Universitat Autònoma de Barcelona

Production of protein nanomaterials in lactic acid bacteria for human and animal medicine

Olivia Cano Garrido

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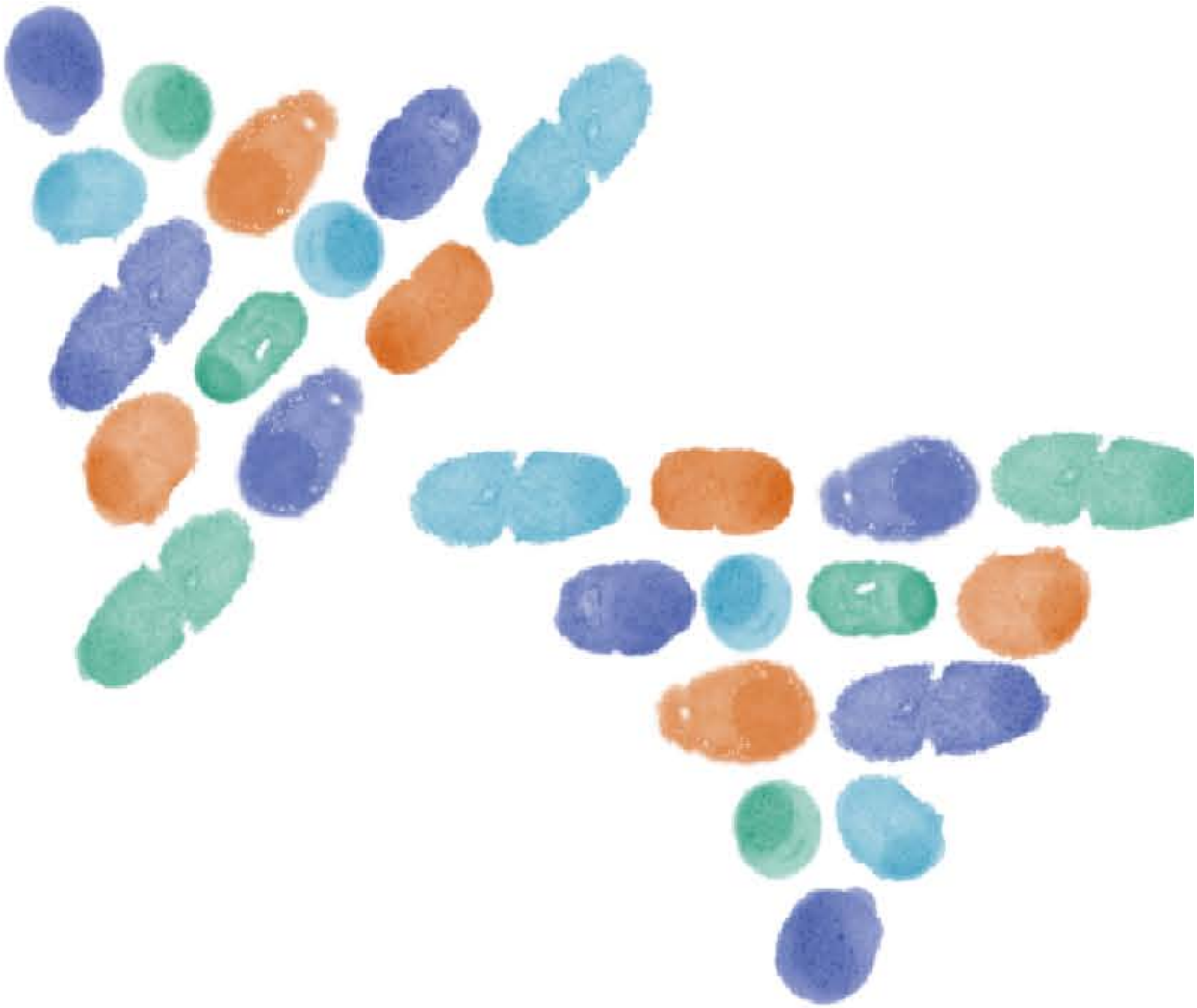
Tesi doctoral 2016



Doctorat en Biotecnologia

Production of protein nanomaterials in lactic acid bacteria for human and animal medicine.

Tesi doctoral- 2016

Departament de Genètica i Microbiologia- Facultat de Biociències

Memòria presentada per Olivia Cano Garrido per optar al grau de doctor en Biotecnologia per la Universitat Autònoma de Barcelona.

Olivia Cano Garrido

Vist i plau dels directors de la tesis:

Elena Garcia Fruitós **Antonio Villaverde Corrales**

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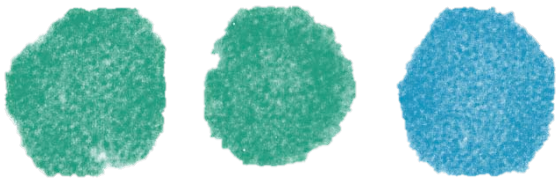
A todos los que me apoyaron y animaron durante la tesis,



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Introduction

The discovery of the DNA structure in 1953 had an enormous impact on the history of science, as the double helix revelation launched the era of molecular biology. Since this discovery, many new procedures and researches came up in different fields, including biomedicine, animal health, agriculture, environmental science and law. During the 60s and 70s, a massive progress was reached in understanding how gens are structured and which are their mechanisms of replication, expression and regulation in organisms such as prokaryotes and phages [1].

All this knowledge has established the basis of the DNA technology. This discipline came into being by Cohen and Boyer experiments. In their first work, a new plasmid was constructed *in vitro* from fragments of two different plasmids (each one carrying a different antibiotic resistance) and, also, it was demonstrated that once incorporated into *Escherichia coli* bacterial cells, the plasmid was still functional providing the bacteria resistance against both antibiotics [2]. In summary, at that time, the first recombinant bacterium of the history was constructed. In their second work, not only an eukaryote gene from a frog was cloned and replicated into a prokaryote host, but the frog protein was also successfully produced in *E. coli* [3]. Thus, all these progresses have made possible to clone and propagate DNA from any organism in a heterologous host.

Biology, as we know it today, was completely revolutionized by four key methodological milestones:

- The discovery of enzymes that on one side were able to form a bond 3'-5'-phospodiester on the linear DNA, such as the ligase and, on the other side, the restriction enzymes which cleave the DNA specifically [4, 5].
- The demonstration that foreign DNA can be transferred and also replicated into host cells [6].
- The genetic material sequencing, a technology which permits to determine a DNA sequence.
- The polymerase chain reaction (PCR), a methodology to amplify DNA from a single copy to millions of it *in vitro*.

The publication of the first method for DNA sequencing in 1977 [7] started a new era of genetic engineering development. The first massive scale sequencing was the Human Genome Project which took 13 years to be completed and cost 2.7 billion dollars. Since that moment, this technology has been profoundly improved, making possible to determine a long sequence of

DNA in a short time and through a cost-effective process. It appears that sequencing a human genome by 1,000 dollars is not far away [8]. Nowadays, 14,700 genomes from 180 different species have been sequenced and displayed in open access at GenBank by the National Center for Biotechnology Information (NCBI) [9].

Today, there is no doubt that recombinant DNA technologies and DNA sequencing have completely modified the current biological know-how. At one time, proteins were extracted from large quantities of animal and plant tissues, being just a good option to obtain small amounts of a desired protein [10]. Scientists now routinely isolate genes from alive or dead organisms, and even create artificial ones. The first recombinant protein, somatostatin, was produced in 1976 [11]. This was the first proof of concept of the overexpression of heterologous proteins in bacterial expression system. Some years later, Genentech Inc. launched the first pharmaceutical product based on a recombinant protein (human insulin) produced in *E. coli* [12].

The biotechnology industry is taking advantage of the recombinant protein technology to develop new commercial goods for human and veterinary medicine such as therapeutic proteins, vaccines, enzymes, prophylactics and diagnostic kits, but also, new products for food processing, bioinsecticides and bioremediation processes, among many others [13]. Besides, the technology to express heterologous proteins in large quantities has also opened a new era on proteomics research. Obtaining purified proteins allows studying the structure, function, posttranslational modifications, and interaction between them. This know-how permits expanding day after day the understanding on cell biology.

Protein production requires a specific environment containing the basic machinery for gene translation and protein folding. As of now, there are basically two main expression systems: cell-free protein expression and living cell factories. In principle, cell-free protein expression is an *in vitro* synthesis using the protein biosynthesis machinery from a cell extract. However, the most critical drawback of this system is the lower protein amount that can be obtained [14]. Among cell factories; bacteria, yeast, fungi and animal cells are commonly used nowadays for the expression of heterologous proteins [13]. The choice of the system is determined by the type of protein, the requirements for its activity and the expected yield. Thus, microbial cell factories are popular due to be easy-to-work, less time-consuming and cost-effective protein expression systems.

1. Protein biosynthesis

Protein biosynthesis is one of the most complex metabolic processes, representing a vast energetic effort for the cell. Protein production is carried out through a process called translation, in which several participants take part: a messenger RNA (mRNA), transfer RNA (tRNA), amino acids, proteins factors and ribosomes. Regardless of the organism of origin, proteins are assembled from a set of 20 amino acids. Protein production occurs in multiple ribosomes at the same time at the cell cytoplasm, being able to synthesize numerous proteins in a short period of time.

1.1 Folding

The covalent protein skeleton, also called primary structure, consists of linking individual amino acids through a peptide bond between an amine ($-NH_2$) and a carboxyl acid (-COOH). Carbons α from adjacent amino acids are separated by three covalent bonds, organised like this: C_α–C–N–C_α. Given that N–C_α and C_α–C bonds can rotate without restriction, protein could adopt an infinite number of conformations [15]. However, each protein has a specific chemical or structural function, hence a unique three-dimensional conformation. Protein three-dimensional structure is stabilised by non-covalent interactions like hydrogen bonds, van der Waals forces, hydrophobic interactions and electrostatics [16]. In fact, the way in which a newly assembled sequence of amino acids folds itself with precision and fidelity depends on the intrinsic characteristics of the amino acid chain, as well as on the influences from the cellular environment [17].

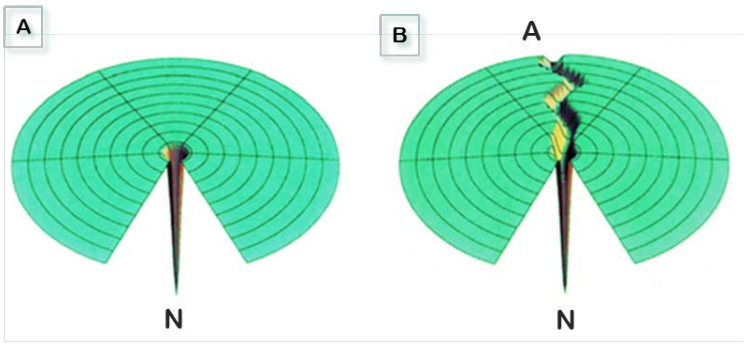


Figure 1.1: Levinthal's argument that random searching would not find the native state. **A.** In this case the polypeptide searches the conformation randomly. **B.** The pathway solution of the polypeptide folding. **(N)** means native conformation and **(A)** means denatured conformation. Adapted from [18].

Anfisen's experiments on ribonuclease renaturation proved that assumption. A denaturalised protein can fold reversibly into their natural conformation without catalytic biomolecules. In this sense, the native conformation of a protein usually correspond to the most thermodynamically stable, being in the minimum free energy state [19]. But some years later, Lenvithal made the argument proposing the "Levinthal paradox". Taking into account that a protein is produced in about 5 seconds, it is impossible that a protein randomly explores all the probable special conformations, because it would take an astronomical length of time (Figure 1.1 A). For this reason, Levinthal exposed the concept of folding pathway though which proteins are able to fold rapidly and efficiently [20]. Figure 1.1 B illustrates how the pathway could work the problem of Figure 1.1 A out. Starting from a non-folded protein **(A)**, the protein finds the native **(N)** structure through a kind of tunnel on the landscape [18].

Due to the complexity of protein folding, many models have been proposed to describe the sequence of events in the folding process such as diffusion-collision [21], nucleation-condensation [22] and stoichiometry models [23]. In this context, the most accepted model to describe protein folding is the "funnel" [24].

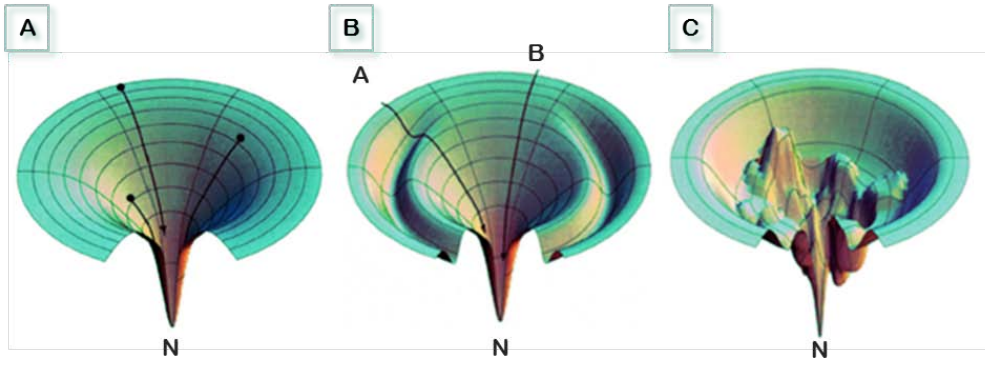


Figure 1.2: Protein folding thermodynamic landscapes. **A.** Smooth landscape. **B.** Moat landscape. **C.** Rugged landscape. **(N)** means native conformation and **(A)** and **(B)** means denatured conformation. Adapted from [18].

The "funnel model" has combined the work from different models. From this hypothesis, proteins go from a high free energy to a low free energy state (satisfying Anfinsen's thermodynamic hypothesis) by multiple pathways (not only one, as pathway folding model suggested). Yet, they do in a directed and rapid way (satisfying Levinthal) [18]. As a novelty, it is introduced that proteins can sometimes adopt intermediate conformations between unfolded and native state. In other words, folding may be effectuated in two or more kinetic phases, sometimes with a fast collapse to compact the whole protein and next, a step of slow reconfiguration to obtain the native structure [25]. Depending on parameters such as the folding

rate, properties, flexibility and the native structure, the folding landscape can be divided into different types [17].

- The smooth landscape: There is no energy barrier for archive the native state (Figure 1.2 A).
- The moat landscape: There are two possible pathways. Route A is a smooth-like, while route B has a folding intermediate trap (Figure 1.2 B).
- The rugged landscape: There are many intermediate conformations. This folding often has kinetic traps and energy barriers for a multi-state folding protein (Figure 1.2 C).

1.2 Protein quality control system

Folding *in vivo* starts whereas the amino acid chain is still attached to the ribosome [26]. Concomitantly, the polypeptide exit channel of the ribosome is too narrow [27] that the complete folding cannot be archived until the entire sequence has left the ribosome. The cellular environment is notably crowded with a protein concentration in a range of 300-400 g/l in *E. coli*, representing about the 20-30% of the cytoplasm composition (Figure 1.3). As a consequence, it is likely that hydrophobic patches of polypeptides tend to aggregate with other molecules, due to the close proximity to another unfolded polypeptides [28].

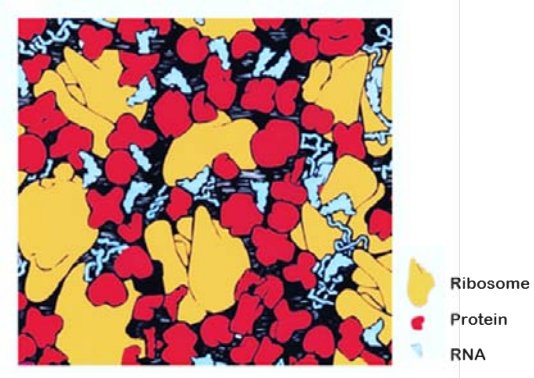


Figure 1.3: A representation of the crowded state of the *E. coli* cytoplasm. Adapted from [28].

Only correctly folded proteins have long-term stability and are able to perform their biological function. To cope with this situation, cells have a sophisticated protein quality control system of chaperones to maximize the correct folding, refolding and protein assembly and of proteases to discard misfolded and unfolded polypeptides [29].

1.3 Chaperones and proteases in prokaryotes

Chaperones are a group of highly conserved proteins dedicate to support proper protein folding. Even though chaperones are constitutively expressed, they are overexpressed under stress conditions; because of this, they are also known as heat shock proteins (HSPs) [30]. Specially, their targets are short and unstructured hydrophobic amino acid sequences that would normally be hidden into the protein *core*. This kind of motifs are very common, and accordingly, chaperones are so promiscuous [31]. Regarding on their mechanism of action, they can be classified in three functional subclasses:

- Folding chaperones assist the folding catalysing structural folding transitions, in an energy-dependent manner. Some examples of foldases are the Trigger factor (TF) [32] and the DnaK-DnaJ-GrpE [33] and GroELS systems [34].
- Holding chaperones act maintaining proteins partially folded in order to prevent improper folding and aggregation. In *E. coli*, some examples are IbpA and IbpB [35].
- Disaggregating chaperones promote the protein solubilisation from protein aggregates. Once misfolded proteins are released from the aggregates can be conducted either to refolding or proteolysis. For instance, ClpB in *E. coli* [36].

The correct management of a cell is accomplished by an accurate regulation of protein levels, maintaining a correct balance between protein synthesis and degradation [37]. Protein degradation guarantees that abnormal proteins are not assembled forming protein aggregates. Particularly, the proteins that finish in the protease system are for instance precipitately terminated polypeptides, proteins kinetically trapped off-pathway and partially folded polypeptides that do not reach a correct conformation after multiple cycle of interaction with chaperones [31]. Furthermore, proteolysis has an important role in other functions such as amino acids recycling, an energy generator during cell starvation and activating proteins. Some examples of widely conserved proteases are ClpP [38], Lon [39] and HtrA [40].

Chaperones and proteases activities must be coordinated to guarantee an efficient protein folding and to prevent or minimize protein misfolding or aggregation. The exact mechanism by which quality control system works is still unsolved. Nevertheless, some plausible models have been presented based on *in vitro* and *in vivo* works and they are summarized in Figure 1.4.

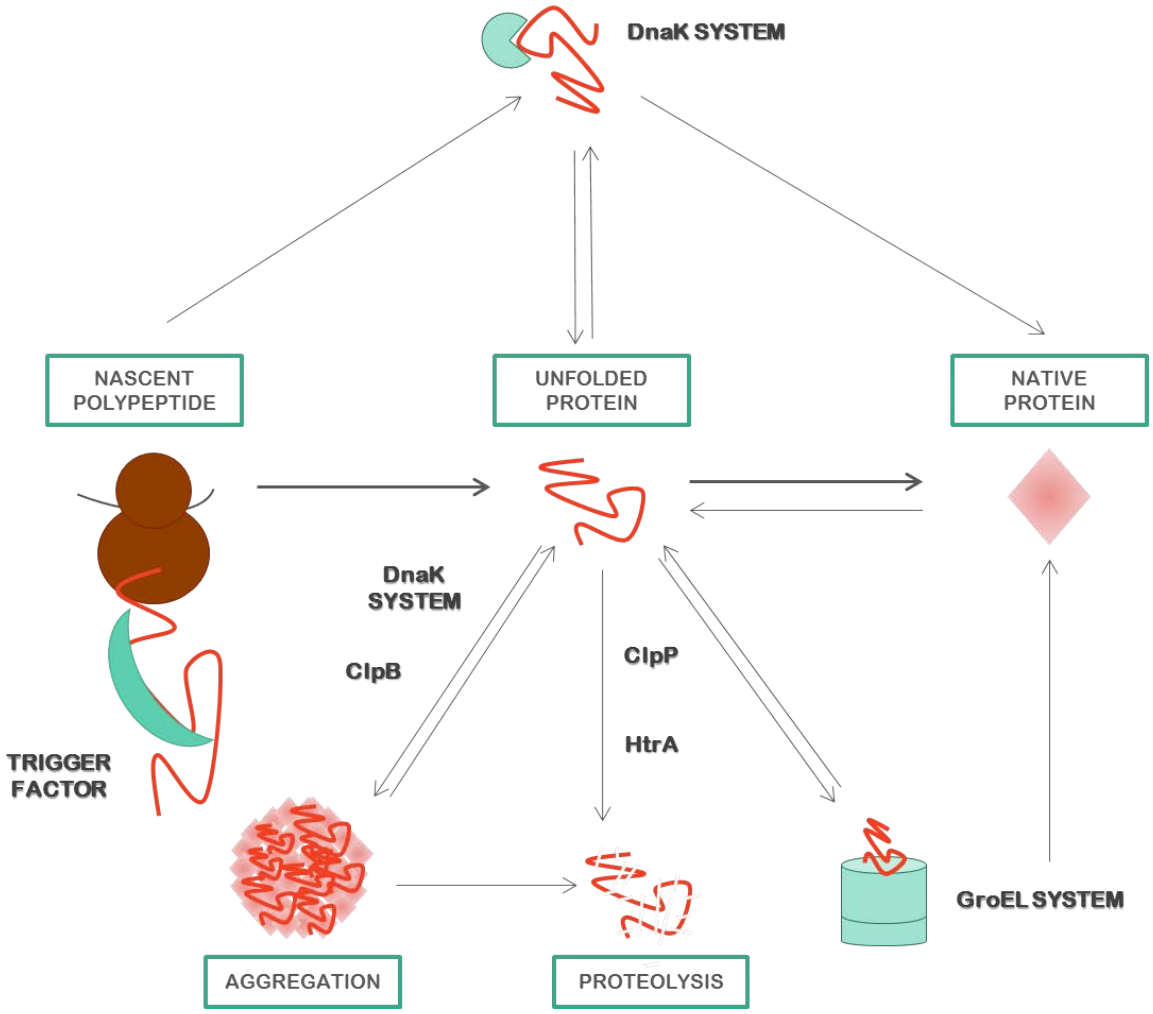


Figure 1.4: Molecular chaperones pathway in *E. coli*. Adapted from [41].

