have been focussed on the development of optimized cell-disruption methods for the isolation of these sub-micron particles lacking bacterial cells (Rodriguez-Carmona et al. 2010). However, the preparation of cell-free IBs, while preventing further bacterial contamination, does not guarantee the absence of free or IB-associated LPS. In fact, cell-wall contaminants are believed to associate with IBs during the disruption and separation processes (Georgiou and Valax 1999). In the present study, we have therefore addressed the question of functional IB production in a recently developed endotoxin-free *E. coli* strain, in order to overcome limitations caused by the potential presence of endotoxin in IB preparations.

Material and methods

Strains, media and plasmids

The *E. coli* K-12 strains used in this study were MC4100 ([*araD139*], (*argF-lac*)169, λ- *relA1*, *rpsL150*, *rbsR22*, *flb5301*, *deoC1*, *pstF25* Strep^R), BW30270 (CGSC#7925 - MG1655; F$^-$, *rph*$^+$, *fnr*$^+$) and its isogenic endotoxin-free strain KPM335 (*msbA52*, Δ*gutQ*, Δ*kdsD*, Δ*lpxL*, Δ*lpxM*, Δ*pagP*, Δ*lpxP*, Δ*eptA*, *frr181*). KPM335 will be described in detail elsewhere. All these strains were transformed with pTr99a-VP1GFP (Ap^R) encoding the fusion protein VP1GFP, an aggregation-prone fluorescent protein previously used as a reporter to study IB formation (Garcia-Fruitos et al. 2007). Both transformed and plasmid-free *E. coli* strains were cultured in Luria-Bertani (LB Miller) media (J.Sambrook et al. 1989).

Competent cells

Overnight cultures of MC4100 and BW32070 grown at 37ºC and 250 rpm were used as a 1/100 inoculum in 50 ml of LB. When the cells reached the early exponential growth phase at an optical density (OD_{550}) between 0.2 - 0.4 at 37ºC, 25-ml aliquots of the cultures were centrifuged (4,000 x *g*) at 4 ºC for 15 min. Pellets were resuspended in 12.5 ml of cold and sterile 50 mM $CaCl_2$ and left for 45 min in an ice bath. Cells were centrifuged again under the same conditions as described above and resuspended in 1.25 ml of cold and sterile 50 mM $CaCl_2$. Finally, aliquots of 200 µl in $CaCl_2$ / glycerol (15 % v/v) were stored at -80ºC.

Electrocompetent cells

KMP335 cells were grown overnight at 37ºC and 250 rpm and diluted 1/50 in 80 ml of LB. The cells were grown to an OD_{550} between 0.2 - 0.4, placed on ice for 20 min and sedimented by centrifugation (3100 x *g*, 4 ºC, 20 min). The bacterial cells were washed successively in 40, 20 and 10 ml of H_2O (ice-cold and sterile), followed by an additional washing step in 5 ml of 10% glycerol (ice-cold and sterile), resuspension of the final cell sediment in 1 ml of 10% glycerol (ice-cold and sterile), and preparation of 50-µl aliquots for electroporation.

Transformation

Transformation of competent MC4100 and BW32070 cells was performed using 40 ng of plasmid DNA. The transformation mixtures were incubated on ice for 30 - 60 min and then warmed up to 42ºC for 45 sec. The mixtures were then immediately placed on ice for 30 sec, and 800 µl of LB were added, followed by incubation of the transformed cells at 37°C for 1 h. Cells were plated on LB-agar plates containing ampicillin (100 µg/ml) and incubated overnight at 37ºC. KPM335 cells were transformed by electroporation in a pre-chilled 0.2-cm gap electroporation cuvette using 50 µl of competent cells and 40 ng of plasmid DNA. Cells were pulsed using a Gene Pulser MX cell electroporator (Bio-Rad, Hercules, CA, USA) at 25 µF, 200 Ω, 2,500 V and 4.7 - 4.8 msec. Immediately after the pulse, 800 µl of LB medium were added, and the mixture was incubated at 37°C for 1 h. The culture was plated on LB-agar plates containing ampicillin (100 µg/ml) and incubated at 37°C overnight.