2. Microbial cell factories

Microorganisms are the most versatile and adaptable living entities. Actually, microbes have colonised diverse ecosystems from glacial to hydrothermal springs. For this reason, they have developed, in addition to all the principal metabolic pathways known in all living entities, unique metabolic processes such as those allowing nitrogen fixation. Thus, it has been taken advantage of their native ability to produce a vast variety of products to manufacture many commercial good [42]. Even though their role in fermentation was not demonstrated until 1857 by Pasteur, microorganisms have been used for fermentation processes in the production of fermented dairy products and alcoholic beverages for thousands of years [43].

Several reasons account for microorganisms as widely used cell factories. To begin with, they are easy to culture in huge amount due to their rapid multiplication. Besides, the production process is cheap, principally, because the used mediums and materials are inexpensive. Moreover, the physiology, biochemistry and genetics of microorganisms have been well defined being possible to genetically engineer them to produce heterologous products. Furthermore, the capacity of microorganisms from the natural sources is not enough to produce products for industrial applications. Therefore, metabolic engineering permits to reduce the overall cost of production by enhancing the productivity [44].

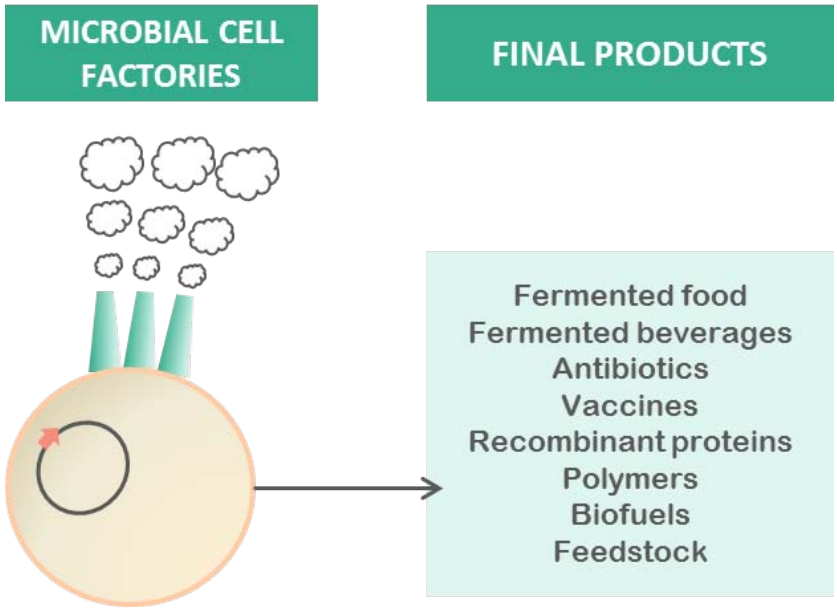


Figure 2.1: Microbial cell factories current applications.

At present, microbial cell factories are routinely used for the production of a broad spectrum of products. Some examples are fermented food and beverages, antibiotics, vaccines,

recombinant proteins, polymers, biofuels and feedstock (Figure 2.1). Biotechnology and biomedicine extensively make use of recombinant proteins as therapeutics or as efficient biocatalysts in industrial processes due to their specific cross-molecular contact [45].

The choice of an appropriate microbial cell factory is crucial to efficiently produce a recombinant protein. Some important issues must be considered in the selection of the expression system: protein characteristics (stability and activity) and desired purity and economic considerations (required yield, cost-effectiveness, time for development, etc.). Based on that, it is possible to conclude that there is no universal cell factory. Each protein is unique and it requires an optimized production strategy [46]. Nevertheless, about 37 % of the recombinant pharmaceuticals approved for human use by the Food and Drug Administration (FDA) and/or by the European Medicines Agency (EMA) are produced by microbial cell factories (bacteria and yeast) [47]. This fact makes clear that microorganisms are a good choice for protein production. Among them, prokaryotes are widely used [13, 48], especially when posttranslational modifications such as glycosylation are not essential for the protein functionality.

2.1 Prokaryotic cell factories

Among all prokaryotic expression systems, the Gram-negative enterobacterium *E. coli* is the workhorse in the recombinant protein production field. The first recombinant DNA methods were developed with *E. coli* and, nowadays, it's still the first-choice microorganism for primarily cloning, genetic modification and small-scale production for research purposes. As a consequence, *E. coli* genetics and physiology are one of the most studied and this provides a wide arsenal of molecular tools adapted to this bacterium [44, 48]. It is easy to find many commercial strains to solve common production protein bottlenecks. For example, there are *E. coli* mutant strains or lineages with reduced protease activity (BL21) [49], others developed to facilitate disulphide bond formation (Origami) [50], some co-express rare tRNAs [51] and/or over-express chaperones [52]. Moreover, there are numerous expression vectors combining different promoters and fusion tags, making the purification easier and enhancing the protein solubility [53].

Noteworthy, *E. coli* is a microbial cell factory used for massive production of a wide number of recombinant products with applications in human and animal medicine and in biotechnology. Insulin, human growth hormone, interferon and plasminogen activator are just some examples

of approved therapeutic recombinant protein-based products [48]. Nevertheless, Gram-negative bacteria have an important limitation for the generation of commercial products, particularly those with therapeutic applicability: the presence of lipopolysaccharide (LPS) in their outer membrane [54] (Figure 2.2).

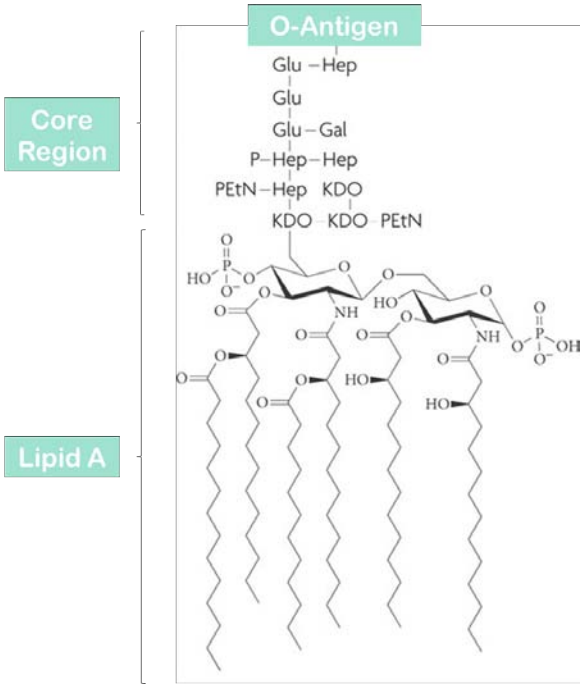


Figure 2.2: The basic structure of *E. coli* lipopolysaccharide (LPS). Adapted from [55].

LPS, also referred to as endotoxin, is formed by three covalently linked parts: the membrane-anchored lipid A, a sugar core and a distal O-specific polysaccharide chain. Every *E. coli* cell contains about 2 x 10^6 LPS molecules [56]. Lipid A is the hydrophobic component of LPS, which can stimulate the Toll-like receptor 4 (TLR4) of the pro-inflammatory pathway in mammals [54, 57, 58]. LPS is a non-specific contaminant of the final product and can promote not only pyrogenicity but also provoke potent inflammatory responses. High concentrations of LPS can even increase heart rate, and lead to septic shock and death [59]. Indeed, the principal regulatory agencies have set a stringent limit of acceptable endotoxin level in order to accept a product as safe for therapeutic purposes [60]. For this reason, *E. coli*-derived products, as well as those derived from other Gram-negative microorganisms, need to be exhaustively purified in order to remove any LPS residue present in the sample. There is not a universal LPS purification method; some examples are ultrafiltration, Triton X phase separation, anion-exchange chromatography, adsorption on activated carbon, treatment with polymyxin B or histamine-

immobilized affinity resins [57, 61] **(Table 2.1)**. These processes show high-associated costs and it has been described that endotoxin removal can also impair protein activity.

Table 2.1: Examples of patented procedures for removal of endotoxins from protein solutions.

PROCEDURE	PRODUCT	REFERENCE
Ultrafiltration	Superoxid dismutase	62
	Myoglobin	63
Anion-exchange chromatography	Cu-Zn-Superoxid dismutase	64
	Urokinase	65
Adsorption on quaternised chitosan	TNF, IL-1	66
Adsorption on non-polar polymers	Cu-Zn-Superoxid dismutase	67
	Catalase	
Hydrophobic interaction chromatography	Hepatitis B surface antigen	68
Sucrose gradient centrifugation	Pertussis vaccine	69
Extraction with bile salts	Immunoglobulins	70
Extraction with detergents and HIC	Lipocortine	71
Pepsin digestion removes endotoxin-binding Fc-fragment	Immunoglobulins	72

2.2 LPS-free expression systems

Given that protein production processes have to combine good cost-effectiveness ratios with high product quality [73, 74], in the last years, the use of cell factories other than *E. coli* are emerging as intriguing alternatives. Specially, LPS-free expression systems are appealing microorganisms because they offer as advantage the absence of endotoxins in the final product.

2.2.1 Endotoxin-free *Escherichia coli*

Considering the limitations associated with the use of *E. coli,* a mutant strain with a modified LPS, which does not trigger immunologic response in mammalian cells, has recently been developed. This strain is characterised by seven genetic deletions in genes involved in the LPS synthesis (*gutQ, kdsD, lpxL, lpxM, pagP, lpxP* and *eptA*). Specifically, they are involved in the pathway in which the endotoxin precursor lipid IV_A is decorated with six acyl chains forming the immunogenic lipid A [75]. This engineered strain has the non-decorated version of the lipid A

(lipid IV_A) and therefore, there is no formation of the activated heterotetrameric hTLR4/MD-2 complex when the recombinant product is administered. Consequently, the endotoxic response is not activated. Moreover, without the acyl chains in lipid $IV_{A,}$ the downstream removal of the rest of LPS is apparently easier [76].

Although the endotoxin-free *E. coli* strains grow slower than wild-type strains, the yield and activity of several model proteins produced in these new recombinant system are similar to those produced in the native strains [57, 77]. Purified proteins were then tested for endotoxin levels by the *Limulus amebocyte* lysate (LAL) method [78] as determined by the FDA. Even though the endotoxic response, determined by the LAL, of the obtained product decreased by 95% compared to the control protein, these values are still too high to be accepted by the FDA. However, some studies have confirmed that these recombinant proteins do not activated the human endotoxic response by using an assay based on Toll-Like receptor activation [79], otherwise, it was mentioned that there is immunologic response when using cells from other mammals such as mouse, Chinese hamster or equine cells (in spite of being clearly lower than the response provoked by LPS standard *E. coli* strains in these cases).

In short, despite these strains have brought a quantum leap, there is still a lot to do to develop fully safe products for therapeutic applications. Mostly, it should be interesting improved production processes without needing specific steps for removing either LPS impurities or modified LPS.

2.2.2 Gram-positive bacteria

Interestingly, Gram-positive bacteria have a considerably thick peptidoglycan layer surrounding the cell membrane that does not contain LPS [80]. The lack of endotoxins in these microorganisms opens an appealing alternative for the production of safe products for both human and veterinary purposes [76]. It should be noted that many Gram-positive bacteria have a long history being used for centuries in food production and preservation [81-83]. In this context, some of them such as *Bacillus*, *Corynebacterium*, Lactic Acid Bacteria (LAB), etc. have been classified as food grade microorganisms. They are Generally Recognized as Safe (GRAS) organisms by the FDA and fulfil criteria of the qualified presumption of safety (QPS) according to the European Food Safety Authority (EFSA). Besides, having a single cellular membrane, these bacteria are an ideal host for heterologous protein secretion purposes [84, 85]. They are also used for cell surface display of therapeutic molecules and antigens of interest [86]. In this context, it is important to emphasise that an exhaustive effort has been done in developing

different tools for the recombinant protein production using Gram-positive bacteria as microbial cell factories [87] (Figure 2.3).

2.2.2.1 *Bacillus* spp.

Among all Gram-positive bacteria, *Bacillus spp*. has traditionally been the host most often chosen for the production of industrial enzymes and some biopharmaceuticals [88]. It was the first endotoxin-free bacteria biochemically and genetically characterised [89], becoming an extremely attractive alternative because of its excellent ability to secrete proteins and safe antibacterial compounds [90]. Nearly all proteins produced in this microorganism have a prokaryotic origin. Some examples are proteases (for detergents), amylases (for starch and baking) and medicinal drugs, such as myo-inositol and scyllo-inositol [91, 92].

Bacillus spp. often secrete endogenous proteases at high levels, requiring of the use of multiple protease-deficient strains [93, 94]. Yet, most of them are protected by companies and often unavailable to academic researchers [95].

2.2.2.2 *Corynebacterium glutamicum*

Considering its traditional use as industrial-scale producer of various L-amino acids, nucleic acids, and vitamins for use in human and animal foods, as well as pharmaceutical products [96-98], *Corynebacterium glutamicum* is considered as a promising emerging host for the production and secretion of recombinant proteins with a safe profile [99-101]. Although the appropriate fermentation conditions are well established, the development of suitable expression systems still has some way to go [101]. For instance, most of *C. glutamicum* expression systems are based on the *E. coli* lac operator–repressor system, regulated by Isopropyl β-D-1-thiogalactopyranoside (IPTG) induction [102]. However, this expression system is likely to present a low level of inducible expression in *C. glutamicum* because of the low permeability of IPTG in this strain [103]. Furthermore, the use of IPTG for industrial purposes shows two important drawbacks: highs cost and potential toxicity [97]. Nevertheless, other suitable alternatives exist such as a heat-inducible [104] and an arabinose-inducible expression system [105]; and also, other endogenous promoters are being currently developed [106].

2.2.2.3 Lactic Acid Bacteria (LAB)

Lactic Acid Bacteria (LAB) are worldwide known for its use in the food industry were they have been extensively used in fermented foods, beverages and animal feed for centuries, being consumed by humans mainly in dairy based products [107]. Besides, some LAB are exploited as

producers of flavouring enzymes, peptides with antibacterial activity, or metabolites that contribute to the flavour, conservation, and texture of foods. Several LAB are also nowadays for sale as probiotic strains with health-promoting capacity [76]. Concomitantly, LAB have been gaining momentum as an alternative for recombinant protein applications [87]. Many strategies have been proposed in order to produce and present different antigens; most of them have been developed in *Lactococcus lactis* [85, 88, 108] but also works in other LAB such as *Lactobacillus pentosus* [109], *Lactobacillus jensenii* [110, 111], *Lactobacillus casei* [112], *Lactobacillus plantarum* [113], *Bifidobacterium bifidum* [114] *and Bifidobacterium breve* [115, 116].

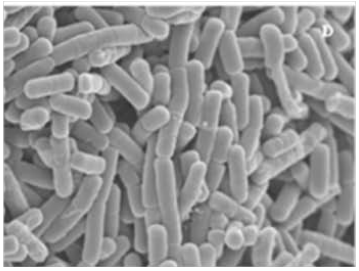
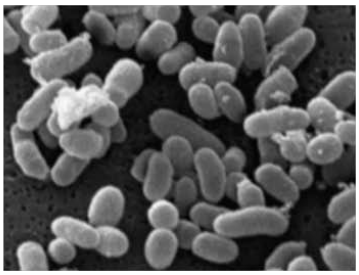
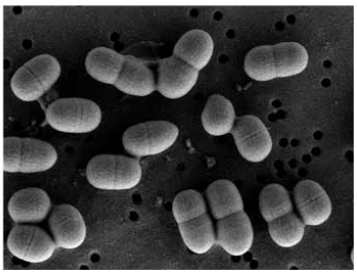
MICROBIAL CELL FACTORY		COMMERCIAL PRODUCTS
Bacillus spp.		Industrial enzymes Poly-γ-glutamic acid Biosurfactants Riboflavin Antimicrobial substances Inosine Hyaluronic acid
Corynebacterium glutamicum		Amino acids Nucleo acids Vitamins Organic acids Polymeric materials γ-glutamyltransferase
Lactococcus lactis		Fermented foods Fermented beverages Flovoring enzymes Antimicrobial substances Food preservatives

Figure 2.3: Commercial goods produced in gram-positive bacteria as a microbial cell factory. *Bacillus spp.* micrograph from [117], *C. glutamicum* micrograph from [118] and *L. lactis* micrograph (not published).

3. *Lactococcus lactis* as a recombinant protein expression system

The characterisation of the physiology, biochemistry and genetics of LAB has significantly evolved and a considerable variety of genetic techniques, transformation protocols, and sophisticated vectors have been developed [119]. In addition, more than 100 LAB species have been sequenced, being the genetic information of these strains publicly available [120]. This has allowed the development of numerous mutant strains and expression vectors that have been successfully applied to use LAB as microbial cell factories. Among all species, *L. lactis* is the most widely used in protein production. Most of the technologies have been developed for *L. lactis,* however, many of them can also be transferred to other LAB bacteria.

3.1 Vectors

So far, several inducible expression systems controlled by environmental elements are available, including the chloride-inducible expression cassette [121], the zinc-inducible expression system [122], the lactate-inducible P170 system [123], the heat shock-inducible system [124], and so on. However, the use of an essential nutrient or metabolite as inductor is still an unsolved issue, because its concentration cannot be completely controlled. Consequently, they show either low efficiency or some basal expression [119, 125] in recombinant protein production processes. Whereas these systems can be useful for protein production in live bacterial vectors in which it is interesting that protein production was triggered at mucosal surfaces.

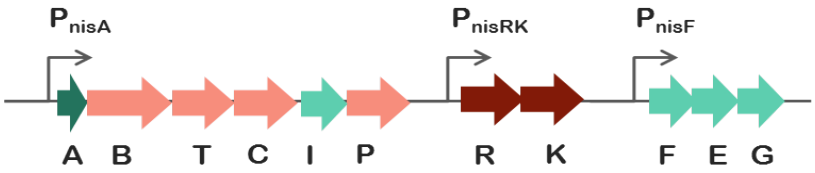


Figure 3.1: Nisin gene cluster. Grey arrows indicated the three promoters that regulate nisin genes expression. A represents nisin *A* structural gene. *B. T, C* and *P* are genes that take part in nisin modification, translocation and processing. *I, F, E* and *G* are genes involved in immunity against nisin. Finally, *R* and *K* genes participate in the regulation of the nisin gene expression cluster. Adapted from [126].

Recently, some expression vectors have been specifically designed to induce the protein production *in situ*. For example the heat-stress (body temperature is some degrees higher than bacteria optimal growth temperature) [127] or the acid-stress (because of the stomach fluids) [128] vectors, which trigger the synthesis once bacteria suffer these environmental stresses, are interesting options. Moreover, *L. lactis* respond to stress by synthesising chaperones such as

GroESL [127], and in this regard, a Stress-Inducible Controlled Expression (SICE) system based on the *groESL* operon promoter has been also developed [128].

The fact of having strong inductions is particularly important in the recombinant protein production context; because of this suitable promoters are required. Such drawback has been overcome with the NICE (Nisin Controlled Expression) system, the best-characterised protein expression system for LAB [126] based on a strong inducible promoter, P_{nisA} (the nisin promoter). Nisin is a bacteriocin produced by some *L. lactis* strains, which is expressed on a pathway encoded by eleven genes (*nis ABTCIPRKFEG*), encoding at the same time all the metabolic pathway and the immunity against nisin **(Figure 3.1)**.

The expression system is basically formed by introducing *nisK* and *nisR* genes in the bacteria of interest, either in the chromosome, as occurs in *L. lactis* NZ9000 strain, or into a plasmid such as pNZ9530 [129]. NisK is a protein kinase anchored in the cytoplasmic membrane, which acts as a receptor for the nisin molecule. The nisin and NisK binding provokes NisK auto-phosphorylation, which phosphorylates NisR, that finally induces gene transcription downstream of PnisA **(Figure 3.1)**. As a bacteriocin, nisin concentration should be tested to find the optimal conditions to reach high production yields, but low toxicity.

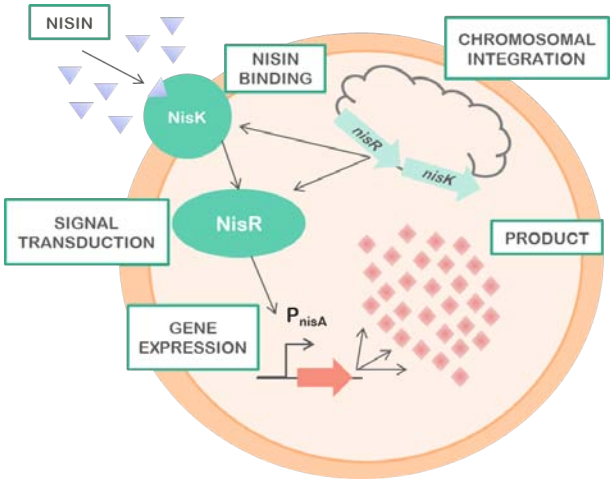


Figure 3.2: Nisin-controlled gene expression. NisK represents a histidine- protein kinase; NisR a response regulator. P_{nisA} is the promoter which activated the recombinant gene expression. Adapted from [126].

Because of the great potential of this safe expression system, the LAB toolbox development does not cease. Recently, an alternative to NICE system have been published, the ACE expression system (agmatine-controlled gene expression). A vector dependable on the transmembrane protein AguR, which responds to extracellular agmatine, and in so doing,

triggers the induction of gene expression via P_{aguB}. Even though, because of its novelty it has not still been well-characterised, the advantage of these systems relies on not requiring additional genes at the chromosome [130].

3.2 Strains

Most of the *L. lactis* strains used for protein production, as for instance, all host strains of the NICE system, derive from *L. lactis* subsp. *cremoris* MG1363, a plasmid-free descendant from a dairy starter strain [131]. Presently, NZ9000 is the most used strain (see 3.1) characterised to have integrated the regulatory genes *nisR* and *nisK* into the *pepN* gene but there is also as alternative the NZ9100 with the genes integrate into a neutral locus.

Apart from intracellular protein production, protein cell wall attachment and protein secretion are two appealing applications of *L. lactis*. However, protein proteolysis is one of the major drawbacks for obtaining stable and high protein yields. Unlike *E. coli* and *B. subtilis*, there are only two proteinases described in *L. lactis*, the cell-wall-anchored proteinase PrtP [132] and the housekeeping membrane-bound proteinase HtrA[133]. Basically, HtrA acts on the degradation of misfolded secreted proteins and on the maturation of pro-proteins [133]. The inactivation of this proteinase gene boosts to increase the stability as well as high production levels of recombinant secreted proteins that were degraded in the native strain [134]. In the context of intracellular protein production, the ATP-dependent serine-protease ClpP has been identified as the principal intracellular protease in *L. lactis* [135]. Therefore, a *L. lactis* harbouring the *clpP* gene deletion was created [136].

However, these mutants have adopted a thermosensivity phenotype, being *clpP*-NZ9000 incapable to form colonies at 37ºC [137] and *htrA*-NZ9000 growth is affected in liquid culture medium at 37 ºC; even the growth is totally stopped at 39 ºC [133]. Interestingly, the double mutant strain *clpP-htrA* NZ9000 strain is more resistant to temperature stress, having also good protein yields [138].

L. lactis has a high commercial potential; at the request of industries, robust strains to work efficiently under stress conditions such as extremes temperatures, pH and high concentrations of weak acids have been developed. There are for example *L. lactis* strains with the capability to produce glutathione in order to resist to the oxidative stress [139] and another strain which tolerate low pH by producing trehalose, a protecting disaccharide [140].

Notwithstanding, these mutant strains have a much lower growth rate than that of the wild type strain. On one side because of the genetic mutation itself, but on the other side due to *L. lactis* growth being susceptible to high antibiotic concentrations, even if having the antibiotic resistance. Moreover, if these microorganisms are employed in human and animal applications, there is necessary to use food-grade marker to avoid antibiotics. Recently, it has been developed a new strategy based on the lethal phenotype of alanine racemase (*arl*), (NZ9130 strain [141]), in which *L. lactis* is unable to grow in the media without the plasmid carrying the *arl* gene.

3.3 LAB expression system applications

3.3.1 Recombinant protein production

Although recombinant protein production in LAB is far from being an extensively explored field, *L. lactis* is an efficient cellular factory able to produce LPS-free recombinant proteins. *L. lactis* is an interesting host to produce proteins for several biomedical and biotechnological applications [126, 142] because its non-strict homofermentative metabolism allows a rapid growth without aeration that facilitates a cheap and easy scale-up protein production [126]. Furthermore, it is a suitable secretor of soluble proteins due to its low extracellular proteinase activity **(Table 3.1)**. Interestingly, some therapeutic proteins of interest have been successfully produced such as the biologically active interferon-gamma (IFN-γ) a cytokine difficult to produce in *E. coli* [143], the *Staphylococcus aureus* Termonuclease [144] and Trefoil factors (TFF) [145] which are cytoprotective peptides involved in epithelial wound healing, among other proteins.

Table 3.1: Recombinant protein production in *L. lactis* as a host

PROTEIN	FUNTION	SOURCE ORGANISM	CATEGORY	REFERENCE
β-lactoglobulin	Antigen cow's milk allergy	*Bos taurus*	Secreted	[146]
IFN-□	Cytokine	*Mus musculus*	Secreted	[143]
Maltogenic amylases	α-amylase-type enzyme	*Lactobacillus gasseri*	Secreted	[147]
IL-10	Cytokine	*Mus musculus*	Secreted	[148]
IL-12	Interleukine 12	*Mus musculus*	Secreted	[149]
IL-2	Interleukine 2	*Mus musculus*	Secreted	[150]
IL-6	Interleukine 6	*Mus musculus*	Secreted	[151]

PROTEIN	FUNTION	SOURCE ORGANISM	CATEGORY	REFERENCE
GLURP-MSP3	Glutamate-rich protein	*Plasmodium falciparum*	Secreted	152
BCV	bovine coronavirus epitope	Bovine rotavirus	Secreted	153
VP8	Capsid protein	Human rotavirus	Secreted	154
E7	Eukaryotic antigen	HPV type-16	Secreted	134
Ara h 2	Peanut allergen	*Arachis hypogaea*	Secreted	144
TTFC	Tetanus toxin fragment C	*Clostridium tetani*	Secreted	155
β-lactamase	Enzyme	*Escherichia coli*	Secreted	156
Crystal protein 5B	Anthelminthic	*Bacillus thuringiensis*	Cytoplasmic/ secreted	157
L7/L12	Ribosomal protein	*Brucella abortus*	Cytoplasmic / secreted	134
hGH	Growth hormone	*Homo sapiens*	Cytoplasmic	107
Lysostaphin	Antibacterial	*Staphylococcus simulans*	Cytoplasmic	142
PEP	prolyl-endopeptidase	*Myxococcus xanthus*	Cytoplasmic	130
NADH oxidase	Nicotinamide adenine dinucleotide	*Streptococcus mutants*	Cytoplasmic	158
Lip	Lipase	*Staphylococcus hyicus*	Cytoplasmic	159
NSP4	Rotavirus Rotavirus non-structural protein 4	Bovine rotavirus	Cytoplasmic	160
LLO	Lysteriolysin O	*Listeria monocytogenes*	Cytoplasmic	161
β-galactosidase	Enzyme	*Clostridium acetobutylicum*	Cytoplasmic	162
Erd2	KDEL-receptor	*Homo sapiens*	Membrane	163
CTP1	Mitochondrial carrier	*Saccharomyces cerevisiae*	Membrane	163
ceQORH	Chloroplast envelope quinone oxidoreductase	*Arabidopsis spp.*	Membrane	163
AAC_{hyd}	Hydrogenosomal carrier	*Neocallimastix patriciarum*	Membrane	164

L. lactis also provides a good environment for the overproduction of membrane proteins [165]. Basically, this is because it has only one single membrane, easily to solubilise with mild

detergents [163] and also because of its low proteolytic activity. Indeed, a number of prokaryotic and eukaryotic membrane proteins have been heterologously expressed in this host [166] **(Table 3.1)**.

Moreover, notwithstanding that any gene involved in disulphide bond (DSB) formation has been found in *L. lactis* by sequence comparison, some recombinant proteins that require a DSB to archive their conformation have been successfully produced [149, 167, 168].

LAB have exclusively been explored as cell factories for the production of soluble recombinant proteins, either intracellularly, anchored in the cell wall or secreted. So far, the possibility to produce protein-based nanostructures in these bacteria has not been explored. It is widely believed that LAB, contrary to what occurs in *E. coli*, are only able to produce soluble proteins, rendering the production of protein aggregates from these GRAS microorganisms rather impossible. However, there are evidences that contradict this generic assumption [169]; but it has not been deeply investigated in these expression systems. Recently, Lu and collaborators have described the presence of highly fluorescent clusters inside *L. lactis* cytoplasm under the overexpression of a fluorescent protein[170], supporting the potential of *L. lactis* to form protein-based clusters within the nanoscale range.

3.3.2 Live bacterial vector

Due to the harmlessness of these microorganisms, LAB are also an ideal alternative to be used as a live bacterial vector. Many studies describe the use of LAB to deliver antigenic and therapeutic proteins through oral, intranasal or vaginal administration [143, 144, 171-173] to treat different gastrointestinal pathologies (GIT), diabetes, cancer and viral infections. Interestingly, the administration of such microorganisms would mean a significant decrease in the production cost of the therapeutic agents, since being live organisms, such vectors are able to autonomously amplify, produce and deliver the protein of interest to a specific target **(Figure 3.3)**.

On account of their great potential, a big effort has been done in the elaboration of constitutive and inducible promoters that do not require the addition of any external inducer. This is applicable not only to *L. lactis*, but also to other LAB species **(Figure 3.1)**. Up to now, the use of LAB as a live vector has been successfully tested in preclinical and clinical trials for a wide variety of medical application such as the Crohn's disease [174], diabetes [175], cervical cancer [176] and so on. Thus, these results consolidate LAB promising as a new vaccine platform. Nevertheless, there is still a lot to be done, since these live vectors are genetically modified

organisms (GMOs). Despite that GMOs are accepted and currently used in food industry, there are still determining regulatory issues to be discussed in depth. Specially, the development of food-grade vectors without antibiotic resistances to be administered to humans and animals [177]. There are a few of evidences that resistance markers may be transferred to the own microbiota. In particular, this is a really unusual phenomenon that has not been described in the field, but due to its significance has to be taken into account for their routinely implementation in health.

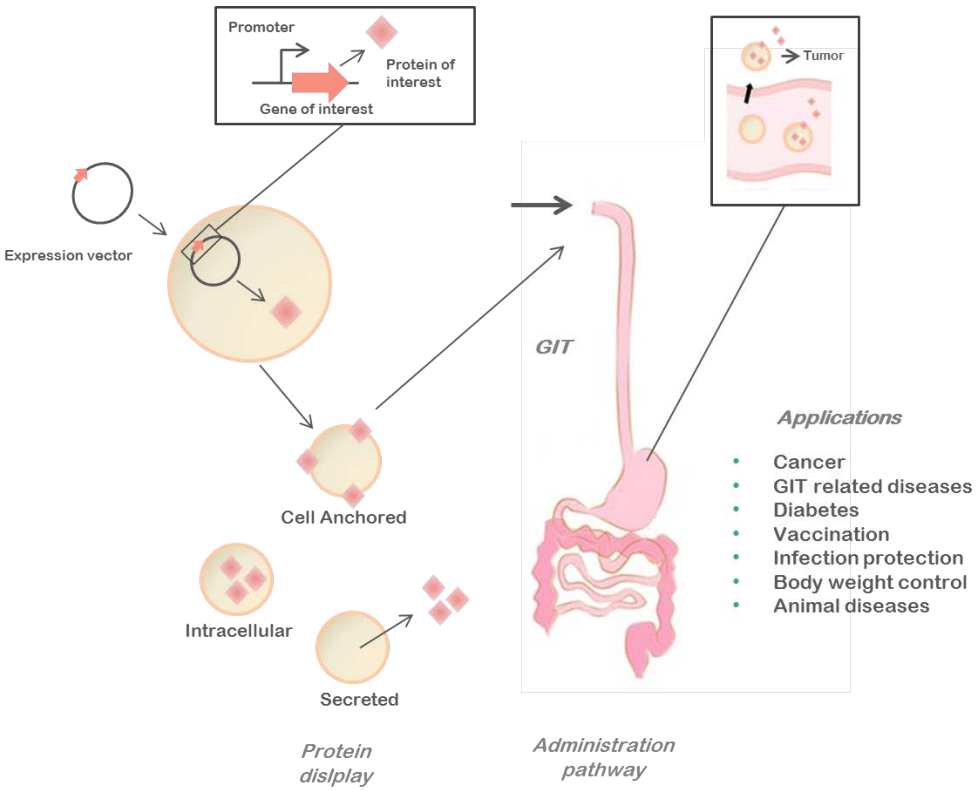


Figure 3.3: Schematic representation of the use of *L. lactis* as a live bacterial vector using the oral administration pathway. Adapted from [86].

4. Recombinant proteins

For a long time, there was a general belief that soluble recombinant proteins were highly functional and properly folded, while proteins that not reach the native conformation were inactive and trapped in the insoluble fraction, forming inclusion bodies (IBs) [31]. However, a long line of experiments have demonstrate that soluble recombinant protein in producing cells occurs in large functional spectrum and wide conformational variety, abounding soluble aggregates [178]. In summary, recombinant proteins can be understood as "a continuum of forms" [179] from soluble to insoluble protein species.

4.1 Inclusion bodies

Protein overproduction could trigger a spectrum of conformational stress responses because of the high abundance of nascent polypeptide and misfolded chains in the host cytoplasm. Under this situation, the protein quality control network tends to saturate and many proteins accumulate in the insoluble fraction as protein aggregates. Aggregates formed in *E. coli* are known as IBs [180, 181]. Even though these protein structures were only described in *E. coli,* lately protein aggregation has been observed in other microorganisms such as the Gram-negative bacteria *Caulobacter crescentus* [182], Gram-positive bacteria such as *C. glutamicum* [183] and *L. lactis* [170] and in yeast [169, 184, 185]. Also, protein aggregation is not restricted to microorganisms. Functional protein aggregates have been also described in insect cells [186] and mammalian cell factories. In the latter, the protein accumulations are called "aggresomes" [187].

Aggregates, and particularly IBs, have long been regarded as protein production by-product; amorphous masses of insoluble material essentially formed by unfolded or largely misfolded polypeptide chains and therefore biologically inert [188, 189]. Nevertheless, contrarily to what was believed, it has been shown that IBs are biologically active [190], in which the recombinant protein itself is their main component [181]. Interestingly, since IBs activity was characterised, many examples of active recombinant proteins expressed as IB have been described from enzymes [190-197] to fluorescent proteins [190, 191, 198], including therapeutic proteins [199-202] **(Figure 4.1)**.

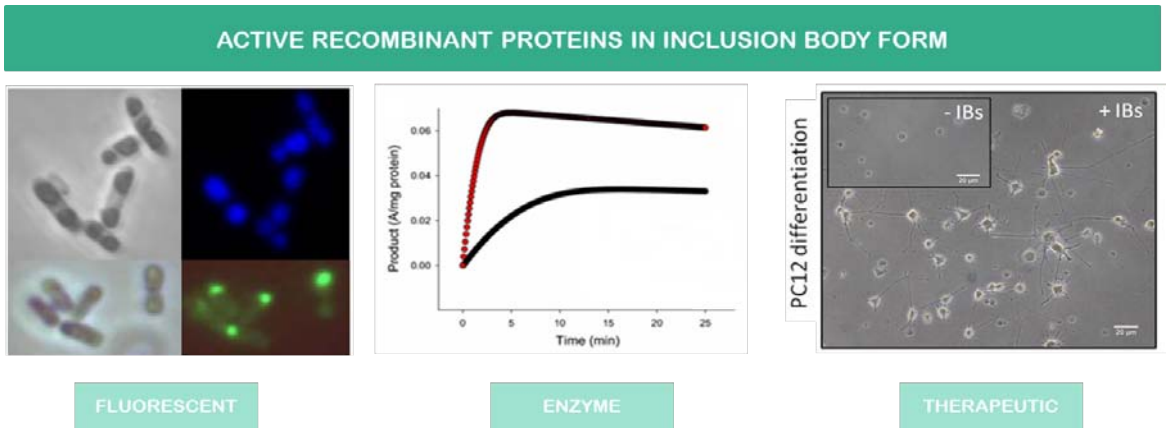


Figure 4.1: **FLUORESCENT**. Optical micrographs of Aβ42(F19D)-BFP (top) and VP1GFP (bottom) inclusion bodies by phase contrast (left) and fluorescent microscopy (right). Adapted from [190]. **ENZYMES**. ONPG hydrolysis representation determined at 414 nm catalysed by soluble β-galactosidase (black symbols) or inclusion bodies of VP1LAC (red symbols). Adapted from [190]. **THERAPEUTIC.** Biological effects of FGF-2 IBs on PC12 cell cultures. Adapted from [201].

4.1.1 IB organisation and structure

Currently, it has been largely accepted that IB formation occurs through an specific process and, as it happens in amyloid fibrils, recombinant protein aggregates have some internal order [203]. IB formation is a dynamic process between protein deposition and protein released from the aggregates [204]. Until IB formation, it is an unequal equilibrium being the rate of incorporation of polypeptides into the IBs higher than the release. Yet, once the IB is formed the equilibrium is achieved. Moreover, it has been described how in absence of protein synthesis, for example either in the stationary-phase of *E. coli* cultures [204] or during the blocking of the protein synthesis by chloramphenicol addition (Figure 4.2), protein solubilisation occurs.

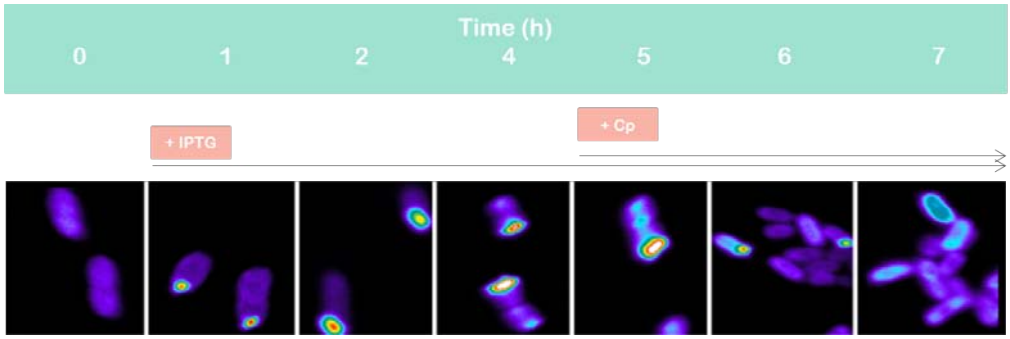


Figure 4.2: Dynamic fluorescence mapping of intracellular *E. coli* IBs, during their formation in different bacterial strains (IPTG) and when protein synthesis was blocked by the addition of chloramphenicol (Cp). Adapted from [190].

It seems that *de novo* synthesised recombinant proteins associate each other through stereo-specific hydrophobic contact, creating a starting seeding nucleus, on which other proteins will progressively settle down [198]. In this regard, the inner architecture of IBs formed in *E. coli* has been examined. As a result, it has been identified both cross β-sheet-based intermolecular and native-like secondary structures [205]. Even though there are no quantitative data available [206] structural studies on IBs have shown that such fibres represent a low proportion of the total IB mass in comparison with their abundance in conventional amyloid fibrils [207]. As a conclusion, contrary to the historical general belief, it seems that, although IBs contain a tightly packed β-sheet organization, it is not its main part. In parallel, the structural analysis supports the presence of properly folded protein [208-210], which explain the biological activity associated with IBs [190, 211, 212]. In fact, functional protein can be released *in vitro* from IBs under mild washing conditions [191, 213, 214]. Nevertheless, how native-like and amyloid-like protein coexists and are organised into these aggregate particles remains still completely unsolved [215]. The last published model suggests that IBs have a cotton-like structure in which there is a firm scaffold where native protein is entrapped into the gaps [216] (Figure 4.3).

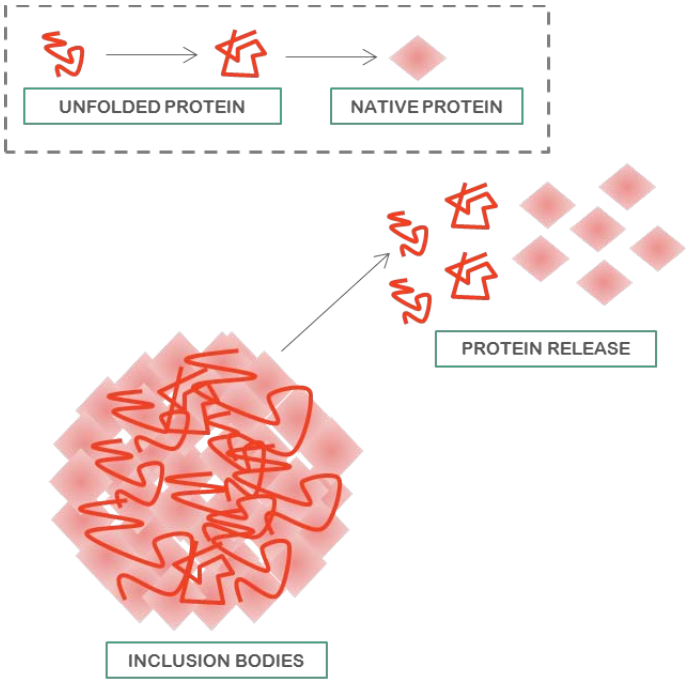


Figure 4.3: Cotton-like structure representation. IBs are composed of unfolded protein scaffold inside which the native protein is embedded. Part of the protein can be released under *in vitro* and *in vivo* conditions. Adapted from [217].

4.1.2 IB properties

IBs are highly porous, hydrated [218], and fully biocompatible particles, which present a spherical or ovoid-shape morphology, ranging from 50 to 500 nm in size [219]. Their mechanical stability and their cost-effective production processes make IBs a promising biomaterial (see section 4.1.3). Unlike soluble protein, IBs conserve their properties upon long-term storage under different conditions, such as lyophilisation [220]. Interestingly, they are extremely stable enough to resist mechanical and enzymatic treatments [221]. This property gives advantage to the production of IBs at industrial-scale and their use under diverse experimental conditions. Besides, IBs display interesting tuneable physicochemical features. At a nanoscale level, properties such as size [220], geometry [198], Z potential [222], stiffness [222], wettability [222] and density [223] are likely to be tailored by the appropriate producing genetic background [222, 224, 225] or by modulating the bacterial culture conditions for instance temperature, harvesting time or also engineering the protein gene [198, 213, 226] (Figure 4.4).