Protein production

VP1GFP was produced at three growth temperatures (37ºC, 25ºC and 16ºC). Overnight cultures were inoculated each in triplicate in three 250-ml shake flasks containing 50 ml of LB media with 100 µg/ml ampicillin (plus 30 µg/ml streptomycin in MC4100) and incubated at 37ºC and 250 rpm until the cells reached the mid-exponential growth phase between 0.5 and 0.6 OD_{550} units. Recombinant gene expression was then induced by the addition of IPTG to a final concentration of 1 mM. Cultures were then incubated for either 3h at 37ºC or overnight at 25°C and 16ºC. At the indicated times, 20 ml of each culture were harvested by centrifugation (10,000 x *g*, 4ºC, 10 min). The pellets were resuspended in 1 ml PBS containing the Complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, USA). Cells were disrupted by 5-min cycles and pulses of 0.5 seconds (power at 40 %) using a Lab Sonic ultrasonicator (B. Braun, Mesulgen, Germany). Each cell lysate was separated into two aliquots of 500 µl for protein quantification by Western blot analysis and fluorescence measurement. Aliquots of the IB samples were centrifuged (15,000 *g*, 4ºC, 15 min), and both the IB pellets and the supernatants were stored at -20ºC.

IB purification

Overnight cultures of MC4100, BW32070 and KPM335 grown at 37ºC and 250 rpm were used as a 1/100 inoculum in 500-ml of LB and VP1GFP IBs were produced at 37 ºC. After production, Lysozyme (1 μg/ml), phenylmethylsulfonyl fluoride (0.4mM) and EDTA-free protease inhibitor cocktail (Complete EDTA-free Roche Diagnostics, Indianápolis, USA) were added and cultures were incubated for 2 h at 37ºC and 250 rpm. Mixtures were stored at -80ºC. Cultures were thawed at room temperature, and 2 ml of Triton X-100 (Roche Diagnostics, Indianapolis, USA) were added and left at room temperature for 1 h. As a bacterial viability control, 100 µl of the culture were plated on LB-agar without antibiotics, and the samples were stored again at -80ºC. Freeze-thaw cycles were repeated until no colony was found on the plates. Once the absence of viable bacteria was confirmed, 125 µl of nonyl phenoxypolyethoxylethanol (NP-40) (Roche Diagnostics, Indianapolis, USA) were added, followed by incubation of the samples at 4ºC for 1 h. After incubation, $MgSO_4$ 0.6 M and DNase 0.6 μg/ml were added (Roche Diagnostics, Indianapolis, USA). The samples were incubated at 37ºC for 1 h with shaking and centrifuged (15,000 x *g*, 4ºC, 15 min). The pellets were subsequently resuspended with 25 ml of lysis buffer (50 mM Tris HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA containing 0.5% Triton X-100) containing 0.5 % Triton X-100 (Roche Diagnostics, Indianapolis, USA). 100 µl of each sample were plated on LB-agar without antibiotics, and the plates were incubated at 37ºC overnight. The samples were centrifuged again under the same conditions as described above. The pellets were resuspended in PBS, and 100 µl-aliquots were plated to monitor bacterial viability. Finally, the resuspended pellets containing the IBs were separated into 5-ml aliquots and sedimented again by centrifugation (15,000 x *g*, 4ºC, 15 min). The supernatants were discarded, and the pellets were stored at -80 ºC.

SDS-PAGE and Western blot analysis

Protein quantification was performed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels according to Laemmli's method (Laemmli 1970). For quantification, the GFP-H6 protein at 500, 250, 125, 75 and 37.5 ng (Vazquez et al. 2010) was used as a standard on each gel. Soluble protein samples were denatured by incubation at 98ºC for 5 minutes, whereas IBs were incubated for 40 min. For Western blot analyses, protein samples were electrotransferred to nitrocelulose membranes (GE Healthcare, Buckinghamshire, England) using 10% glycine, 20% methanol in H_2O as a transfer buffer. The membranes were blocked with 5% milk powder in PBS overnight, followed by

incubation of the membranes with anti-GFP antibody as recommended by the manufacturer (sc-8334, Santa Cruz Biotechnology, CA, USA). HRP-conjugated anti-rabbit IgG (H+L) antibody (Bio-Rad, Hercules, CA, USA) at a dilution of 1/2000 was used as a secondary antibody. The membranes were developed in a solution consisting of 25% cold methanol, 0.2% H_2O_2 and 0.65 mg/ml of 4-chloronaftol in PBS. Images of the membranes were obtained using a GS800 Calibrated Densitometer scanner (Bio-Rad, Hercules, CA, USA). The protein amount of the bands was analyzed from the standard curve fitting equation of GFP-H6 using the Quantity One software.

Determination of GFP fluorescence

To determine the fluorescence of GFP, IBs were resuspended in 1 ml PBS, whereas supernatants (0.5 ml of soluble protein) were mixed with 0.5 ml PBS. Measurements were performed in a fluorescence spectrometer Cary Eclipse (Varian, Mulgrave Australia) using 1-ml cuvettes at 450 nm of excitation wavelength and 510 nm of emission wavelength.

Statistical analysis

The data were analyzed using a GLM model under a completely randomized design with a factorial array of 4 x 3 (4 strains x 3 production temperatures) according to the following equation:

$Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha_i\beta j) + E_{ij}$

Where:

Y_{ij} = Protein quantity, fluorescence, specific fluorescence (soluble and IBs)

μ = Total mean of each variable

$\alpha_{i:}$ = Strain effect

β_j = Temperature effect

$(\alpha i\beta_j)$ = Interaction strain-temperature effect

$E_{ij=}$ Experimental Error

Moreover, a least square comparison test of each production was made for all variables.

Light microscopy

For visualization of bacterial cells and IBs, 1 ml of each sample was centrifuged at 10,000 x *g* and 4ºC for 10 min. Pellets were resuspended in 1 ml of 0.1 % formaldehyde in PBS. Images were examined at 100 x magnification in the dark field with fluorescence and phase contrast using a Leica DRMB microscope (Leica Microsystems, Wetzlar, Germany). Images were digitalized using the Leica LAS 3.0 software.

Scanning electron microscopy

Purified IBs were resuspended in PBS-Tween-20 buffer and filtered using a 0.2 µm Nucleopore Whatman membrane (GE Healthcare, Buckinghamshire, England). Membranes were fixed with 2.5 % glutaraldehyde-phosphate at 4ºC for 1 h. After fixing, the samples were dehydrated through sequential washes with ethanol (Sharlau Microbiologics, Barcelona, Spain) at increasing concentrations from 50 to 99 % during 15-30 min. Remaining Ethanol was evaporated in a K850 CDP desiccator (Emitech, Ashford, UK), and dry membranes were covered with gold using a Sputter Coater k550 (Emitech, Ashford, UK). Examination of the specimens was performed at accelerations between 0.5 and 30 kV using a Hitachi s-570 scanning electron microscope (Hitachi Ltd, Tokio, Japan).

Cell proliferation assay

Human skin fibroblast cells (1BR3.G) were grown at 37ºC and 10% CO_2 on IB-decorated surfaces. For that, 1 µg of VP1GFP IBs were added to each well of an untreated Costar 3370 plate and incubated with Dulbecco's Modified Eagle's Medium (DMEM) at 4ºC overnight. After incubation, the medium was replaced, and non-attached IBs were washed out three times using 200 ml of filtered and sterile PBS. 1BR3.G cells were added (5,000 cells per well) in DMEM medium with 2% of Fetal Bovine Serum (FBS). Plates were incubated at 37 ºC for 24, 48 and 72 hours, and the MTT proliferation cell assay was performed using the Nonradioactive Cell Proliferation and Cytotocity Assay Kit EZ4U as recommended by the manufacturer (Biomedica Medizinprodukte GmbH, Wien, Austria). Absorbance was measured in a multilabel

reader VICTOR3 V1420 (Perkin Elmer) at 450 nm (and 620 nm as reference), and the values were standardized with respect to the negative control.

IB internalization test

HeLa cells (60,000 cells per well) were seeded in treated 12 well plates (Nunclon surface, Nunc 150628) in the presence of MEM-α medium supplemented with 10% FBS and 2 mM Glutamax (Gibco, Rockville MD) . After incubation at 37ºC and 5% CO_2 for 24 h, the medium was removed, and the cells were washed with DPBS. Then, 5 µg of VP1GFP IBs were suspended in MEM-α containing 10% FBS and 2 mM Glutamax, and added per well. After 48h, cell samples were treated with trypsin (1 mg/ml) in DPBS for 15 min, and samples were analyzed on a FACSCanto system (Becton Dickinson) using a 15 W air-cooled argon-ion laser at 488 nm excitation for GFP. Fluorescence emission was measured with a 530/30 nm band pass filter

LPS detection assay

The LPS detection assay using tenfold serial dilution steps of VP1GFP IBs was performed with HEK-Blue™ hTLR4 cells in accordance with the specifications of the supplier of the cell lines (InvivoGen, Toulouse, France). HEK-Blue™ Null2, the parental cell line of HEK-Blue™ hTLR4, was used as a control.