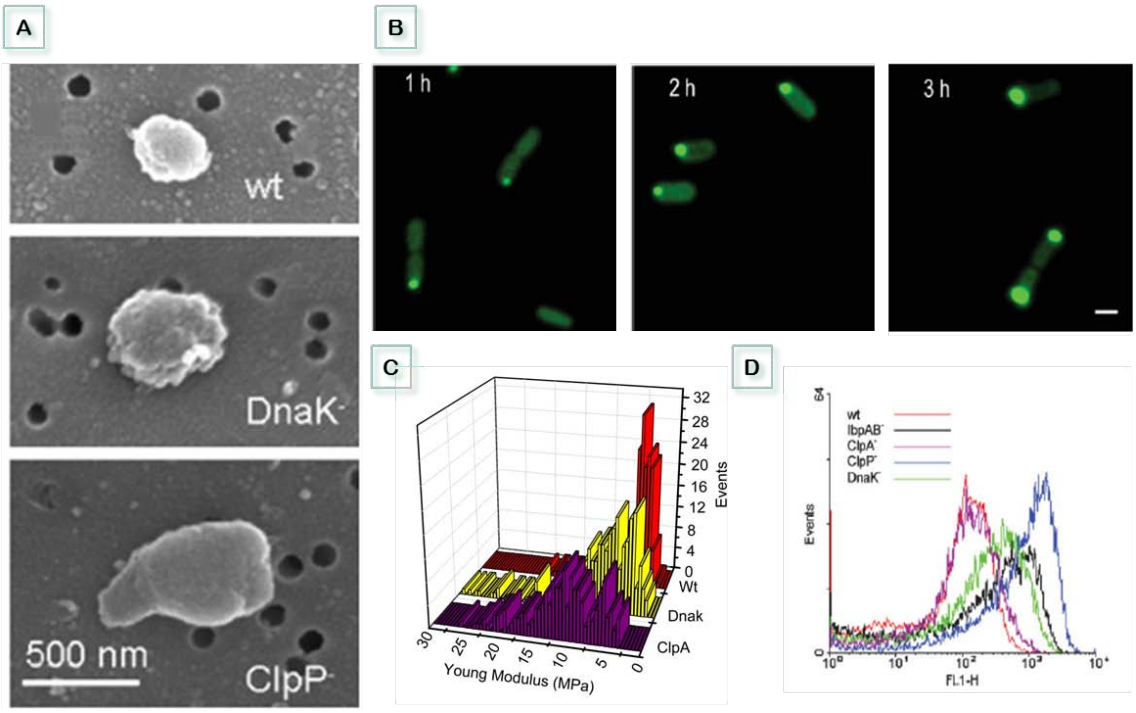


Figure 4.4: Morphological and functional characterisation of IBs. **A.** SEM micrographs of morphologically representative IBs purified from different genetic backgrounds. Adapted from [198]. **B.** Confocal microscopy micrographs of *E. coli* cells overproducing IBs. From top to the bottom, cell sample at 1 h, 2 h and 3 h after protein induction showing different IBs size. Scale bar: 1µm. Adapted from [227]. **C.** Histogram representation of different stiffness of IBs produced in different *E. coli* strains . Adapted from [222]. **D.** Distinct activities of IBs produces in different *E. coli* strain analysed by flow cytometry. Adapted from [202].

4.1.3 Biotechnological applications of IBs

4.1.3.1 Source for soluble protein extraction.

Protein aggregation in bacteria is the major bottleneck for the production of soluble and stable polypeptides for industrial and research purposes. Many approaches have been pursued to minimize the IB formation either by the coproduction of chaperones [228], protein engineering, adjusting gene dosage and the transcription rate, reducing the production temperature or adding fusion proteins [229] such as gluthathione-S-transferase (GST), maltose-binding protein (MBP), small ubiquitin modified (SUMO), N-Utilization substance (NusA), thioredoxin and so on [230]. However, these strategies are particularly inefficient and protein-dependent (Figure 4.5).

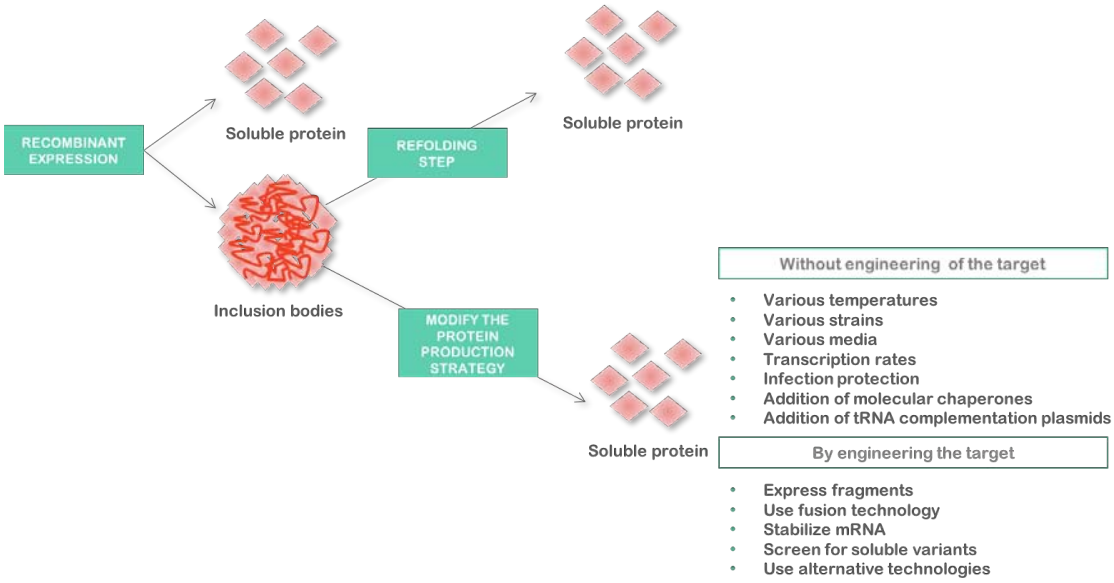


Figure 4.5: Summary of the downstream methods employed to obtain soluble protein. Adapted from [228].

However, because IBs are mainly formed by the overproduced recombinant protein, they are a convenient source of relatively pure protein. In this context, many protocols aiming to extract these proteins have been developed [231]. Traditionally, protein recovery from IBs was performed by adding high concentration of denaturants such as 6-8 M urea and 6 M guanidine hydrochloride (GdnHCl) with the aim to completely denaturalise the protein structure [232, 233]. Nevertheless, in some cases, the use of these harsh denaturants can even favour protein aggregation during the refolding process [234]. In this context, a mild solubilisation protocol has recently been reported to extract native-like proteins from IBs, using non-denaturing buffer with low concentration of organic solvents and detergents, without requiring any refolding step [231, 234, 235].

4.1.3.2 Immobilized enzymes in biocatalysis

The functional nature of IBs provides them an appealing potential for their use in purposes in which protein aggregation fundamentally is not a major obstacle [236]. Therefore, the catalytic activity of many recombinant enzymes produced as IBs have been deeply explored, concluding that IBs are an effective, new kind of biocatalysts [237]. So far, a wide variety of enzymes in IB format have been tested such as phosphatases [226], oxidases [193], lipases [238], aldolases [194, 239], phosphorylases [195], and kinases [192], opening a wide range of possibilities for the industry.

Importantly, the use of IB as catalysts has arisen as a noteworthy strategy in order to solve the issues related to enzyme immobilisation traditionally used for biocatalysis purposes [240]. As previously mentioned, recombinant proteins can be difficult to produce in soluble format. Moreover, although proteins are immobilised with the aim of increasing the protein half-life and stability, it has been described that some soluble enzymes suffer a decrease of their activity when immobilised [193]. Besides, some studies claim that the production of IBs is at least 20 times cheaper than the production of their soluble counterpart [226]. Thus, IBs are an appealing alternative due to their high stability, being also likely to be reused in repeated-batch reactions [195]. In addition, IB porosity allows the substrate transfer through the IB scaffold and the rugose nature of their surface would benefit the IB immobilisation.

4.1.3.3 Tissue engineering

In the last decades, tissue engineering and regenerative medicine have become fields of growing interest and the demand of new biomaterials as scaffolds for cell growth is significantly increasing [241]. Thus far, most of the materials used for these purposes have been obtained through synthetic processes. However, these synthetic materials (metals, ceramics and polymers) show important drawbacks for human health [242]. In this regard, biological fabrication of materials using microorganisms offers an interesting opportunity, being produced through a cost-effective and fully scalable process to industrial standards. In this context, bacterial IBs offer intriguing possibilities as biomaterial for tissue engineering because of being a biocompatible and mechanically stable protein-based nanoparticle. In addition, contrarily to what occurs with other materials, IBs, once purified, are “ready to be used” [243]. Therefore, any cross-linking step, which can affect proteins function and structure, is required.

A perfect biomaterial should be able to mimic the natural mammalian cell environment, formed by a dense and overcrowded extracellular matrix rich in both mechanical and biological signals, controlling important cellular processes such as cell adhesion, migration, apoptosis,

proliferation and differentiation [244, 245]. Nowadays, it has been described that the three main properties of materials to control cell growth, proliferation and differentiation are chemistry, stiffness and topography [241].

Two principal approaches exist for the development of biocompatible surfaces, named top-down and bottom-up technologies [246]. In the first case, the topography of the desired surface is modified by either groove or pit using lithographic methods [247]. Otherwise, bottom-up strategy is based on the surface decoration by using materials like nanotubes [248, 249] or spherical particles [250-252] made of different materials. Although top-down approaches are usually used for *in vitro* research, it is necessary a fabrication process, being not suitable to construct scaffolds for bioengineering and nanomedicine applications [241]. Using a bottom-up approach, IBs formed by irrelevant proteins have been used to randomly decorate surfaces, rendering a significant stimulation of cell adhesion and proliferation in several mammalian cell lines without any sign of cytotoxicity [220, 253] **(Figure 4.6)**.

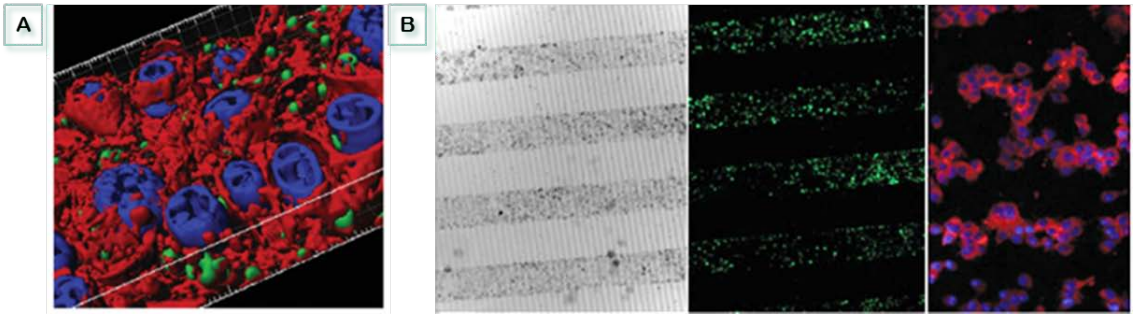


Figure 4.6: IBs-stimulated mammalian cell proliferation **A.** Confocal stack of 22 sections of BHK cell growing onto GFP bacterial IBs and processed with Imaris 3D. **B.** Micrographs of 50 µm lined IB-patterned surfaces taken by conventional (top), confocal microscopy of the IBs (middle) and BHK cell distribution after 48 of growth on the decoration (bottom). Adapted from [202].

Interestingly, it has been described that biologically active protein can be extracted *in vitro* from IBs under mild washing conditions [213] without losing their structural scaffold. In the manner of the secretory granules from the endocrine system, native protein is slowly released to the medium from protein accumulations [254, 255]; being a potential protein format to control recombinant drug administration. IBs formed by specific proteins with a key role in cell proliferation and differentiation such as growth and morphogenic factors [199, 202] have been successfully used to decorate scaffold material, creating a multifunctional cell adhesive surface able to influence on cell behaviour and as well, favouring cells with the biological activity of the released protein from IBs [256, 257].

Importantly, as previously mentioned, nanoscale properties of IBs such as wettability, Z-potential, rigidity, contact angle, roughness, morphology, stiffness and/or geometry [198, 220, 222] can be easily modulated using different bacterial genetic backgrounds. Thus, it is possible to create a library of nanoparticles able to cover all cell requirements.

4.1.3.4 Protein delivery system

The natural liberation of functional proteins from IBs in aqueous environment [191, 213], but also their uptake by mammalian cells and their biocompatibility with cell interfaces [198, 222, 258] (Figure 4.7), empowers IBs as promising advanced protein-based nanoparticles for cell therapy (known as Nanopill technology) [199, 202, 259, 260]. In recent studies, an important phenotypic effect was noticed upon mammalian cells exposure to IBs formed by therapeutic proteins (Heat shock protein 70 (Hsp70), catalase (CAT), dihydrofolate reductase (DHFR), keratin 14 (K14) and leukemia inhibitory factor (LIF)) administrated in suspension *in vitro* [199, 202, 217].

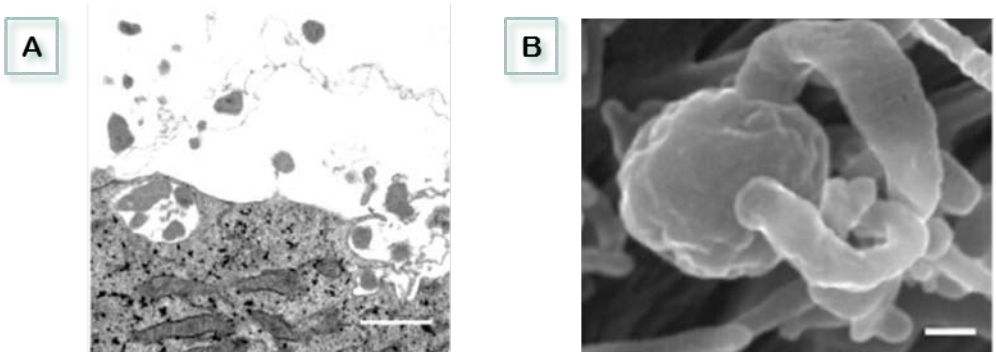


Figure 4.7: IBs cell uptake. **A.** TEM micrographs illustrating the IB endocytic pathway in HeLa cells. Scale bar: 1 µm. **B.** SEM images showing in detail the contact between IB and cell filopodia. Scale bar: 200 nm Adapted from [261].

Intriguingly, in spite of being preliminary *in vivo* studies, it has been observed that the oral administration of high doses of IB was harmless to mice models [258]. In particular, mice did show neither intolerance, physical discomfort nor any histologic damage in the intestine. Therefore, despite their amyloid structure, IBs do not present any cytotoxicity and side-effect *in vivo*.

4.2 Soluble self-assembling proteins

In the current situation, genetic engineering techniques allow the incorporation of different specific activities into the same heterologous protein. Modular proteins are based on different functional domains in the same single polypeptide [262], conferring for example DNA

condensation, a therapeutic domain, cytosolic trafficking, endosomal space, cell targeting, internalisation, nuclear localisation, blood brain barrier crossing ability or systemic stability [263, 264] which could offer intriguing possibilities in gene therapy.

In the context of therapeutic proteins or those intended as vehicles for drugs, it has been described that monomeric proteins are not stable enough and do not present a correct biodistribution [265], being in general soluble protein nanoparticles more advantageous to be used in nanomedicine in comparison with single proteins due to their stability and high penetrability in cells.

Soluble proteins which are able to organise themselves into a supramolecular structure within the nanoscale are true protein nanoparticles. Self-assembling proteins spontaneously associate to form thermodynamically stable, soluble and ordered constructions. Basically, these structures are conformed by cross-molecular interactions between proteins and stabilized by weak non-covalent interactions between protein monomers [266-268]. Nevertheless, there is still a lot to do in order to know about the process that drive and control self-assembling and nanoparticles features.

Whereas size and morphology have a significant role in nanoparticle biodistribution, a rational control of them in self-assembling proteins is a requisite for their application in nanomedicine. It has been described that a stable conformation above 10 nm permits to escape from the renal filtration while nanoparticles bigger than 200 nm could be cleared by spleen [265]. Besides, size and morphology are also decisive for cellular uptake, toxicity and immunogenicity [269].

In the lasts years, soluble self-assembling proteins such as virus-like particles (VLPs) [270, 271], bacterial microcompartments (BMCs) [272, 273], modular proteins [274] and eukaryotic vaults [275] have been deeply studied as delivery vectors, being a safer alternative to viral gene therapy. Our group has made many efforts in the development of architectonic tags [276] to promote soluble protein self-assembling. The combination of cationic and poly-histidine peptides at both the N and C-terminal drive the spontaneous protein self-assembling in monodisperse protein nanoparticles [276]. According to structural models, the extreme of a protein interacts with the opposite extreme of the adjacent conforming a rod-shaped or disk-shaped supramolecular structure [262] (Figure 4.8).

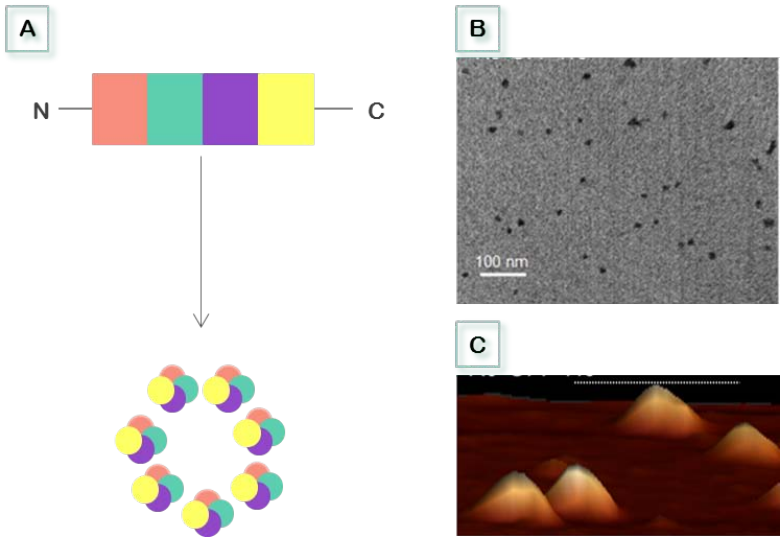


Figure 4.8: Self-assembling modular proteins. **A.** Schematic representation of modular protein. Adapted from [277]. **B.** TEM micrographs of protein nanoparticles formed by modular proteins. Adapted from [265] **C.** AFM images of protein nanoparticles formed by modular proteins .Adapted from [265].

Up to now, these modular self-assembling proteins has only been explored in different *E. coli* genetic backgrounds such as LPS-free, non-disulphide bridge formation and in the protein quality system mutant strains. Interestingly, no important differences in the size and superficial charge have been observed between soluble self-assembling. However, when tested in cancer cell lines penetrability and biodistribution was dramatically influenced by the producer strain. This fact allows choosing the most suitable nanoparticle among a variety of them [278].

5. Nanobiotechnology for animal and human medicine

The nanoparticle configuration, either IB or soluble self-assembling particles, permit to resolve problems that were associated to the use of soluble recombinant proteins. On one side in veterinary, it is necessary to develop inexpensive treatments, being IBs a costless alternative with a great potential and versatility. On the other side, the soluble configuration poses some problems on stability, pharmacokinetics and cost-effectiveness; therefore, it is also indispensable to look for drug alternatives in human medicine. In this case, the self-assembling soluble nanoparticles are a promising approach showing an enormous functional flexibility because they can be pre-designed and engineered. These self-assembling proteins nanoparticles produced in a LPS-free microbial cell factory are highly-effective, non-hazardous, safe and cost-effective [279], being an ideal product for animal and human medicine. In our study, we have chosen the following models as examples to test our nanoparticles: in the veterinary field, the cow dry period (a relevant issue in the animal production sector) and in the human medicine field, the colon cancer model (the third most common cancer type and the fourth cause of cancer death in the world).

5.1 Veterinary

Prevention and control of animal diseases are crucial in livestock farming. Some of them cause animal mortality, but in other cases such diseases, although they do not regularly result in the death of the animal, provoke a drastic reduction of their productivity, causing considerable economic losses. In many cases, antibiotics are used to treat or prevent many of these diseases. Particularly in livestock, the proportion of antibiotics used is much greater than in humans, besides the fact that, many of the antibiotics used are deemed medically necessary for human health. Bacterial resistances can disseminate through many routes, being significantly the most concerning the food chain because the presence of antimicrobial resistant bacteria in food and animals can be acquired by human consumers. Therefore, in 2001, the European Commission launched a strategy aiming to combat the excessive use of antibiotics which is causing an alarming increase in the antibiotic resistances.

Recently, in China and in some European countries, a colistin-resistant *E. coli* has been detected in animals, but also in people. Colistin is a last resort antibiotic against multidrug resistant Gram-negative bacteria in humans, as well as an antibiotic routinely administrated to animals. This fact strongly confirms that the antibiotic resistance was acquired due to colistin

use in animals, showing the danger of treating animals with valuable antibiotics for humans. Thus, given that alarming situation, it is fundamental to find alternatives in order to reduce the antibiotic administrations.

In this context, it is widely accepted that antibiotic use should be limited to treat specific diseases, but not for preventive purposes [280]. Experts suggest that an easy solution should be establishing a limit of antibiotics use in farming and banning the animal treatment with critical antibiotics for human health. Besides, the development of antibiotic alternatives, massive vaccinations and rapid diagnostics should be carefully considered to find a solution to this complex problem.

5.1.1 Cow dry period

Antibiotics are routinely administrated to foresee animal diseases and therefore increase the animal production, being the cow dry period a concrete case of preventive use of antibiotics. In dairy cows, a non-lactating period is necessary between lactations to ensure cell turnover in the secretory epithelium of mammary gland for optimal milk production in the next lactation. A 60-day dry period has been standardised for most dairy cow farms, which significantly reduce the duration of the productive period of cows. However, shortening the dry period has been described to provoke important milk production losses [281]. Dry period approaches are based on either reduce or eliminate the animal milking, being the most usual the abrupt dry off. Basically, the dry period is characterised by two principal stages: the mammary gland involution and the tissue regeneration [282] (Figure 5.1). Physiologically, milk stasis together with protein and hormonal factors prompt the active gland involution and also stop milk secretion.

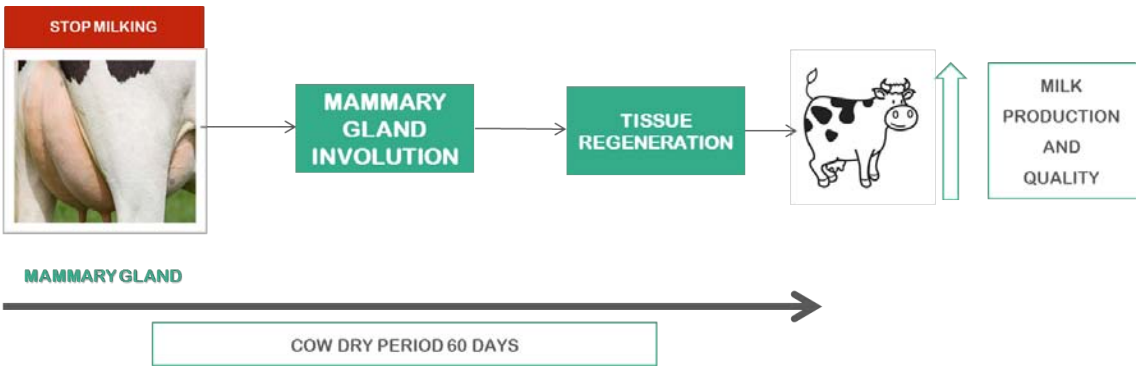


Figure 5.1: Schematic representation of the typical cow dry period.