Results

To investigate the formation of IBs by the aggregation-prone VP1GFP protein, wild-type and endotoxin-free *E. coli* strains carrying the pTr99a-VP1GFP plasmid were grown aerobically in LB medium containing 100 µg/ml ampicillin at 37°C, 25°C and 16°C. As shown in Figure 1, the optical densities at 550 nm (OD_{550}) of all cultures following induction of recombinant gene expression for three hours did not differ considerably from each other, although the endotoxin-free strain KPM335 consistently reached lower OD_{550} values than its parental wild-type strain BW30270 and the MC4100 control strain at all three temperatures.

Production and separation of VP1GFP into soluble and insoluble fractions revealed that the amounts of recombinant VP1GFP obtained from MC4100 and BW30270 were usually similar, but about twice the amount than in the endotoxin-free strain KPM335 at 37ºC (Fig. 2). In KPM335, the total amount of soluble VP1GFP was higher than in wild type strains. Strikingly, no insoluble protein was detected in KPM335 at 16ºC. The intensity of GFP fluorescence of both the soluble and the insoluble fraction (Fig. 3A) was in accord with the protein amount obtained from the *E. coli* strains (Fig. 2). As expected, specific fluorescence of insoluble VP1GFP was in general lower than that of the soluble protein, with very low levels in MC4100 and BW30270 at 37ºC (Fig. 3B). As a general trend, specific fluorescence of the soluble protein increased with lower culture temperatures, indicative of improved protein folding.

Interestingly, insoluble VP1GFP produced in KPM335 showed the highest specific fluorescence at each temperature when compared with the insoluble fraction from the wild-type strains (Fig. 3B), suggesting a higher conformational and, therefore, functional quality of IBs produced in the LPS-free strain. this fact could be of bionanotechnological relevance if in this strain, the insoluble protein is organized as inclusion bodies (IBs). IBs, as sub-micron protein particles, are gaining relevance in biotechnology (Garcia-Fruitos et al. 2012) and biomedicine (Vazquez and Villaverde 2013) because of their penetrability in mammalian cells in absence of toxicity and their ability to release therapeutic proteins inside receiving cells, in either substitutive protein therapies (Liovic et al. 2012; Talafova et al. 2013; Vazquez et al. 2012) or in tissue engineering (Seras-Franzoso J et al. 2013; Cano-Garrido et al. 2013; Seras-Franzoso et al. 2013b; Seras-Franzoso et al. 2013a). Interestingly, they act as natural biomimetics of hormone-releasing secretory granules of the endocrine system (Villaverde 2012), what offers a plethora of therapeutic opportunities.

The formation of fluorescent IBs in KPM335 was confirmed by light microscopy (Fig. 4C). Like IBs produced in MC4100 and BW30270 (Figs. 4A and 4B) and commonly observed in IB-producing bacteria (Villaverde and Carrio 2003), the IBs in KPM335 appeared as refractile particles adopting a polar distribution. The fluorescent intensity was also highest here in KPM335 (Fig. 4C) and, thus, in good agreement with measurements using fluorometry (Fig. 3B). However, the pseudo-spherical morphology and size between 300 and 500 nm of the IBs formed in MC4100, BW30270 and KPM335 were indistinguishable from each other (Fig. 5) and similar to IBs isolated from other *E. coli* strains (Garcia-Fruitos et al. 2010).

The endotoxic activity of the IBs produced in *E. coli* strains MC4100, BW30270 and KPM335 was investigated using the HEK-Blue™ hTLR4 LPS detection assay. As shown in Figure 6, the hTLR4 stimulating activity of IBs from KPM335 was of several orders of magnitude lower compared with the ability of IBs from the wild-type strains to stimulate the hTLR4 signaling pathway.

Apart from the highest specific fluorescent intensity and the extremely low endotoxic activity, IBs formed in KPM335 were indistinguishable from those obtained from its parental strain or the common MC4100 laboratory strain. However, we wanted to ensure that these particles still retained their ability to (i) mechanically stimulate the growth of mammalian cells when used as surface-decorating topographies in cell culture settings as described previously (García-Fruitós et al. 2009; Seras-Franzoso et al. 2013c; Diez-Gil et al. 2010; Tatkiewicz et al. 2013; Seras-Franzoso et al. 2013b), and (ii) release functional proteins when internalized by mammalian cells (Liovic et al. 2012; Vazquez et al. 2012; Seras-Franzoso J et al. 2013; Seras-Franzoso et al. 2013b). The comparative analysis of cell proliferation on IB-decorated surfaces revealed similar properties of all tested IBs (Figure 7). Noteworthy, the ability of KPM335 IBs to penetrate into mammalian cells with fluorescent activity was fully conserved (Fig. 8), thus confirming a similar molecular architecture, mechanical stability and cell penetrability of IBs from the endotoxin-free strain. In fact, penetrability of these IBs was slightly but significantly higher than that of conventional, wild-type IBs ($p<0.05$ when measuring fluorescence and $p<0.01$ when determining the percentage of uptaking cells). Taken together, IBs lacking endotoxic activity hold promise for their use in biomedical applications (Garcia-Fruitos et al. 2012).

Discussion.

E. coli is the most common cell factory for the production of recombinant proteins (Ferrer-Miralles and Villaverde 2013), including biopharmaceuticals for use in humans (Ferrer-Miralles et al. 2009). Like in other Gram-negative bacteria, the outer membrane of *E. coli* contains LPS as a main constituent that is released upon cell disruption to obtain intracellular products of biotechnological interest. In the human body, the LPS, also known as endotoxin, is a potent activator of inflammatory responses as it promotes the release of several of pro-inflammatory cytokines, which may cause high fever, severe tissue damage and death (Erridge et al. 2002). Therefore, many procedures for removal of LPS from recombinant protein preparations have been developed and adapted to specific processes to obtain products with different levels of purity (Magalhaes et al. 2007; Liu et al. 1997; Petsch and Anspach 2000). However, elimination of LPS requires considerable efforts and adds significant cost to downstream purification steps of proteins.

Lately, *E. coli* and other cell factories are being used to produce protein products of potential use in medicine, that are more complex than soluble proteins, including phage components, virus like particles and diverse nanostructured protein materials such as fibers, regular nanoparticles and IBs (Rodriguez-Carmona and Villaverde 2010; Vazquez and Villaverde 2013; Neus Ferrer-Miralles et al. 2013). Being in general considered as waste materials in protein production processes, bacterial IBs have gained interest as they are formed by significant fractions of properly folded, functional polypeptides (Garcia-Fruitos et al. 2005; Garcia-Fruitos et al. 2012; Gonzalez-Montalban et al. 2007). Due to their mechanical stability, the ability to penetrate mammalian cells in the absence of cellular damage and the release of functional protein, in a way similar to the release of functional hormones from amyloid repositories (Villaverde 2012), bacterial IBs became unexpectedly promising materials in drug delivery and in regenerative medicine (Garcia-Fruitos and Villaverde 2010; Liovic et al. 2012; Talafova et al. 2013; García-Fruitós et al. 2009; Seras-Franzoso et al. 2012; Seras-Franzoso J et al. 2013; Seras-Franzoso et al. 2013c; Seras-Franzoso et al. 2013b; Seras-Franzoso et al. 2013a; Tatkiewicz et al. 2013; Vazquez et al. 2012; Villaverde et al. 2012; Villaverde 2012). Although not determined quantitatively, contamination of IBs with bacterial LPS is a result of cell debris formation during cell disruption and separation (Neubauer et al. 2006; Georgiou and Valax 1999). Being highly porous and structurally more complex than soluble protein, LPS removal from IBs would necessarily pose technical problems. Here we have explored, as an alternative to commonly used LPS removal methods, the biofabrication of functional

IBs in a newly developed endotoxin-free *E. coli* strain. We were particularly interested in whether these IBs produced in an endotoxin-free *E. coli* strain would maintain their mechanical and biological properties as the bacterial LPS could be a structural component of IBs supporting their key properties as functional biomaterials.