The extracellular matrix degradation is the key signal that triggers the involution and cellular apoptosis [283], being the matrix metalloproteinases (MMPs) the essential enzymes in this

process [284]. Furthermore, it has been described that MMPs stimulate the release of cytokines and growth factors, modulating the immunity system and the cell growth [284].

Importantly, milk stasis during this non-productive phase is associated to higher risk to contract intramammary infections and acute pain [285]. This affects seriously the animal welfare but also, incurs in milk production losses and in a reduction of milk quality in the next lactation [286]. Bovine mastitis is the most common diseases of cows, causing economic losses of about 1.5 billion euros each year in the dairy sector. At present, the preventive use of antibiotics is the usual protocol to prevent the risk of infections (Figure 5.2), since no other therapies are available. However, understanding that the European Commission promotes its reduction, many studies have aimed at finding an alternative against antibiotics by stimulating the immune system recruiting phagocytes to fight infections in a time when the mammary gland health is compromised. In this regard, nanomedicine could have an important role to find alternatives to the preventive use of antibiotics.

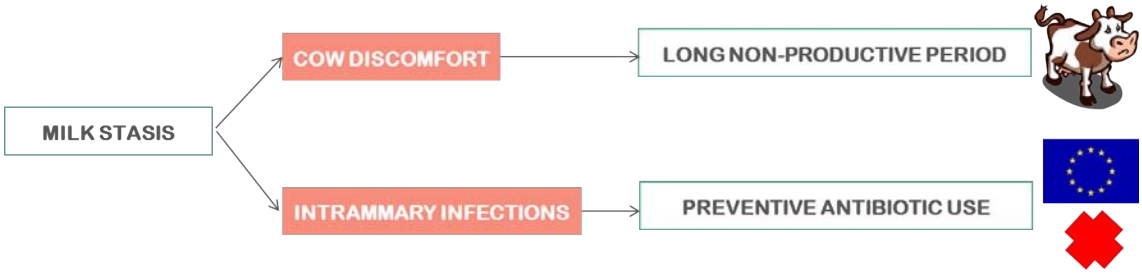


Figure 5.2: Schematic representation of the typical cow dry period.

Some studies have suggested the potential of the *Panax ginseng* root extract as immunomodulatory and immune response adjuvant. Even though the subcutaneous injection at the beginning of the cow dry period show an increase of the phagocytic and neutrophil activity during one week treatment, only a decrease of the bacterial growth is only observed in *Staphylococcus aureus* infected mammary glands [287]. About the immune stimulation, the LPS administration has also been explored. LPS infusion generates a massive leukocytes response and an increasing of immune cells tissue infiltration. Nevertheless, the correlation between LPS stimulation and the acceleration of the mammary gland involution is not clear enough [288]. Finally, there are many *in vivo* studies focused on the soluble recombinant cytokines administration to fight against intramammary infections by immune system stimulation. The infusion of recombinant IL-2 produced in *E. coli* protects the mammary gland against *S. aureus* and *E. coli* infection [289]. Nevertheless, its use during the cow dry period is related to an abort tax increase; probably a side-effects of a either LPS or endotoxin contamination. Moreover, the

recombinant IL-8 intramammary injections immediately diminish the *S. aureus* infection [290] and the immunotherapy with soluble recombinant IFN-γ has been tested in cattle, presenting a high potential against *S. aureus* and *E. coli* without side-effects [291].

Although these studies present some evidences indicating that the exogenous administration of recombinant cytokines is an appealing strategy, there are not still conclusive results. In this sense, the nanomedicine, as before mentioned, can play an important role to fill this gap. In our study we have developed nanoparticles to get a stronger immune system response and a fast tissue regeneration to eventually replace the antibiotic use in cow dry period.

5.2 Human medicine

Cancer is the second cause of death worldwide. Current cancer therapies are principally surgery, chemotherapy and radiotherapy that far from being effective, present high systemic toxicity, strong side-effects and low patient survival rates when patient are diagnoses at late state of the disease. The existing drugs are also ineffective enough at non-toxic administrable doses, on account of this, more effective drugs improving target specificity, lower toxicity, good pharmacokinetic and high therapeutic activity are needed. In this regard, the development of targeted treatments is a promising approach to overwhelm the restrictions of the conventional therapies.

Some targeted drug therapies based on small molecule inhibitors and monoclonal antibodies have been proved. Although those drugs have prolonged patient survival in general terms, there is still a lot of work to do to cure most of patients in any stage of illness. Those drugs have many limitations to overcome for example: the small molecules easily diffuse (being therefore not specific enough) and monoclonal antibodies just target protein in the surface whereas many cancer targets are intracellular. In this context, the number of peptides and proteins described as tumor-peptides is growing because contrary to other therapies, they have the ability to distinguish between healthy and sick cell and even between specific tumor types. In this regard, multimeric self-assembling proteins are a promising therapy due to their high biocompatibility, biosafety, low toxicity and tuneability [263]. Those self-assembling protein-based nanoparticles could specifically deliver therapeutic component in the desired cells, increasing the local drug concentration and reducing the side effects [292, 293].

5.2.1 Colorectal cancer

Colorectal cancer is the second most common cancer in the developed countries with an average of 5-years survival on the USA [294]. This cancer sometimes begins with a sub-mucosal compartment that is simply removed by surgery. Nevertheless, in the 25 % of colon cancers metastatic foci can be found, decreasing life survival of less than 10%.

As other cancer therapies, current treatments have high toxicity and attempts to target drugs with antibodies have not being so effective because of the low uptake into the tumor. Thus, there is a critical necessity of therapies to target metastatic cancer cells. For this reason, targeted self-assembling soluble protein nanoparticles are very promising tools biocompatible and highly specific.

In particular, our group has made self-assembling soluble protein-based nanoparticles targeted to bind to CXCR4 receptor (a cell surface marker associated to cancer colorectal) which present an excellent *in vitro* performance and *in vivo* accumulation principally in tumor and metastasis in colorectal cancer mice model; showing also a robust targeting and biological behaviour of these protein nanoparticles [265].

6. Model proteins

The principal features and functions of the proteins used in this work are presented below.

6.1 Bovine metalloproteinases 2 (MMP-2) and 9 (MMP-9)

Metalloproteiases (MMPs) are zinc-dependent endopeptidases that are secreted as proenzymes and subsequently activated by the plasminogen activator (PA) system [284]. According to the structure, MMPs can be divided into 8 groups. Among all MMPs, MMP-2 and MMP-9 are two of the most widely active metalloproteinases in cattle [295], specially these proteins have an important role in the mammary gland involution [284]. Therefore, their endogenous administration could accelerate the gland involution, being their use promising in order to short and optimize the cow dry period.

Although recombinant bovine MMP-2 and MMP-9 protein production have not been described yet, their homologous human proteins have been previously produced in their active form in baculovirus [296, 297]. The common structure of MMPs is a multidomain, characterised by a zinc-dependent catalytic domain linked to a fibronectin type II-like triple repeat motif to bind and degrade a type of collagen IV (the major component of basement membranes) or denatured collagen (also known as gelatine) [296]. Besides, MMPs also contains a flexible hinge region and then at C-terminal a haemopexin-binding domain in which regulatory proteins binds (Figure 6.1).

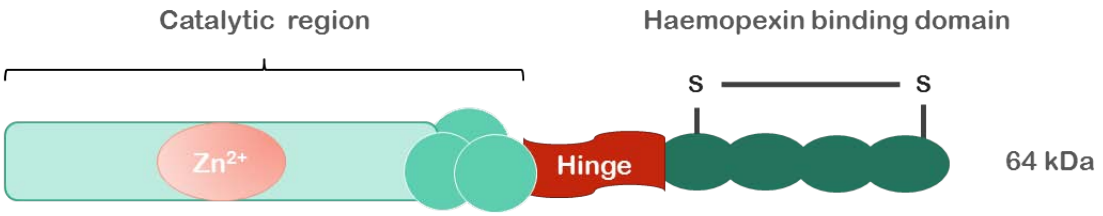


Figure 6.1: Schematic representation of MMP-9 and MMP-2 protein domains. The Zn-catalytic domain (pink) fused to three fibronectin motifs (light green spheres). Following, the hinge motif and at C-terminal the haemopexin binding domain. Adapted from [298].

By sequence analysis, a high homology between the human and the bovine form has been confirmed, being about a 99% for MMP-2 and 79% for MMP-9. Considering the published information about the human MMP-2, we have synthetized the fragment comprised between Tyr110 y Asp452 (NCBI, NM_174745), which codifies the catalytic domain with the fibronectin domain of this peptidase (39.19 KDa). On the other side, from bovine MMP-9, we have cloned

the fragment from Phe107 y Pro449 (NCBI, NM_174744.2), which also codifies the catalytic domain with the fibronectin domain (39.19 KDa).

6.2 Bovine interferon gamma (IFN-γ)

Interferon gamma (IFN-γ) is a cytokine from group II of IFNs. IFN-γ has been described as the main modulator of the immune and inflammatory stimuli [299] by activating neutrophils and the lymphocytes expansion. As mentioned before, the immunotherapy with soluble IFN-γ has been tested and presents encouraging results [291]. However, the production of this kind of protein in soluble format is associated to instability and to prohibitive production costs to be implemented in animal health.

Native IFN-γ is a 166 amino-acid protein, including a 23 amino-acid signal sequence at C-terminal. Moreover, it suffers some post-translational modification such a two glycosylations [300] and the conversion of the last amino acid into a pyroglutamate [301]. The tertiary structure consists of six α-helix forming two domains a N-terminal one with four of the α-helixes and a C-terminal one with only two of them [302]. However, the biologically active protein is the dimer, being the presence of two copies of the proteins in an anti-parallel inter-locking quaternary conformation necessary in order to bind with two other copies to finally bind to its high-affinity receptor [303] (Figure 6.2). In our study we have produced the mature protein from Glu23 to Tre101 (NM_173925).

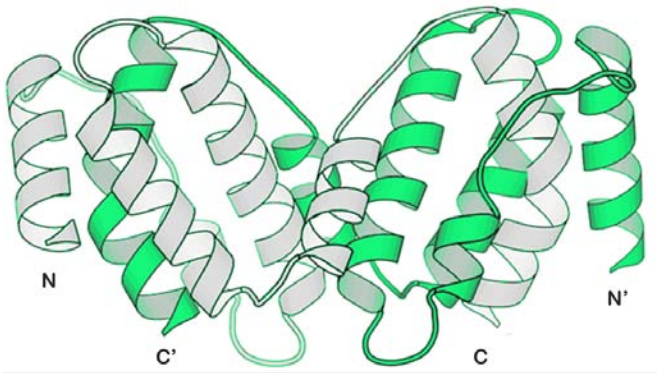


Figure 6.2: Representation of the bovine IFN-γ. Adapted from [302]

6.3 Green fluorescent protein (GFP) fusion

Even though green fluorescent protein (GFP) exists in many organisms, nowadays the most widely used is the GFP from *Aequorea victoria* [304]. Interestingly, the GFP genetic sequence contains all the necessary information for the proper formation of the chromophore which does not need any specific enzyme or cofactor to emit fluorescence. Nowadays a GFP mutant, also called enhanced GFP (EGFP) with higher fluorescence is extensively being used. EGFP has only one absorption maximum at about 488 nm and one emission maximum around 507 nm and, also, it is resistant against photobleaching [305].

About the three-dimensional structure, GFP is composed by 11-stranded β- barrel (42 Å long and 24 Å diameter) surrounding a single α-helix where the chromophore is attached, being placed in the middle of the cylinder [306] (Figure 6.3.A). Regarding the chromophore, the residues 65-67 (Ser-Tyr-Gly) fold forming a *p*-hydroxybenzylideneimidazolinone [307].

6.3.1 VP1GFP

In our work we have used a GFP fused to the aggregation-prone VP1 capsid of foot-and-mouth disease virus (FMDV), as a reporter of protein aggregation. VP1 is one of the 4 proteins that forms the virion of foot-and-mouth disease virus (FMDV) [308] which is wedge-shaped and formed by eight-stranded β- sandwiches [309]. One of the VP1 particularities is its G-H-loop which contains the major antigenic site of the virus between residues 138 and 150 and as well, the highly conserved sequence Arg-Gly-Arg (RGD) to interact to integrin receptors [310] (Figure 6.3 B). Studies reveal that when GFP is fused with VP1, 80% of the proteins go to the insoluble fraction [190].

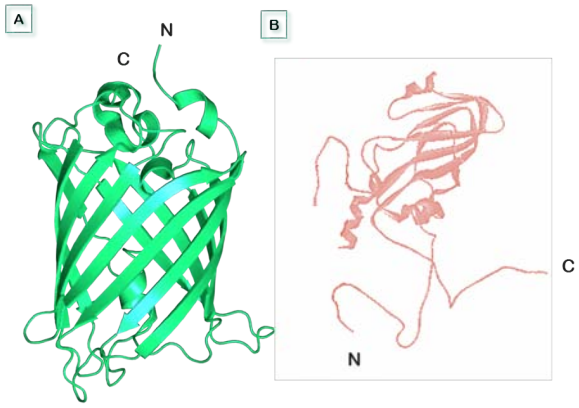


Figure 6.3: Schematic representation of the proteins forming VP1GFP **A.** GFP from *Aequorea victoria.* **B.** VP1 from the foot-and-mouth disease virus.

6.3.2 T22-GFP-H6

T22-GFP-H6 is a multimeric protein formed by the T22 ligand at C-terminal, also known as, polyphemusin II peptide from horseshoe crabs' blood. Particularly, T22 has three amino acid mutations (Tyr5, Lys7 and Tyr12) in order to enhance their natural affinity to CXCR4 receptor [311, 312]. Moreover, at N-terminal there is a 6 histidine tag for self-assembling and purification purposes

When produced in *E. coli,* it has been described that these proteins spontaneously self-assembles into soluble nanoparticles of about 15 nm. The nanoparticle formation is due to the interactions between the amino terminal T22 and the carboxy terminal H6 [264].

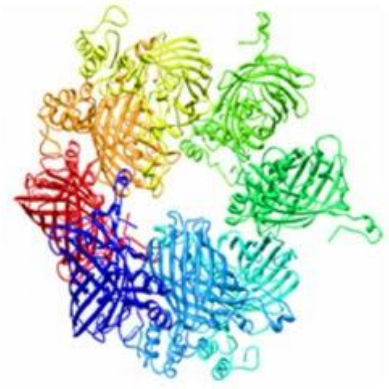
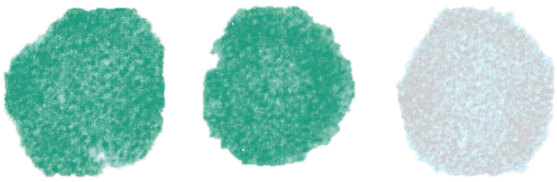


Figure 6.4: Possible organization of T22-GFP-H6 as octamers in which T22 supports the electrostatic self-assembling on the complex. Adapted from [263].



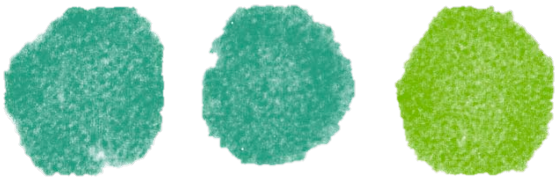
Objectives

Up to now, it has been detected that IBs are formed by relative amounts of functional and non-functional proteins species of a same protein. The aim of the first part of this thesis is to explore how these protein species are organised forming mechanical stable IBs by a time course Proteinase K digestion kinetic. In order to reach these goals, several specific issues have been addressed:

- To analyse and characterise potentially different IB protein populations.
- To explore the architecture of IB produced in different *E. coli* genetic backgrounds. Specifically, strain deficient in genes of the protein quality control network, for instance DnaK$^-$, ClpA$^-$and ClpP$^-$.
- To determine which part of the IB structure is responsible for the mechanical stimulation of mammalian cell growth when used as topographies in cell culture.

On account of the protein nanoparticle great potential in human and animal medicine, the aim of the second part of this thesis is to set up and explore their production in the LPS expression system *L. lactis*, more appropriate for their future applicability. For this purpose, we have set the following objectives:

- To explore different protein production conditions and different *L. lactis* genetic backgrounds.
- To analyse the conformational quality and solubility of produced proteins in the previous conditions.
- To isolate and characterise the main morphometric and physico-chemical features of these protein-based nanoparticles.
- To produce relevant proteins in veterinary medicine as protein nanoparticles (aggregates) and test their activity.
- To produce modular proteins and test the self-assembling soluble nanoparticle formation.
- To compare the behaviour of *L. lactis* and *E. coli* nanoparticles *in vitro*.
- To test their functionality *in vivo*.



Results

Article 1

Supramolecular organization of protein-releasing functional amyloids solved in bacterial inclusion bodies

Cano-Garrido O*, Rodríguez-Carmona E*, Díez-Gil C, Vázquez E, Elizondo E, Cubarsi R, Seras-Franzoso J, Corchero JL, Rinas U, Ratera I, Ventosa N, Veciana J, Villaverde A, Garcia-Fruitós E.

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*Equal contribution

Since the biological activity associated to IBs was demonstrated, several studies have deeply explored their physicochemical properties and diverse promising applications as a protein-based functional biomaterial.

According to the architecture, the inner structure of IBs was regarded as fully amorphous for a long time. Nevertheless, several studies have revealed amyloid-type characteristics on different IB-forming proteins such as their ability to bind to Thioflavin T and Congo red, and as well, intermolecular β-structures detected by Fourier transform infrared spectroscopy (FTIR). Simultaneously, the biological activity has evidenced that IBs are formed also by an important percentage of native or native-like proteins. Actually, it has been tested the efficacy of mild-washing protocols to liberate functional protein from IBs.

In this context, the way in which amyloid-like and native-like polypeptides are organized remains still unsolved. Therefore, in this work, we have analysed *E. coli* IBs produced in different bacterial genetic backgrounds by using diverse analytic approaches with the aim of determine their supramolecular organization.

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Supramolecular organization of protein-releasing functional amyloids solved in bacterial inclusion bodies

Olivia Cano-Garrido [a,b,c,1], Escarlata Rodríguez-Carmona [a,b,c,1], César Díez-Gil [c,d], Esther Vázquez [a,b,c], Elisa Elizondo [c,d], Rafael Cubarsi [c,e], Joaquin Seras-Franzoso [a,b,c], José Luis Corchero [c,a,b], Ursula Rinas [f,g], Imma Ratera [c,d], Nora Ventosa [c,d], Jaume Veciana [c,d], Antonio Villaverde [a,b,c,*], Elena García-Fruitós [a,b,c]

[a] Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain
[b] Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain
[c] CIBER en Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Bellaterra, 08193 Barcelona, Spain
[d] Departament de Nanociència Molecular i Materials Orgànics, Institut de Ciència de Materials de Barcelona (ICMAB-CSIC), Bellaterra, 08193 Barcelona, Spain
[e] Departament de Matemàtica Aplicada IV, Universitat Politècnica de Catalunya, Jordi Girona 1-3, 08034 Barcelona, Spain
[f] Institute of Technical Chemistry-Life Science, Leibniz University of Hannover, D-30167 Hannover, Germany
[g] Helmholtz Centre for Infection Research, Inhoffenstraße 7, D-38124 Braunschweig, Germany

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ABSTRACT

Slow protein release from amyloidal materials is a molecular platform used by nature to control protein hormone secretion in the endocrine system. The molecular mechanics of the sustained protein release from amyloids remains essentially unexplored. Inclusion bodies (IBs) are natural amyloids that occur as discrete protein nanoparticles in recombinant bacteria. These protein clusters have been recently explored as protein-based functional biomaterials with diverse biomedical applications, and adapted as nanopills to deliver recombinant protein drugs into mammalian cells. Interestingly, the slow protein release from IBs does not significantly affect the particulate organization and morphology of the material, suggesting the occurrence of a tight scaffold. Here, we have determined, by using a combined set of analytical approaches, a sponge-like supramolecular organization of IBs combining differently folded protein versions (amyloid and native-like), which supports both mechanical stability and sustained protein delivery. Apart from offering structural clues about how amyloid materials release their monomeric protein components, these findings open exciting possibilities for the tailored development of smart biofunctional materials, adapted to mimic the functions of amyloid-based secretory glands of higher organisms.

1. Introduction

Bacterial inclusion bodies (IBs) are mechanically stable protein aggregates commonly observed in bacteria during the synthesis of recombinant proteins [1]. Since their production is cost-effective and fully scalable, and the embedded proteins retain significant levels of biological activity [2,3], interest in the use, exploration and further adaptation of IBs as nanostructured functional materials has recently emerged [4–10]. The biological activity of the protein forming a given IB can be tailored by selecting the appropriate genetic background of the producer *Escherichia coli* strain [11,12]. Also, several nanoscale features of IBs can be tuned by genetic approaches or by manipulating bacterial culture conditions [5,13,14]. However, the supramolecular organization of IB polypeptides still remains unsolved. As IBs were initially considered as fully amorphous protein deposits [15], a certain extent of amyloid-like organization has been noted [16] and repeatedly confirmed through independent studies on different IB-forming proteins [9,17–22]. Therefore, IBs have been adapted as convenient models to monitor amyloid-like protein aggregation and to examine the molecular biology of prion diseases [19,20,23–25]. In fact, when treating IBs with proteinase K, to which amyloids are generically resistant, the amyloid fibers remain intact after massive protein degradation [22].

In parallel, evidence has been accumulated showing that IBs are also composed of significant fractions of functional polypeptides, forming a native or native-like secondary structure [26–29], which is indicative of proper protein folding and supports the biological activity associated with IBs [3,17,30]. In fact, functional protein can be released in vitro from IBs under mild washing conditions [31–33]. Also, in emerging therapeutic applications, IBs spontaneously internalized by mammalian cells release sufficient amounts

* Corresponding author at: Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain. Tel.: +34 935813086; fax: +34 935812011.
E-mail address: antoni.villaverde@uab.cat (A. Villaverde).
[1] These authors contributed equally.

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of functional protein to render a potent biological effect (acting as nanopills) without losing their mechanical integrity [34]. The ability of IBs to release functional proteins could be related to the slow delivery of monomeric building blocks, recently identified in a large set of protein hormones [35] which accumulate in secretory granules in form or amyloids. How these hormones are mechanistically removed from repository amyloid material is an issue that is still to be resolved [35–37].

The relative amounts of functional and non-functional protein species in IBs seems to be highly variable [3], linked to the conformational status of the bulk protein and therefore dependent on the particular polypeptide. Most importantly, it is not known how amyloid-like and native-like versions of a same protein species coexist in IBs, permitting their use as mechanically stable materials but also as slow drug release systems. Due to the broad applicability of IBs in tissue engineering [13,14,38] and in protein-based medicines by mimicking protein hormone secretion [34,39–41], it would be of extreme interest to finely dissect the IB material organization, and to understand how the architectural traits conferring structural stability and allowing the slow release of the functional material could be engineered during IB biofabrication. Therefore, here we have approached the dissection of the supramolecular organization of IB protein by the combined use of diverse analytical approaches. The obtained data, which reveals a tunable matrix-like organization of these protein particles and a bimodal protein organization, offers intriguing possibilities for the further engineering of these complexes as smart nanomaterials for efficient drug release in advanced therapies.

2. Materials and methods

2.1. Strains and plasmids

The *E. coli* strains employed in this study were MC4100 (*araD139 Δ(argF-lac) U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*, Str^R) [42] as a wild type (wt) and its derivatives JGT4 (*clpA*::*kan*), JGT20 (*dnak756 thr*::Tn10) and JGT19 (*clpP*::*cat*), deficient in the ATPase ClpA, chaperone DnaK and protease ClpP, respectively [43]. All these strains were transformed with the IPTG-inducible plasmid pTVP1GFP (Amp^R) [44], which encodes a green fluorescent protein (GFP) fused to the VP1 capsid protein of the foot-and-mouth disease virus [45]. IBs formed by the following proteins were produced in the indicated *E. coli* strains: R9-GFP-H6, a double-tagged green fluorescence protein [46,47], in Rosetta BL21 (DE3) (Cm^R)/pET21b-R9GFPH6 (Amp^R); the his-tagged human chaperone Hsp70 [34] in BL21(DE3)/pReceiver-B01 (EX-R0068-B01, from GeneCopoeia, (Amp^R)); the human leukaemia inhibitory factor (LIF) [34], fused to the C-terminus of thioredoxin (TRXLIF), in BL21(DE3)/pET32b-TRX (Amp^R); and the human basic fibroblast growth factor (FGF-2) in BL21(DE3)/pET29c(+)-hFGF-2 (Amp^R) [48].

2.2. Bacterial culture conditions

Bacteria were cultured in shake flasks at 37 °C and 250 rpm in LB medium [49,50], supplemented with required antibiotics. Recombinant gene expression was induced by 1 mM IPTG when the OD_{550nm} reached 0.5. At 3 h post-induction (4 h for JGT19/pTVP1GFP and 5 h for BL21(DE3)/pReceiver-B01), culture samples of 20 ml were taken for IB purification.

2.3. IB purification

IBs were purified as described elsewhere [51]. Briefly, culture samples were harvested by centrifugation at 4 °C and 5000*g* for 5 min, resuspended in 20 ml of lysis buffer and frozen at overnight −80 °C. After that, 100 μl of 100 mM protease inhibitor phenylmethanesulphonyfluoride (PMSF; Roche) and 400 μl of 1 mg ml^{-1} lysozyme (Roche) were added. After 2 h at 37 °C, 100 μl of Triton X-100 was added and incubated at room temperature for 1 h. Next, samples were ice-jacketed and sonicated at 40% amplitude under 0.5 s cycles until all viable cells were eliminated. Then 5 μl of Nonidet P40 (NP-40, Roche) was added to the rest of the suspension, and incubated at 4 °C for 1 h. Finally, samples were incubated with 15 μl of 1 mg ml^{-1} DNase and 12 μl of 1 M $MgSO_4$ for 45 min at 37 °C. After the incubation, the material was washed once with 1 ml of lysis buffer and 0.05% Triton X-100, and centrifuged at 4 °C and 15,000*g* for 15 min. Pellets were stored at −80 °C until analysis. All incubations were carried out under agitation.

2.4. Digestion with proteinase K

Pure IBs were resuspended in 10 ml of phosphate-buffered saline (PBS) and sonicated for 3 min in order to obtain a homogeneous suspension. Upon diluting all suspensions to an OD_{350nm} of 1 for comparison, proteinase K (to 0.01 mg ml^{-1} final concentration) was added and incubated at 37 °C under gentle shaking for 1 h. During the digestion process, OD_{350nm} was monitored every 5 min. When necessary, proteolysis was immediately arrested by adding 1 mM PMSF to the mixture. After that, samples for Western blot and cytometry analyses were centrifuged at 4 °C and 15,000*g* for 15 min before the soluble (protein released from IBs) and insoluble (IBs) fractions were separated.

2.5. Quantitative protein analysis

Both soluble and insoluble fractions were resuspended in denaturing buffer (1.28 g of Tris base, 8 ml of glycerol, 1.6 g of sodium dodecylsulphate (SDS), 4 ml of β-mercaptoethanol and 9.6 g of urea) at appropriate ratios, boiled for 5 or 45 min, respectively, and loaded onto SDS–polyacrylamide gel electrophoresis (10% acrylamide) denaturing gels. Protein bands were identified by Western blot, using a commercial polyclonal serum against GFP (Santa Cruz Biotechnology) and a monoclonal antibody against VP1. Secondary antibodies were an anti-rabbit (Bio Rad) when determining GFP and an anti-mouse (Bio Rad) when determining VP1. Gels were scanned at high resolution and bands were quantified with Quantity One Software (Bio Rad, Hercules, CA) using known concentrations of recombinant GFP as references.

2.6. Microscopic analysis

Cryo-transmission electron microscopy (cryo-TEM) of IBs was performed with a JEOL JEM-2011 transmission electron microscope (JEOL Ltd., Tokyo, Japan) using a 120 kV voltage. A sample drop was placed in a copper grid coated with a perforated polymer film. Excess solution was dried by blotting with filter paper. Immediately the film had been prepared, the grid was submerged in liquid ethane at a temperature just above its freezing point (−179.1 °C). The vitrified sample was then analysed with the microscope. To prevent sample perturbation and ice crystal formation, the specimens were kept cool (−196.15 °C) during both the transfer and viewing procedures. For confocal microscopy, samples were resuspended in PBS + 0.5% Tween, sonicated for 2 min in order to prevent the formation of big aggregates and observed under a Leica SP2 AOBS confocal fluorescence microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany), using a 488 nm excitation wavelength and an emission wavelength between 500 and 600 nm (×63, NA 1.4 oil). Micrographies were analysed using Adobe Photoshop software.

2.7. Dynamic light scattering (DLS)

IB size was measured by DLS (Malvern Nanosizer Z). Particles were resuspended in PBS (pH 7.4) and sonicated for 1 min to make the suspension homogenous. DLS measurements were performed at 37 °C in a 1 ml plastic cuvette. Every sample was analysed in triplicate, averaging 30 single measurements.

2.8. Flow cytometry

Samples were analysed with FACS Canto flow cytometer (BD Biosciences) equipped with an argon laser exciting at 488 nm and a red diode (emission wavelength 635 nm). Fluorescence emission was measured with 502LP channel (530/30 nm filter pass band). Data were analysed by WinMDI 2.8 Software.

2.9. Numerical modeling

The IB digestion process was described through a mixture of decreasing exponential functions with up to three populations. Hence, the protein amount y at time x is given as

$$y = \sum_{i=1}^{n} a_i \exp(-b_i x)$$

where n is the number of populations, a_i is the population fraction and b_i is the exponential factor, whose inverse is the expected life of the population. The half-life of the ith population is obtained from

$$T_i = \ln 2 / b_i$$

Given a set of data, $y_j = y(x_j)$; $j = 1, \ldots, m$, we estimated the number of parameters to be $p = 2n$ (eventually $p = 2n - 1$, assuming the existence of a constant nth population with $b_n = 0$). The optimal non-linear fit is then carried out by minimizing the sum of the m residuals squared

$$S^2 = \frac{1}{m-p} \sum_{i=1}^{m} (y_i - y(x_i))^2$$

so that S^2 is expected to be a χ^2 distribution with $(m–p)$ degrees of freedom. We chose the optimal fit that corresponded to the smallest S^2 value among the approximations carried out with a number of parameters from one to six. When we include a flexible exponential decay for all populations in the model, such a degree of freedom does not substantially improve the goodness of fit in any of the tested cases, according to the χ^2 probability of the squared error.

2.10. Mammalian cell culture and proliferation assay

VP1GFP IBs produced in a *E. coli* DnaK$^-$ strain for 3 h were resuspended in sterile PBS, and 8 μg of the material was used to coat non-treated Costar 3370 96-well plates, which were left overnight at 4 °C for binding. Wells were washed in PBS and blocked with 3% bovine serum albumin in PBS for 1 h at 37 °C. Afterwards, 2.5×10^3 newborn hamster kidney (BHK) cells, generously provided by E. Domingo, were seeded per well and incubated in Dulbecco's modified Eagle's medium supplemented with non-essential amino acids, foetal calf serum (5%), gentamicin and antimycotics at 37 °C for 48 and 72 h. Blank wells underwent exactly the same treatment described above but in the absence of IBs.

After incubation, cell proliferation was determined using the EZ4U kit (Biomedica, GmbH) following the manufacturer's instructions, and analysed in the multilabel reader VICTOR^{3}V 1420 (PerkinElmer). The reading absorbance was 450 nm, with 620 nm as a reference, and the values were standardized with respect to medium-containing wells. A pre-test to select the incubation time before saturation was carried out with the kit reagents. All assays were done in triplicate. Data were expressed as the mean ± standard error of the mean of the values obtained per condition and evaluated statistically by a one-way analysis of variance test, followed by Bonferroni's post-hoc analysis.

3. Results

Although it is known that IBs contain proteinase K-resistant material organized as amyloid fibers [22], the relative amount of this protein version within the particles had been never investigated. To approach this issue, we determined the material in VP1GFP IBs that remained resistant to digestion by using a time-course approach. For that, we selected as the starting material IBs formed in different *E. coli* mutant strains in which several key genes of the protein quality control network had been knocked down (*dnaK*, *clpA* and *clpP*). As observed (Fig. 1), a small fraction of IB protein (usually less than 20%) was progressively lost in the absence of proteinase K, indicative of spontaneous protein release under mild conditions, as previously described [31,33]. More interestingly, the disintegration kinetics promoted by proteinase K was highly strain dependent. This indicates that, as expected, the conformational status of the IB protein (and eventually the IB amyloid content) is regulated by the activities of the cell's protein quality control. When estimating the amount of IB protein that is fully resistant to proteinase K, we observed dramatic differences when comparing model IBs, ranging from 9% to 33% (in ClpA$^-$ cells and in ClpP$^-$ cells, respectively; Table 1, population 3). On the other hand, between around 15% and 60% of the IB protein was immediately degraded (t_{50} below 3 min, population 1), while an additional protein population ranging from 20% to 70% of the total protein content exhibited intermediate resistance (t_{50} between 10 and 17 min, population 2). The heterogeneity in the conformational protein states (with three distinguishable populations) was clearly influenced by the cell's genetic background. Interestingly, in DnaK$^-$ IBs and ClpA$^-$ IBs, in contrast to wt and ClpP$^-$ IBs, more than 50% of the protein was highly sensitive to the protease, what could suggest a greater tendency to be released and/or stronger native-like organization of the embedded protein when compared to wt IBs.

To evaluate the architecture of the proteinase K-resistant core and its interactions with proteinase-resistant material, the size of the DnaK$^-$ IB particles and the fluorescence of the remaining protein were dynamically monitored during protein digestion. DnaK$^-$ IBs are of particular interest regarding biotechnological and biomedical applications as they are enriched with highly active polypeptides [12]. Therefore, these particles perform well as enzymatic biocatalysts [52] and as nanopills for protein replacement therapies [34,40]. As observed (Fig. 2A), the DLS analyses of these particles show a major peak at around 400 nm, fully compatible with previous observations [11], plus a minor fraction of the protein organized as smaller (100 nm) particles. Such a size distribution is not altered upon IB storage in buffer alone, but, surprisingly, it also remains stable upon incubation with proteinase K (Fig. 2A), under conditions in which the IB protein is effectively digested (Fig. 2B, IB; Fig. 2C). Furthermore, fluorescence of IBs during digestion progressively declines, in parallel with protein amounts (Fig. 2D), a fact that confirms the efficient performance of the protease. Interestingly, the intensity of the IB fluorescence remaining after long-term digestion represents less than 7% of the initial value (Fig. 3), a value much lower than that of the protein fraction resistant to proteinase K (30%, Fig. 1). This indicates that the proteinase K-resistant version of the recombinant protein is essentially not fluorescent, showing only a residual emission capacity, while the protein removed by the protease from IBs was highly fluorescent. In this context, very minor amounts of full-length fusion were observed in the residual IB material, which was mainly composed of degradation fragments (Fig. 2C). Although

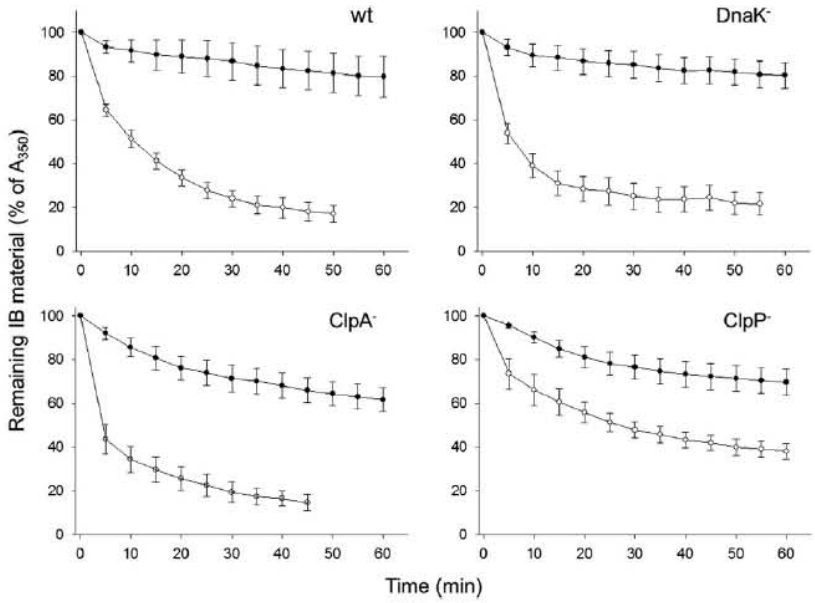

Fig. 1. Time-course stability of IB protein during proteinase K attack monitored by the amount of remaining IB material. IBs produced in different strains (relevant phenotypic traits are indicated at the top of each plot) were digested in three independent experiments, from which the average values are shown (empty symbols). The evolution of IB samples in the absence of proteinase K is also shown as controls (filled symbols).

Table 1
Distribution of IB protein populations according to their half-life under proteinase K treatment.

		wt	DnaK-	ClpA-	ClpP-
Population 1	Percentage (%)	16.2	58.8	52.0	17.5
	t_{50} (min)[a]	4.03E-05	2.89	1.24	3.93E-04
Population 2	Percentage (%)	69.9	20.2	38.8	49.0
	t_{50} (min)	10.8	13.3	16.1	17.3
Population 3	Percentage (%)	13.9	20.9	9.16	33.5
	t_{50} (min)	∞	∞	∞	∞
Fit[b]	S^2, q, $P(S^2, q)$	0.19, 6, 1	0.77, 7, 1	0.11, 5, 1	0.07, 8, 1

[a] $t_{50} = \ln 2/b_i$ indicates the time needed to for half of the IB material to disintegrate. This value was estimated by the approximation $a_1 \exp(-b_1 x) + a_2 \exp(-b_2 x) + a_3 \exp(-b_3 x)$.
[b] Goodness of the fit: squared error, S^2; degrees of freedom, q; and X^2 probability, $P(S^2, q)$.

GFP-immunoreactive material was observed in the soluble fraction of the digestion reaction (Fig. 2B, D), we were unable to determine any fluorescence emission (not shown), indicative of protease-mediated inactivation of the protein. No VP1 was observed among the soluble digestion products, which could be compatible with selective and partial degradation of the modular VP1GFP. On the other hand, small amounts of full-length fusion protein were observed in the supernatant of non-treated IB samples (Fig. 2B). This was in agreement with previous observations [31,33], and indicates the occurrence of fully soluble protein versions that are spontaneously released from IBs upon incubation in plain buffer.

The conservation of IB volume during proteolytic protein removal and fluorescence extinction (Fig. 2) was wholly unexpected. To discard any artifact during DLS determination, we analysed IBs by microscopy during digestion. Confocal fluorescence maps of isolated IBs were generated before the addition of the protease and at different times upon incubation with the enzyme. As observed, proteinase K digestion indeed ablated the fluorescence emission of IBs, though it did not produce significant reductions in the IB volume or detectable effects on the morphology (Fig. 4A). Cryo-TEM analysis of partially digested IBs (Fig. 4B) fully confirmed the morphological integrity of IBs during digestion and, in addition, revealed a progressive loss of density in proteinase K-treated IBs. In combination with data from Table 1, these images are also supportive of an architectonic organization of bacterial IBs in which the proteinase K-resistant amyloid fibers act as a mechanically stable scaffold, the gaps of which are filled with fluorescent, proteinase K-sensitive protein species (Fig. 4C).

The Western blot data presented in Fig. 2B, showing the presence of GFP but not of VP1 or VP1GFP in the supernatant fraction of the reaction mixture (D), could be indicative of the VP1 moiety of the protein organizing as IB amyloid fibers, while overhanging GFP, in a globular form, could be cleaved and released by the enzyme. This organization has been previously proposed and experimentally supported in the case of prion filaments formed by Ure2p fusions, in which the Ure2p itself acts as the fiber backbone [53]. However, the absence of a VP1 band signal in the insoluble fraction of protease-treated IBs upon digestion for 35 min (Fig. 2B, IB; Fig. 2C) opposed this possibility, although the immunodetection of GFP at this time would instead be compatible with GFP-containing protein versions adopting both globular and proteinase K-resistant conformations. On the other hand, to evaluate if the

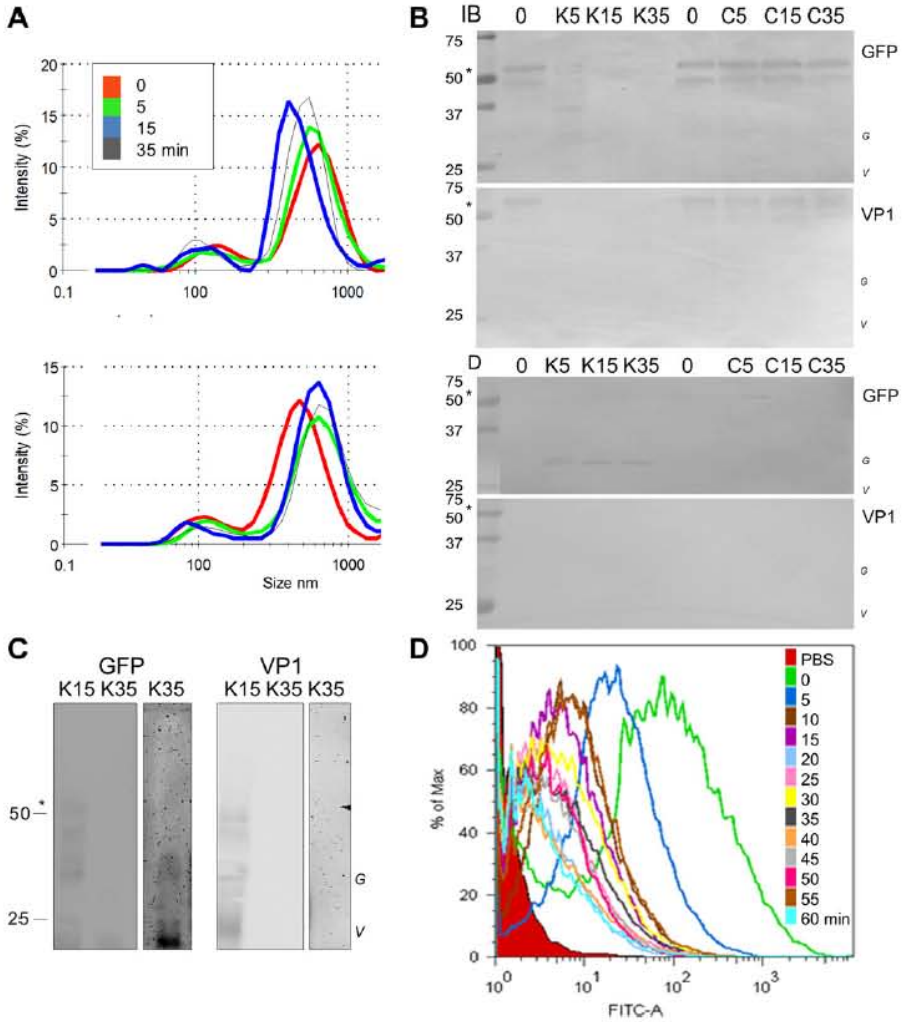

Fig. 2. Morphological and functional characterization of IBs isolated from the DnaK⁻ strain. (A) Size distribution of control IBs (upper image) and IBs treated with proteinase K (lower image). (B) Western blot of IB samples during proteinase K digestion and of digested material (D) released to the soluble fraction of the digestion reaction. K5, K15 and K35 correspond to IBs treated with proteinase K for 5, 15 and 35 min, respectively. C5, C15 and C35 correspond to the control IBs incubated in absence of the protease for 5, 15 and 35 min, respectively. The material was immunodetected using both anti-GFP and anti-VP1 antibodies, as indicated. The electrophoretic position expected for the full-length form of the protein is indicated by an asterisk, while that of truncated GFP is indicated by a *G* and that of VP1 by a *V*. Note the presence of a truncated, GFP-alone protein version already in the starting material. (C) Highly sensitive chemiluminiscent Western blot analysis of the IB protein fraction during proteinase K digestion, immunodetected using either anti-GFP or anti-VP1 antibodies. The reaction time was 1 min, but the lanes with a higher background (K35, right) derive from blots developed for up to 30 min. (D) Fluorescence emission of IBs analysed by flow cytometry at different time points of the digestion process.

protein partitioning observed in VP1GFP IBs would be restricted to only fusion proteins, we determined the proteinase K-mediated disintegration kinetics of IBs formed by several alternative non-fused polypeptides. As observed (Fig. 5), the decay of IB material follows the same pattern and population separation than that found in the case of VP1GFP (Fig. 1 and Table 1). Therefore, although the distribution of protein domains into globular and fibril-like conformations cannot be discarded, in no case would this be dependent on the eventual modular character of the protein-forming IBs.