Here we show that, although the endotoxin-free strain KPM335 grew slower than the control strains (Fig. 1), the yield of the model protein produced in KPM335 was even slightly higher compared with the wild-type controls (Fig. 2). Remarkably, the specific fluorescence of VP1GFP deposited as IBs in KPM335 was significantly higher than in the wild-type strains (Fig. 3). This could be due to the lack of normal LPS as a contaminant in the material, which may result in a better protein folding with reduced quenching of fluorescence. Also, IBs from KPM335 adopted the pseudo-spherical geometry common in the material (Garcia-Fruitos et al. 2010), being morphologically indistinguishable from standard particles (Figs. 4 and 5). In the same context, endotoxin-depleted IBs used as topographies for mammalian cell proliferation (Seras-Franzoso et al. 2013c) stimulated, as conventional IBs, cell spread (Fig. 7). This fact indirectly indicates that the LPS-free material has the same surface chemical properties and mechanical stability as wild-type protein particles and that LPS is not a structural element significantly contributing to the mechanical stability of the bacterial amyloids. Interestingly, the capability of LPS-free IBs to penetrate mammalian cells is slightly but significantly higher than that of the control material (Fig. 8B). Irrespective of the precise mechanism, this fact, together with their higher specific fluorescence (Fig. 3B), points out LPS-free IBs as valuable biocompatible materials for the delivery of protein drugs. On the other hand, among all bacterial cell factories (Ferrer-Miralles and Villaverde 2013) and non-bacterial protein production hosts developed thus far (Corchero et al. 2013), endotoxin-free *E. coli* strains seem to be an appealing alternative to minimize the biological risk of contaminations in the final products. This is especially relevant in the context of new systems biotechnology tools for this species (Lee et al. 2012) that further develops to become a true industrial factory, and makes biological fabrication of soluble proteins and nanostructured materials highly competitive with synthetic chemistry (Chen 2012; Vazquez and Villaverde 2013).

In the context of potential applications of IBs in drug delivery either *in vitro* for regenerative medicine (Seras-Franzoso J et al. 2013), *in vivo* in oral administration (Vazquez et al. 2012) or envisaging transdermal or cell-targeted delivery (Talafova et al. 2013; Liovic et al. 2012), endotoxin-free IBs hold promise for the development towards a powerful tool that ensures both functionality and additional biosafety, being particularly attractive regarding cost-effective production and regulatory issues.

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Figure legends

Figure 1. Optical densities at 550 nm (OD_{550}) of the cell suspensions of *E. coli* strains MC4100/pTr99a-VP1GFP, BW30270/pTr99a-VP1GFP and KPM335/pTr99a-VP1GFP 3h post-induction of *VP1GFP* gene expression at different temperatures (37ºC, 25ºC and 16ºC).

Figure 2. Amount of soluble and insoluble VP1GFP produced in *E. coli* strains MC4100/pTr99a-VP1GFP, BW30270/pTr99a-VP1GFP and KPM335/pTr99a-VP1GFP 3 h post-induction of *VP1GFP* gene expression at different temperatures.

Figure 3. Green fluorescence of soluble and insoluble fractions in VP1GFP-producing *E. coli strains* MC4100, BW30270 and KPM335 (A) and specific fluorescence of soluble and insoluble VP1GFP produced in MC4100, BW30270 and KPM335 (B) at different temperatures.

Figure 4. Light microscopy images of VP1GFP-producing *E. coli* strains MC4100 (A), BW30270 (B), and KPM335 (C) 3h post-induction. Left to right: phase contrast microscopy at 100 X magnification; fluorescence microscopy at 100 X magnification; merged images using Image J software.

Figure 5. Scanning electron micrographs of VP1GFP IBs isolated from *E. coli* strains MC4100 (left), BW30270 (middle) and KPM335 (right).

Figure 6. Stimulation of the hTLR4 signaling pathway by IBs produced in *E. coli* strains MC4100, BW30270 and KPM335. Relative NF-κB induction was measured following stimulation of HEK-Blue hTLR4 cells with tenfold serial dilutions of IBs.

Figure 7. Proliferation of 1BR3.G cells on matrices formed by IBs from *E. coli* strains MC4100, BW30270 and KPM335 at 37ºC monitored at several times after seeding.