To evaluate to which extent the IB skeleton is responsible for the mechanical stability of IBs, we tested partially digested IB material to see if its topography challenged the mechanical stimulation if mammalian cell growth in conventional cultures. In previous studies, we have proved that decoration of flat surfaces with IBs dramatically stimulated mammalian cell proliferation [11,13,14] through enhanced adhesion and further mechano-transduction events [38]. IBs digested with proteinase K for 5, 15 and 35 min were able to enhance proliferation of BHK cells just as well as non-treated IBs, when used as surface topographies (Fig. 6). A slight improvement on cell proliferation promoted by partially digested IBs (asterisk in Fig. 6) might be indicative of an enhanced nanorugosity of the material. These findings confirm the integrity of the IB architecture, which is essentially supported by proteinase K-resistant forms of the recombinant protein, and that remains essentially unaffected by the removal of the proteinase K-sensitive functional forms. These second protein species, which do not have a scaffolding role in the IB organization, are highly fluorescent and probably represent those protein species responsible for the native-like secondary structure repeatedly observed in IBs [17]. Such a matrix-like organization accounts for the homogeneous distribution of fluorescence previously reported in GFP IBs by fine confocal analysis [5,33].

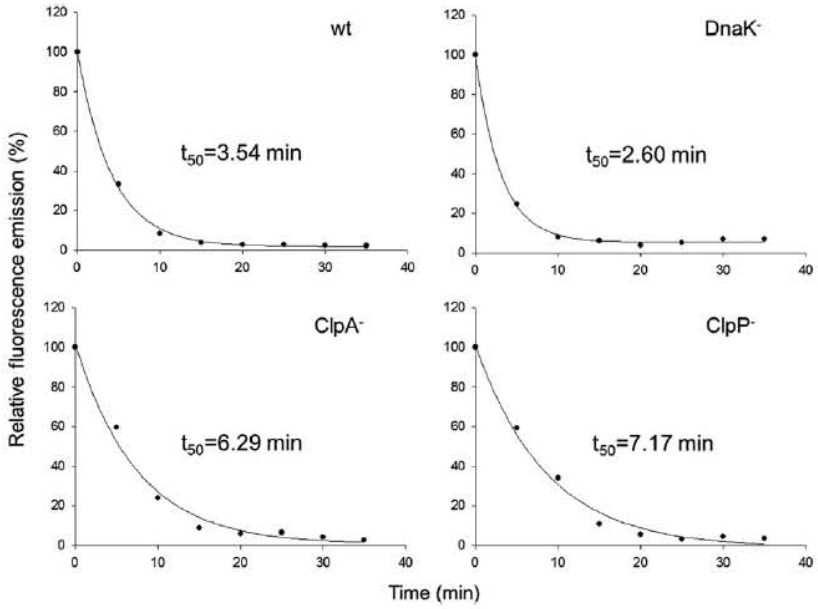

Fig. 3. Comparative kinetics of fluorescence extinction during proteinase K digestion. Fluorescence emission of IBs produced from different genetic backgrounds during proteinase K digestion analysed by flow cytometry. The digestion time at which the fluorescence emission was reduced by a half is indicated as t_{50}.

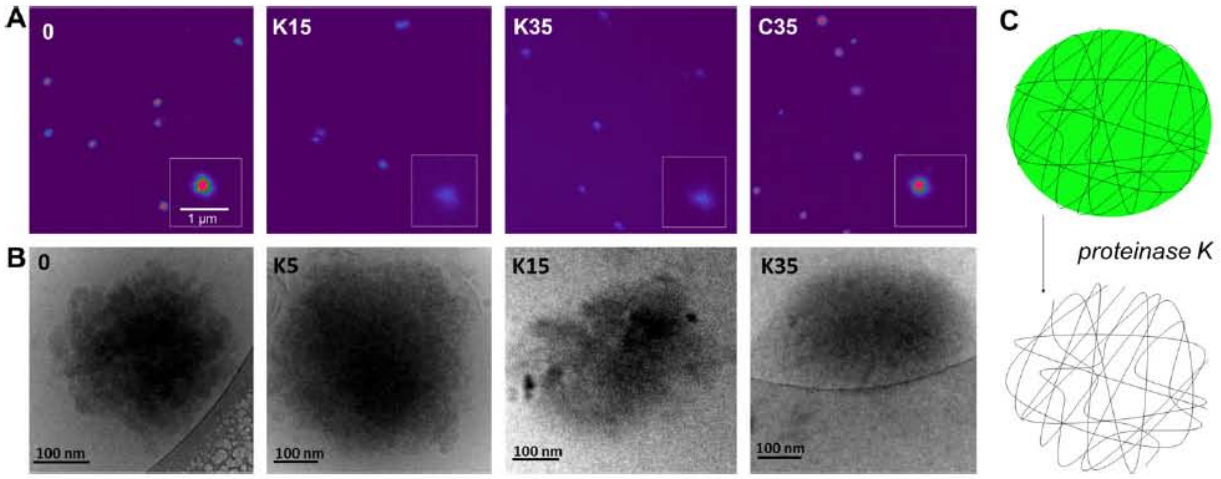

Fig. 4. Morphology and fluorescent maps of DnaK⁻ IBs during proteinase K digestion. (A) Confocal microscopy images using the Metamorph reference table of purified DnaK⁻ IBs exposed to proteinase K. The scale bar in the inset represents 1 µm. (B) Cryo-TEM examination of IBs produced in DnaK⁻ and exposed to proteinase K. (C) Architectonic model for bacterial IBs with a proteinase K-resistant scaffold (black lines), filled with biological proteinase K-sensitive protein species (green material). The nomenclature is as in Fig. 2.

4. Discussion

Protein aggregation is intimately associated with the conformational stress suffered by cell factories [54], and has represented a consistent bottleneck to the bacterial production of soluble proteins in both the biotechnology and pharmaceutical [1,55,56]. Several strategies have been developed to minimize the in vivo formation of IBs [57], which are large protein aggregates with exceptional mechanical stability [58]. Most of these approaches are based on the co-production of molecular chaperones along with the recombinant protein. These folding modulators are believed to be limiting for the non-physiological high amounts of recombinant proteins produced in recombinant bacteria to reach their native conformation [59]. However, the complexity of the cells' protein control system and the paradoxical effects of chaperone co-expression often result in undesired side effects [12,60,61], in failure to prevent IB formation and, eventually, in significant protein yield reduction [12,62]. In the context of the increasing needs of new natural and biocompatible materials in nanomedicine [7], IBs, like other natural amyloids [9,63,64], are seen as self-assembling protein-based materials [8,65]. In addition, IBs can be produced at high yields and can be isolated from producing bacteria by simple procedures based on cell wall destruction, sedimentation and repeated washing [66]. The biological activity

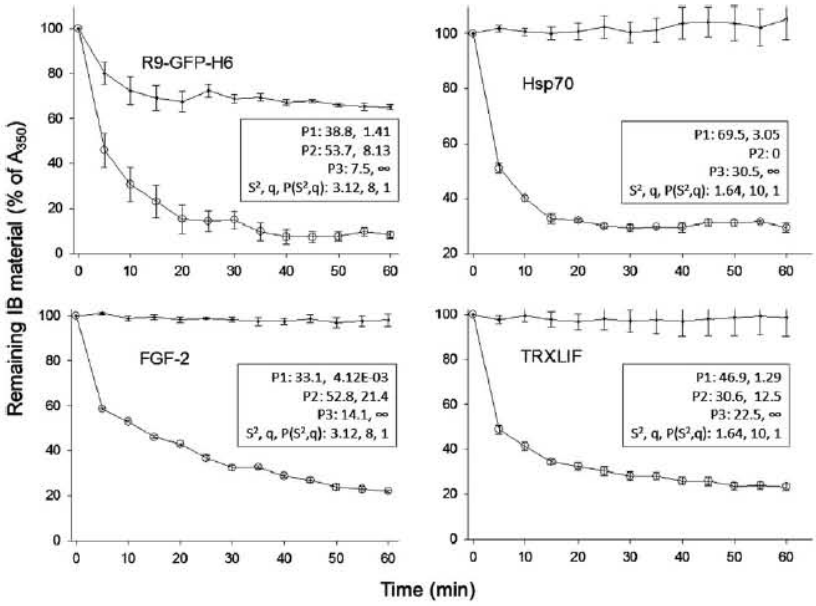

Fig. 5. Time-course stability of different IBs protein during proteinase K attack. IBs formed by different non-fusion proteins (R9-GFP-H6; Hsp70 and FGF-2) and a fusion protein (TRXLIF) were digested in three independent experiments, from which the average values are shown (empty symbols). The evolution of IB samples in the absence of proteinase K is also shown as controls (small filled symbols). Relevant parameters derived from the numerical analysis are also shown. In P (population), both the percentage and t_{50} (in min) are given.

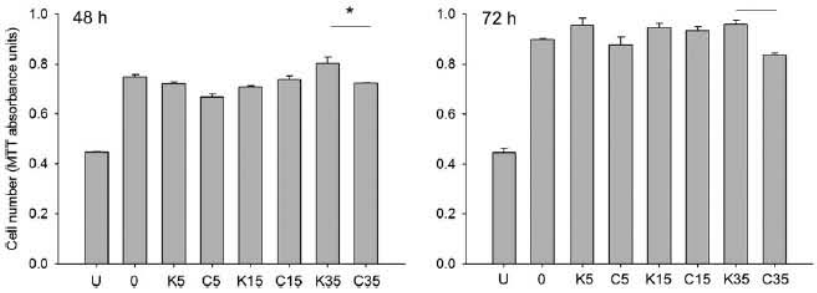

Fig. 6. BHK cell proliferation on IBs. Cells were cultured on IB-free wells (U) or on wells decorated with DnaK⁻ IBs treated (K) and non-treated (C) with proteinase K for different times. The nomenclature is as in Fig. 2. The experiment was performed in triplicate. The differences in cell densities comparing IB-decorated wells and IB-free wells (U) were significant in all cases ($p < 0.01$). In addition, the number of viable cells was also different ($p < 0.001$) when comparing the effect of topographies created with control and proteinase K-treated IBs for 35 min (indicated by an asterisk).

retained by IB proteins [2] makes them useful as immobilized biocatalysts for industrial processes [4] but also as nanopills for protein drug delivery [34], as they are not toxic to either cultured mammalian cells [38] or to model animals upon oral delivery [34]. Furthermore, the fusion of aggregation tags can favour the natural tendency of recombinant proteins to form IBs [67–70], enabling essentially any desired polypeptide to be produced in such form. In addition, the appropriate selection of the fusion tag could be used to modulate the conformational status of the protein in IBs [71], which might result in a tool for tuning the ability to release functional protein species from IBs when used as nanopills [34] or in other therapeutic contexts.

Protein–protein contacts in IBs proteins are based on stereospecific interactions [72], which confer sequence specificity, define a seeding pattern in the aggregation process and constrain the compositional heterogeneity of the resulting aggregates [16,22]. Stereospecificity involves a native-like secondary structure, as fully demonstrated in IBs through infrared spectroscopy [26] and solid-state NMR spectroscopy [73], which coexists with the amyloidal format of an IB protein fraction organized as proteinase K-resistant protein fibrils through cross-molecular beta-sheet interactions [9,18,19,21,22]. In the present study, we provide data supporting the hypothesis that the mechanical stability of bacterial IBs is conferred by a proteinase K-resistant fibril-like scaffold, while the biological activity (fluorescence in our GFP reporter model) is associated with protease-sensitive forms of the protein (Table 1 and Fig. 3). The loss of this sensitive and functional material during controlled digestion does not affect the morphology or size of IBs (Figs. 2 and 4A), although cryo-TEM analyses reveal an important loss of IB density concomitant to the reduction of protein content and fluorescence emission (Fig. 4B). Since IBs are porous and highly hydrated materials [74], Peternel and Komel [10] have recently proposed a cotton-like model for the inner IB organization that accounts for the irreversible contractibility observed at low pHs [75].

Our results experimentally confirm such a model, in which an amyloidal fibrilar scaffold acts as a sponge for native or native-like protein species that fill in the gaps of the matrix (Fig. 4C). In fibril-forming fusions of the Ure2p protein, the prion protein forms the core of the fiber while fused globular domains overhang it, expanding its diameter [53]. This prion-oriented model is not straightforwardly compatible with the data presented here on bacterial IBs, which suggest that the full-length VP1GFP or GFP-containing fragments, present in both released and residual IB material (Fig. 2B and C), might occur as the scaffold component but also as releasable polypeptides. On the other hand, the digestion analysis of other IBs formed by non-fused polypeptides (Fig. 5) indicates that the fractioning of IB polypeptides into populations with different proteinase K sensitivity does not depend on the nature of the fusion protein. This result is again in line with the hypothesis that conformational isoforms of the same protein can be present in IBs in form of a fibrillar supportive scaffold and as properly folded protein. In this context, a significant part of IB protein is spontaneously released from the particles just by buffer incubation (Fig. 2B), proving its full solubility. The proteinase K-sensitive population of IB protein would then be composed of soluble and quasi-soluble functional protein species, while the IB scaffold would be formed from amyloidal forms (fibers or other types of oligomers), among which fibrils have been effectively identified already [22]. The isoform model of IB organization is also supported by the release of functional non-fused proteins from IBs once internalized by mammalian cells [34], and by the equal digestion pattern of fusion and non-fusion IB proteins (Fig. 5). Interestingly, disintegration of Hsp70 can be accounted for by only two protein populations, being the type of IBs that had been produced in bacteria over a longer period (5 h).

This dual organization of proteins within IBs explains the mechanics of IBs that act as intracellular drug delivery systems in the form of nanopills [34]. Interestingly, the relative amounts of scaffold and functional polypeptides depend on activities of the cells' protein quality control system, with can in turn be regulated by the appropriate selection of the genetic background of the *E. coli* cells used for the biofabrication of the IBs (Table 1). Although the complexity of this cellular network (consisting of chaperones and proteases with overlapping, synergistic and antagonistic activities) will probably prevent the rational linking of specific genetic traits and IB architectonic phenotypes, empirical observations derived from experimentally simple procedures should enable us to select the most appropriate mutants for tailoring the IB architecture through a trial-and-error process.

In a time when the role of non-toxic amyloids in nature is being investigated [35,64] and functional amyloids are foreseen as intriguing biomaterials in nanomedicine [5,9,37,63,76], the controlled protein packaging of bacterial IBs offers a valuable tool for both the precise elucidation of the architecture of functional amyloids and the design and controlled production of amyloidal nanoparticles, usable as smart nanopills for the controlled release of drug monomers [39].

5. Conclusions

The recognition of amyloids as functional materials composed of releasable proteins opens an intriguing spectrum of exciting possibilities in nanomedicine. The analysis of the supramolecular architecture of bacterial amyloids reported here has revealed a sponge-like organization of IBs, combining proteinase K-resistant (inactive) and proteinase K-sensitive (functional) protein forms. Such a bimodal architecture offers intriguing clues to how bacterial amyloids self-assemble into functional entities, and why recombinant bacteria are appropriate biofactories for the in vivo controlled packaging of recombinant drugs in a form suitable to be released in biological targets.

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Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 2 and 4 are difficult to interpret in black and white. The full color images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2012.11.033.

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Article 2

Expanding the recombinant protein quality in *Lactococcus lactis*

Cano-Garrido O*, Rueda FL, Sánchez-García L, Ruíz-Ávila L, Bosser R, Villaverde A, Garcia-Fruitós E.

Microbial Cell Factories. 2014 Dec 4;13:167

Among all microbial cell factories, *E. coli* is the dominant expression system for recombinant protein production. However, the presence of lipopolysaccharides (LPS) in the membrane of such Gram-negative bacteria is an important drawback for the synthesis of BioPharma goods. Essentially, endotoxins can contaminate the final product and, nowadays, there is not yet any cost-effective and efficient protocol for their removing, in particular, for complex products such as protein nanoparticles. Precisely because of that, it is necessary to set up the use of endotoxin-free expression systems for the production of proteins for biomedical and veterinary purposes.

Particularly, in this work we have explored *L. lactis* as one of these alternatives, due to their extensive use as a food-grade bacteria, being classed as a Generally Recognized as Safe (GRAS) specie for a wide range of applications. Although a considerable work has been done to extend the *L. lactis* toolbox, in the last years, the conformational quality and solubility of proteins produced in this expression system has not been explored so far. In this study, with the aim of exploring such parameters, we have investigated how protein solubility and conformational quality of a model protein produced in this LPS-free microbial cell factory is influenced by diverse protein production conditions.

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MICROBIAL CELL FACTORIES

TECHNICAL NOTES Open Access

Expanding the recombinant protein quality in *Lactococcus lactis*

Olivia Cano-Garrido[1,2,3], Fabian L Rueda[1,2,3], Laura Sànchez-García[1,2,3], Luis Ruiz-Ávila[4], Ramon Bosser[4], Antonio Villaverde[1,2,3] and Elena García-Fruitós[1,2,3*]

Abstract

Background: *Escherichia coli* has been a main host for the production of recombinant proteins of biomedical interest, but conformational stress responses impose severe bottlenecks that impair the production of soluble, proteolytically stable versions of many protein species. In this context, emerging Generally Recognized As Safe (GRAS) bacterial hosts provide alternatives as cell factories for recombinant protein production, in which limitations associated to the use of Gram-negative microorganisms might result minimized. Among them, Lactic Acid Bacteria and specially *Lactococcus lactis* are Gram-positive GRAS organisms in which recombinant protein solubility is generically higher and downstream facilitated, when compared to *E. coli*. However, deep analyses of recombinant protein quality in this system are still required to completely evaluate its performance and potential for improvement.

Results: We have explored here the conformational quality (through specific fluorescence emission) and solubility of an aggregation-prone GFP variant (VP1GFP) produced in *L. lactis*. In this context, our results show that parameters such as production time, culture conditions and growth temperature have a dramatic impact not only on protein yield, but also on protein solubility and conformational quality, that are particularly favored under fermentative metabolism.

Conclusions: Metabolic regime and cultivation temperature greatly influence solubility and conformational quality of an aggregation-prone protein in *L. lactis*. Specifically, the present study proves that anaerobic growth is the optimal condition for recombinant protein production purposes. Besides, growth temperature plays an important role regulating both protein solubility and conformational quality. Additionally, our results also prove the great versatility for the manipulation of this bacterial system regarding the improvement of functionality, yield and quality of recombinant proteins in this species. These findings not only confirm *L. lactis* as an excellent producer of recombinant proteins but also reveal room for significant improvement by the exploitation of external protein quality modulators.

Keywords: *Lactococcus lactis*, Solubility, Recombinant protein quality, Conformational quality, GRAS

Introduction

Obtaining proteins of biotechnological and biomedical interest from their natural sources is hampered by severe economic constraints. The emergence of recombinant DNA technologies allowed developing different gene expression systems (cell factories) adapted to produce functional versions of the desired proteins, becoming recombinant protein production a routine practice in the BioPharma industry [1-3]. Among these expression systems, the bacterium *Escherichia coli* has been the principal working horse, because of its well-known genetics and physiology, cost-effective culture and easy scaling up. However, being *E. coli* a Gram-negative bacterium, cell wall lipopolysaccharides (LPSs) are generic contaminants of the final product and promote not only pyrogenicity but also activation of acute inflammatory responses. The presence of bacterial endotoxins is then one of the major concerns by regulatory agencies [4], and the need of adding steps for endotoxin removal turns otherwise simple processes into practices with high associated costs. Besides, protocols for endotoxin removal can also impair or

* Correspondence: Elena.Garcia.Fruitos@uab.cat
[1]Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, Bellaterra 08193, Cerdanyola del Vallès, Spain
[2]Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Bellaterra 08193, Cerdanyola del Vallès, Spain
Full list of author information is available at the end of the article

destroy protein function [5]. Since protein production processes have to meet not only good cost-effectiveness ratios but also high product quality [6-8], the use of cell factories other than *E. coli* is becoming an increasingly recognized need [3]. In this context, other bacterial groups are emerging as intriguing alternatives that offer advantages over the use of *E. coli* regarding protein quality, absence of endotoxins, disulfide bridge formation and solubility [9,10]. Lactic Acid Bacteria (LAB) are classified as Generally Recognized As Safe (GRAS) organisms and represent appealing possibilities for the production of safer therapeutic proteins [11,12]. Specifically, *Lactococcus lactis*, which has been used for long in food industry, has emerged as a cost-effective protein cell factory [12]. In this regard, a wide range of genetic tools adapted to LAB make nowadays possible to successfully produce an increasing number of fully LPS-free recombinant proteins [5,11,13-16]. More specifically, it is worth mentioning that three versatile gene expression systems, named NICE (nisin-controlled expression system) [17], P170 [18,19] and zinc systems [20], have been developed for use in *L. lactis*. Interestingly, *L. lactis* is also currently used as a life vector for drug, DNA and other molecules delivery to mucosal surfaces [15,21-23], proving their huge potential for its use in human medicine [11]. In addition, GRAS organisms are being considered for the production of diverse bacterial products, not only soluble proteins but also biopolymers, polymeric nanoparticles and self-assembling protein-based nanoparticles, among others [24]. For instance, polyhydroxibutirate (PHB) inclusions produced in *L. lactis* are purer than those obtained in *E. coli* [5] and have lower production costs due to the reduction of the number of downstream processing steps [5]. In this context, producing endotoxin-free biopolymeric beads is critical for use in medical applications [25,26].