Figure 8. Internalization of IBs by HeLa cells. The HeLa cells were incubated with 5 µg of VP1GFP IBs from *E. coli* strains BW30270 and KPM335. Internalization of IBs was monitored by measuring the intensity of fluorescence inside the cells (A) and the fraction of fluorescent cells (B).

Reference List

Badtke MP, Hammer ND and Chapman MR. (2009). *Sci Signal,* **2,** e43.

Cano-Garrido O, Rodriguez-Carmona E, ez-Gil C, Vazquez E, Elizondo E, Cubarsi R, Seras-Franzoso J, Corchero JL, Rinas U, Ratera I, Ventosa N, Veciana J, Villaverde A and Garcia-Fruitos E. (2013). *Acta Biomater,* **9,** 6134-6142.

Chen GQ. (2012). *Microb Cell Fact,* **11,** 111.

Corchero JL, Gasser B, Resina D, Smith W, Parrilli E, Vazquez F, Abasolo I, Giuliani M, Jantti J, Ferrer P, Saloheimo M, Mattanovich D, Schwartz S Jr, Tutino L and Villaverde A. (2013). *Biotechnol Adv,* **31,** 140-153.

Diez-Gil C, Krabbenborg S, Garcia-Fruitos E, Vazquez E, Rodriguez-Carmona E, Ratera I, Ventosa N, Seras-Franzoso J, Cano-Garrido O, Ferrer-Miralles N, Villaverde A and Veciana J. (2010). *Biomaterials,* **31,** 5805-5812.

Erridge C, Bennett-Guerrero E and Poxton IR. (2002). *Microbes Infect,* **4,** 837-851.

Ferrer-Miralles N, Domingo-Espin J, Corchero JL, Vazquez E and Villaverde A. (2009). *Microb Cell Fact,* **8,** 17.

Ferrer-Miralles N and Villaverde A. (2013). *Microb Cell Fact,* **12,** 113.

Garcia-Fruitos E, Gonzalez-Montalban N, Morell M, Vera A, Ferraz RM, Aris A, Ventura S and Villaverde A. (2005). *Microb Cell Fact,* **4,** 27.

Garcia-Fruitos E, Martinez-Alonso M, Gonzalez-Montalban N, Valli M, Mattanovich D and Villaverde A. (2007). *J Mol Biol,* **374,** 195-205.

Garcia-Fruitos E, Seras-Franzoso J, Vazquez E and Villaverde A. (2010). *Nanotechnology,* **21,** 205101.

Garcia-Fruitos E, Vazquez E, Diez-Gil C, Corchero JL, Seras-Franzoso J, Ratera I, Veciana J and Villaverde A. (2011). *Trends Biotechnol,* **doi:10.1016/j.tibtech.2011.09.003**.

Garcia-Fruitos E, Vazquez E, ez-Gil C, Corchero JL, Seras-Franzoso J, Ratera I, Veciana J and Villaverde A. (2012). *Trends Biotechnol,* **30,** 65-70.

Garcia-Fruitos E and Villaverde A. (2010). *Korean J Chem Eng,* **27,** 385-389.

García-Fruitós E, Rodríguez-Carmona E, Díez-Gil C, Ferraz RM, Vázquez E, Corchero JL, Cano-Sarabia M, Ratera I, Ventosa N, Veciana J and Villaverde A. (2009). *Advanced Materials,* **21,** 4249-4253.

Georgiou G and Valax P. (1999). *Methods Enzymol,* **309,** 48-58.

Gonzalez-Montalban N, Garcia-Fruitos E and Villaverde A. (2007). *Nat Biotechnol,* **25,** 718-720.

J.Sambrook, EF.Fritsch and T.Maniatis. (1989). *Molecular Cloning, a laboratory manual.* New York.

Laemmli UK. (1970). *Nature,* **227,** 680-&.

Lee SY, Mattanovich D and Villaverde A. (2012). *Microb Cell Fact,* **11,** 156.

Liovic M, Ozir M, Bedina ZA, Peternel S, Komel R and Zupancic T. (2012). *Microb Cell Fact,* **11,** 67.

Liu S, Tobias R, McClure S, Styba G, Shi Q and Jackowski G. (1997). *Clin Biochem,* **30,** 455-463.

Magalhaes PO, Lopes AM, Mazzola PG, Rangel-Yagui C, Penna TC and Pessoa A, Jr. (2007). *J Pharm Pharm Sci,* **10,** 388-404.

Maji SK, Perrin MH, Sawaya MR, Jessberger S, Vadodaria K, Rissman RA, Singru PS, Nilsson KP, Simon R, Schubert D, Eisenberg D, Rivier J, Sawchenko P, Vale W and Riek R. (2009). *Science,* **325,** 328-332.

Mankar S, Anoop A, Sen S and Maji SK. (2011). *Nano Rev,* **2**.

Neubauer P, Fahnert B, Lilie h and Villaverde A. (2006). pp. 237-292.

Neus Ferrer-Miralles, Escarlata Rodriguez-Carmona, Jose Luis Corchero, Elena Garcia-Fruitos, Esther Vazquez and Antonio Villaverde. (2013). *Crit Rev Biotechnol,* **in press**.

Petsch D and Anspach FB. (2000). *J Biotechnol,* **76,** 97-119.

Rodriguez-Carmona E, Cano-Garrido O, Seras-Franzoso J, Villaverde A and Garcia-Fruitos E. (2010). *Microb Cell Fact,* **9,** 71.

Rodriguez-Carmona E and Villaverde A. (2010). *Trends Microbiol,* **18,** 423-430.

Seras-Franzoso J, Peebo K, Corchero JL, Tsimbouri, P. M., Unzueta U, Rinas, U., Dalby, M., Vazquez E, García-Fruitós, Elena, and Villaverde A. A nanostructured bacterial bio-scaffold for the sustained bottom-up delivery of protein drugs. Nanomedicine (Lond) . 2013.
Ref Type: In Press

Seras-Franzoso J, Diez-Gil C, Vazquez E, Garcia-Fruitos E, Cubarsi R, Ratera I, Veciana J and Villaverde A. (2011). *Nanomedicine (Lond),* **doi: 10.2217/NNM.11.83**.

Seras-Franzoso J, ez-Gil C, Vazquez E, Garcia-Fruitos E, Cubarsi R, Ratera I, Veciana J and Villaverde A. (2012). *Nanomedicine (Lond),* **7,** 79-93.

Seras-Franzoso J, Peebo K, Garcia-Fruitos E, Vazquez E, Rinas U and Villaverde A. (2013a). *Acta Biomater*.

Seras-Franzoso J, Steurer C, Roldan M, Vendrell M, Vidaurre-Agut C, Tarruella A, Saldana L, Vilaboa N, Parera M, Elizondo E, Ratera I, Ventosa N, Veciana J, Campillo-Fernandez AJ, Garcia-Fruitos E, Vazquez E and Villaverde A. (2013b). *J Control Release,* **171,** 63-72.

Seras-Franzoso J, Tsimbouri PM, Burgess KV, Unzueta U, Garcia-Fruitos E, Vazquez E, Villaverde A and Dalby MJ. (2013c). *Nanomedicine (Lond)*.

Talafova K, Hrabarova E, Chorvat DA and Nahalka J. (2013). *Microb Cell Fact,* **12,** 16.

Tatkiewicz WI, Seras-Franzoso J, Garcia-Fruitos E, Vazquez E, Ventosa N, Peebo K, Ratera I, Villaverde A and Veciana J. (2013). *ACS Nano*.

Vazquez E, Corchero JL, Burgueno JF, Seras-Franzoso J, Kosoy A, Bosser R, Mendoza R, Martinez-Lainez JM, Rinas U, Fernandez E, Ruiz-Avila L, Garcia-Fruitos E and Villaverde A. (2012). *Adv Mater,* **24,** 1742-1747.

Vazquez E, Roldan M, Diez-Gil C, Unzueta U, Domingo-Espin J, Cedano J, Conchillo O, Ratera I, Veciana J, Daura X, Ferrer-Miralles N and Villaverde A. (2010). *Nanomedicine (Lond),* **5,** 259-268.

Vazquez E and Villaverde A. (2013). *Nanomedicine (Lond),* **8,** 1895-1898.

Villaverde A. (2012). *Nanomedicine (Lond),* **7,** 1277-1279.

Villaverde A and Carrio MM. (2003). *Biotechnol Lett,* **25,** 1385-1395.

Villaverde A, Garcia-Fruitos E, Rinas U, Seras-Franzoso J, Kosoy A, Corchero JL and Vazquez E. (2012). *Microb Cell Fact,* **11,** 76.