Despite reports on protein production in *L. lactis* abound [27], further analyses of protein quality and solubility in this system are required to deeply understand the host performance regarding protein quality control and to fully exploit and ever expand, its well-known properties as a factory for soluble, highly functional proteins. Besides, considering that, although *L. lactis* is a facultative anaerobe with a fermentative metabolism, this microorganism is also able to undergo respiratory growth when hemin is added to aerated cultures [28], both growth conditions have also been explored in this study.

Results

To explore recombinant quality in *L. lactis* we have studied the influence of production time and growth conditions of a wild type strain in the production of an aggregation-prone fluorescent protein (rVP1GFP) that has been previously used as a convenient solubility model in *E. coli* [29]. The specific emission of GFP has been successfully used as a marker of conformational quality of GFP-containing misfolding-prone proteins as there is a positive linear dependence between conformational quality and the presence of native-like conformations [30,31].

Growth conditions

Under anaerobic fermentation but not under hemin-stimulated respiration, protein solubility was compromised rendering fluorescent protein deposits (Figure 1). This pattern was coincident with that of the formation of PHB inclusions that occurs only under anaerobiosis [26]. In fact, cells were not fluorescent under aerobic conditions (Figure 1), in agreement with previously studies indicating that hemin-induced cell respiration does not support the production of functional proteins [32,33]. Therefore, fermentative growth was established as standard conditions for subsequent experiments.

Production time

The fluorescence emission (Figure 2) and the ratio of soluble versus insoluble protein (Figure 3) fractions were determined by fluorimetry and western blot (Figure 4), respectively. The fluorescence emission does not present significant differences at different production times. However, in general terms, it is possible to conclude that GFP activity increases at longer production times, being this effect more marked in the soluble fraction (Figure 2). Besides, it is worh mentioning that at 3 h post-induction, protein solubility reached 67%, a yield much higher than that reached in *E. coli* for the same protein under comparable conditions (10-18%, [34]). In this context, the potential of the system for the soluble protein production was confirmed with a difficult-to-express human catalase, which under these conditions majorly occurred at the soluble cell fraction (75%) (data not shown). On the other hand, the conformational quality, namely the ratio between protein activity and protein yield, of soluble rVP1GFP (estimated through its specific fluorescence) (Figure 5) evolved contrarily to the resulting protein amount in cells (Figure 2). This means that those conditions that favour protein solubility are those giving non-optimal results regarding conformational quality. Interestingly, such a divergence between protein yield and quality has been previously described in recombinant *E. coli* [29,35], but the present finding confirms this fact as a generic event.

Growth temperature

Finally, we determined how the production at suboptimal temperatures might influence solubility and conformational quality, through fluorescence determination

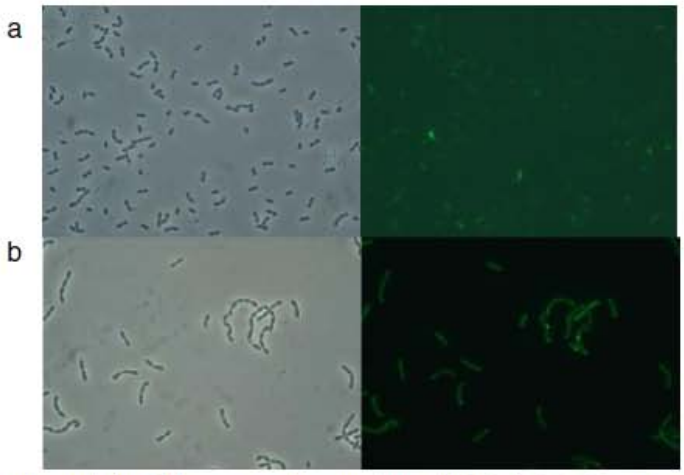

Figure 1 Optical microscopy images (left) and fluorescence microscopy images (right) of *L. lactis* NZ9000 overproducing rVP1GFP protein at 30°C 3h post-induction under a) hemin-stimulated respiration and b) anaerobic conditions. Fluorescent protein aggregates are observed as highly fluorescent dots in the cell cytoplasm.

(Table 1). The fluorescence of the soluble and insoluble protein produced at 25°C was similar to that obtained at 30°C. However, the specific fluorescence at 30°C was notably higher than that observed at 25°C, showing in this last condition a 6-fold increase and 1.6-fold increase for the soluble and insoluble fraction respectively. At 16°C we observed a decrease in the fluorescence of both soluble and insoluble protein fractions. On the other hand, while the specific activity of the insoluble protein at 16°C was practically the same than that obtained at 30°C, the specific fluorescence of the soluble fraction at 16°C improved. Thus, decreasing the production temperature did not result in any improvement of fluorescence emission of insoluble protein, which was highly fluorescent at 30°C. In this direction, we also observed residual enzymatic activity in the insoluble fraction of catalase-producing cells (6.5 μmol/min/μg) (data not shown).

Discussion

Under the need to obtain LPS-free bacterial-derived products for biomedical applications, the use of Gram-positive microorganisms as a recombinant cell factory has progressively gained relevance [12]. In this context, an increasing number of approaches using LAB and other Gram-positive microorganisms [9], have proven the huge potential of these cell factories in recombinant protein production [12,36-38]. Although *L. lactis* has been widely explored as a cell platform for the production of both homologous and heterologous recombinant proteins [39-41], as far as we know, the solubility and

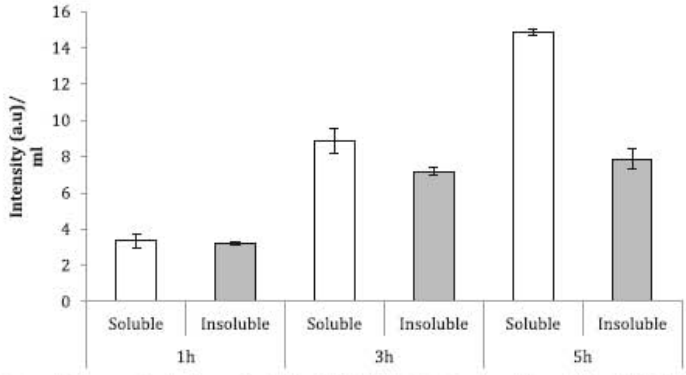

Figure 2 Fluorescence at 1, 3 and 5 h post-induction of soluble rVP1GFP (white bars) and insoluble rVP1GFP (grey bars) produced in *L. lactis* NZ9000 at 30°C.

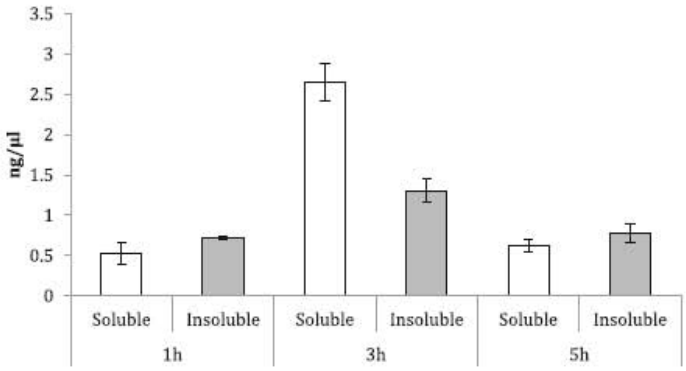

Figure 3 Yield of soluble (white bars) and IB rVP1GFP (grey bars) produced in *L. lactis* NZ9000 at 30°C.

fine conformational quality of both soluble and insoluble recombinant protein has been never studied in detail in this microorganism.

In the present study, we provide data in line with previous studies that describe that fermentative growth is the optimal condition to successfully produce recombinant proteins in *L. lactis* (Figure 1) [32,33]. Thus, although hemin-stimulated respiration has been described as an alternative growth condition inducing an increase in biomass and reducing media acidification [42], our results clearly show that under this condition protein production is highly compromised in both soluble and insoluble protein fractions. These data are in agreement with previous results published. Specifically, the authors describe the reduction of recombinant protein production under aerobic conditions in the presence of hemin, when compared to that obtained under similar conditions without aeration [32,33].

Growth temperature also plays a key role regulating protein solubility and conformational quality. In this regard, protein activity is negatively affected at low temperatures, but the conformational quality of soluble protein reaches higher values at suboptimal temperatures, especially at 16°C. On the contrary, specific activity of insoluble protein is poorly influenced by the temperature.

Conclusions

Recombinant protein quality in *Lactococcus lactis* is largely influenced by the metabolic regime, growth conditions and temperature. By controlling these parameters, the conformational quality of both soluble and insoluble protein fractions is widely modulated, what offers a desirable high versatility of this system for the production of endotoxin-free conventional soluble protein and protein aggregates of biomedical interest. Beyond the well-recognized ability of this system as a producer of soluble proteins, the wide range of conformational quality of the model protein prompts the further exploitation of external effectors as efficient solubility modulators for optimal yields.

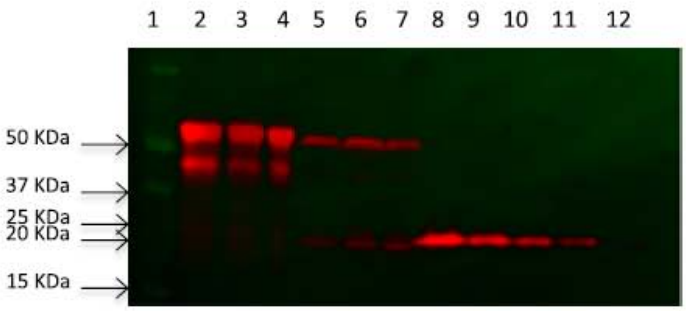

Figure 4 Western blot of soluble and insoluble rVP1GFP fractions produced in *L. lactis* NZ9000. 1: Marker, 2: insoluble rVP1GFP (replica 1), 3: insoluble rVP1GFP (replica 2), 4: insoluble rVP1GFP (replica 3), 5: soluble rVP1GFP (replica 1), 6: soluble rVP1GFP (replica 2), 7: soluble rVP1GFP (replica 3), 8-12: standard curve (750, 500, 250, 125 and 75 ng of R9-GFP). The band around 50 Kda corresponds to VP1GFP , while the band observed around 20 KDa corresponds to GFP.

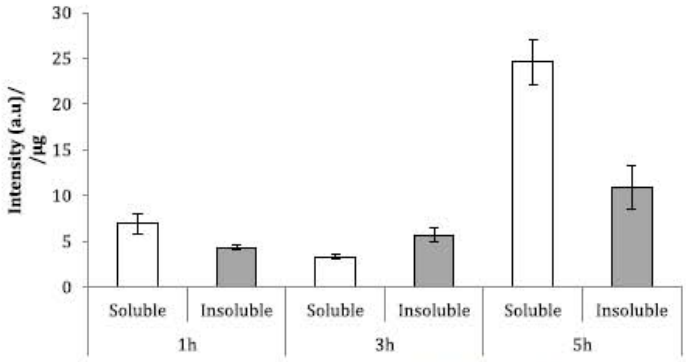

Figure 5 Specific fluorescence of soluble (white bars) and insoluble (grey bars) rVP1GFP produced in *L. lactis* NZ9000 at 30°C.

Materials and methods

Bacterial strain, plasmids and growth conditions

The *Lactococcus lactis* strains used in this study was NZ9000 (*pepN::nisRnisK*) (NIZO). A model recombinant protein rVP1GFP (a fusion between the VP1 capsid protein from the food-and-mouth disease virus and the Green Fluorescent Protein) and human catalase (hcatalase) were produced by expressing the encoding gene from the Cm^R pNZ8148 plasmid (NIZO) under *nis*A promoter control.

Plasmid construction

The rVP1GFP and hcatalase sequences were codon optimized (*Geneart*). In the sequence design we added a *NcoI* restriction site at 5' followed by nucleotides CA to restore the reading frame and an *PstI* restriction site at 3' for rVP1GFP and *XbaI* for hcatalase. rVP1GFP gene was digested by *Nco*I and *PstI*, and ligated into the *NcoI-PstI* fragment of the expression plasmid pNZ8148. hcatalase gene was digested by *Nco*I and *XbaI*, and ligated into the *NcoI-XbaI* fragment of the expression plasmid pNZ8148. Ligation products were transformed by electroporation into *L. lactis* competent cells and positive colonies were isolated by antibiotic selection.

Table 1 Effect of growth temperature on protein solubility and conformational quality

		Fluorescence (A.U./ml · OD) %	Specific fluorescence (A.U./µg) %
30°C	Soluble fraction	18,32	59,40
	Insoluble fraction	81,68	40,60
25°C	Soluble fraction	15,19	26,26
	Insoluble fraction	84,81	73,74
16°C	Soluble fraction	0,02	68,09
	Insoluble fraction	99,98	31,91

Preparation of *L. lactis* competent cells

A protocol to prepare competent *L. lactis* was developed based on previous data [43]. *L. lactis* was growth O/N at 30°C in 50 ml M17 broth media enriched with 0.5% glucose, 2% glycine, 0.5 M sucrose (G-SGM17B) and appropriated antibiotics. Then, 400 ml of G-SGM17B medium were inoculated with an aliquot of the overnight culture to reach an $OD_{550\ nm} = 0.05$ and they were grown at 30°C during around 3h until the $OD_{550\ nm}$ reached 0.2-0.3. Then, cells were harvested by centrifugation at 10,000 g for 20 min at 4°C and the pellet was resuspended in 400 ml of 0.5 M sucrose plus 10% glycerol. Samples were harvested again at 10,000 g for 10 min at 4°C, and the pellet was washed in 200 ml of 0.5 M sucrose plus 10% glycerol and 50 mM EDTA and formed again at 10,000 g for 10 min and 4°C. Cells were resuspended in 100 ml of 0.5 M sucrose plus 10% glycerol, and pelleted by last time at 10,000 g for 10 min at 4°C. Finally, the sediment was suspended in 4 ml of 0.5 M sucrose plus 10% glycerol, aliquoted and stored at -80°C until used in transformation experiments.

Transformation

Electroporation was performed using Gene Pulser from Bio-rad fitted with 2500V, 200 Ω and 25 µF in a pre-cooled 2 cm electroporation cuvette. Following, samples were supplemented with 900 µl restorative medium (M17 broth with 0.5% Glucose, 20 mM Mg_2Cl_2 and 2 mM Ca_2Cl_2) and incubated for 2h at 30°C. The electroporation mix was centrifuged for 10 min at 10,000 g at 4°C and the pellet was resuspended in 100-200 µl of M17 media and plated.

Recombinant protein production

A pre-inoculum was prepared from a fresh colony of the recombinant cells the pre-inoculum was used to inoculate 250 ml shake flasks. The cells were cultured as

described in [44]. Specifically, chloramphenicol (5 μg/ml) was used for plasmid maintenance and an agitation of 250 rpm was used for those cultures grown under hemin-stimulated respiration.

Expression of the target gene was induced when the optical density reached a value of 0.5 with nisin, as described in [17,44]. Growth temperatures in the production phase were 30, 25 or 16°C. Samples were taken at 1, 3 and 5 h in cultures at 30°C and at 5 h post-induction in cultures grown at 25°C and 16°C.

All the experiments have been run in triplicate (replica 1, replica 2 and replica 3).

Protein fractioning

Samples of 10 ml were taken in triplicate from bacteria cultures. Cells were pelleted by centrifugation at 10,000 g at 4°C for 10 min and the sediment was resuspended in 1 ml phosphate buffered saline (PBS) supplemented with (Complete EDTA-free, Roche) to prevent proteolysis. Then, ice-jacketed samples were disrupted by sonication (3 cycles of 5 min at 40% amplitude under 0.5 s cycles). Total cell extracts were centrifuged at 15,000 g and 4°C for 15 min. Finally, soluble and insoluble fractions were aliquotated until further analysis.

Protein determination

Soluble and insoluble protein fractions were analysed by denaturing SDS-PAGE (10% acrylamide). Samples were resuspended with denaturing buffer (Laemli 4x: Tris base 1.28 g, glycerol 8 ml, SDS 1.6 g, β-mercaptoethanol 4 ml, urea 9.6 g in 100 ml) [45]. Soluble and insoluble protein fractions were boiled for 5 and 45 min respectively. At that time, samples were loaded onto the gel. SDS-PAGE protein bands were electroblotted onto nitrocellulose membranes and identified using a commercial polyclonal serum against GFP (GFP (FL): SC-833, Santa Cruz Biotechnology). The secondary antibody was an anti-rabbit (Goat Anti-Rabbit IgG (H + L)-HRP Conjugate #172-1019, Bio Rad). The amounts of recombinant protein were estimated by comparison with known amounts (usually ranging from 75 to 750 ng) of R9-GFP [46] or commercial hcatalase (Sigma Ref. C9322). Densitometric analyses of the bands were performed with the Quantity One software.

Fluorimetry

Fluorescence emission (510 nm) was determined by fluorimetry using a Cary Eclipse Fluorescence Spectrophotometer (Variant), by using an excitation wavelength of 450 nm, to measure protein fluorescence at soluble and insoluble protein.

Catalase activity assay

Catalase activity was determined by the Catalase Assay kit (Abcam Ref. ab83464). In the assay H_2O_2 was added to the catalase samples in order to measure the unconverted H_2O_2 which reacts with OxiRed™. Reaction final product was measured at Ex/Em = 535/587 nm by Victor 3 Multilabel Plates Reader. Catalase activity is reversely proportional to the signal.

Microscopy

Samples of 1 ml were taken from bacteria cell culture by triplicate and harvested by centrifugation at 10,000 g and 4°C for 10 min. Cells were fixed with 0.1% formaldehyde in PBS and kept at 4°C until microscopy observation. Fixed cells were deposited on a glass slide fixed with a slide cover and observed with a Leica DRMB Microscopy. Microphotographies were taken by phase contrast and epifluorescense.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EGF and AV designed this study and drafted the manuscript. OCG performed most of the experiments and prepared the final data and figures. FR carried out protein production and quantification at 25°C and LSG performed part of protein fractioning and determination experiments. LRA and RB have been involved in the experimental design. All authors read and approved the final version of the manuscript.

Acknowledgments

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Author details

[1]Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, Bellaterra 08193, Cerdanyola del Vallès, Spain. [2]Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Bellaterra 08193, Cerdanyola del Vallès, Spain. [3]CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Bellaterra 08193 Cerdanyola del Vallès, Spain. [4]Spherium Biomed S.L., Avda. Joan XXIII, 10, 08950, Esplugues de Llobregat Barcelona, Spain.

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Since the following studies continuing this work have not been still accepted for publication, they will be presented in the Annex 1 of this PhD thesis.

- **Manuscript 1:** **(Annex I, page 109)**

  Functional protein-based nanomaterial produced in GRAS microorganism: a new platform for biotechnology.

- **Manuscript 2:** **(Annex II, page 133)**

  In vivo study to determine the potential of soluble and insoluble recombinant metalloproteinases for the optimization of the cow dry period.

- **Manuscript 3:** **(Annex III, page 139)**

  Systems-trimmed properties of $CXCR4^{+}$-targeted protein nanoparticles produced in the food-grade bacterium *Lactococcus lactis.*

- **Manuscript 4:** **(Annex IV, page 157)**

  Genetic engineering of *Lactococcus lactis* towards a suitable protein production platform with biomedical interest

In the following pages you will find a short abstract of each of those works. Please look up the corresponding annex to find the whole text.

Other works not tightly related to the thesis and not discussed are presented in Annex 2.

Manuscript 1

Functional protein-based nanomaterial produced in GRAS microorganism: a new platform for biotechnology

Cano-Garrido O, Sánchez-Chardi A, Parés S, Giró I, Tatkiewicz W, Ferrer-Miralles N, Ratera I, Natalello A, Curbasi R, Veciana J, Bach A, Villaverde A, Arís A, García-Fruitós E.

Submitted to Acta Biomaterialia

In the Article 2, our group has described the conformational quality of a protein produced in *L. lactis* under overexpression conditions. The detailed study done in both soluble and insoluble protein fractions revealed the presence of protein nanoclusters in the bacteria cytoplasm.

Even though IBs formation has only been characterized in *E. coli*, protein clusters, resembling IBs, have also been described in other organisms. Thus, taking into account the advantage of using recombinant protein-based nanoparticles produced in a LPS-free environment, we decided to further explore the aggregates observed in *L. lactis* under protein overexpression conditions.

In this study, we have done a detailed characterization of these aggregates. This has allowed us to conclude that *L. lactis* protein clusters have properties comparable with those observed before in other recombinant systems such as *E. coli*. This finding opens a wide range of possibilities for both human and animal medicine.

Manuscript 2

In vivo study to determine the potential of soluble and insoluble recombinant metalloproteinases for the optimization of the cow dry period.

Parés S*, Cano-Garrido O*, Fàbregas F, Bach A, Ferrer-Miralles N, Terré M, Villaverde A, Garcia-Fruitós E and Arís A

*Equal contribution

Experiments carried out in collaboration with the Department of Ruminant Production at the "Institut de Recerca i Tecnologia Agroalimentàries" (IRTA, Caldes de Montbui).

In Manuscript 1, we have isolated and characterised the main morphometric and physico-chemical properties of protein nanoparticles produced in *L. lactis* as a microbial cell factory. In particular, we have produced three proteins with relevance in the dry period of dairy cows (MMP-9, MMP-2 and IFN-γ). Determinations show that these protein-based nanoparticles are fully functional *in vitro*. These results evidence that *L. lactis* protein nanoparticles could have a great potential in veterinary medicine.

Among all the tested proteins, MMP-9 nanoparticles produced in the *L. lactis clpP-htrA* strain were the ones which presented higher activity levels. For this reason, in this work, we have performed an *in vivo* study in which we have infused MMP-9 nanoparticles and soluble MMP-9 (as a control) in cow mammary glands (n =12) with the aim of studying its potential at dry-off.

Manuscript 3

Systems-trimmed properties of CXCR4^{+}-targeted protein nanoparticles produced in the food-grade bacterium *Lactococcus lactis*

Cano-Garrido O*, Cespedes MV, Unzueta U, Saccardo P, Roldan M, Sánchez-Chardi, Curbasi R, Vasquez E, Mangues R, Garcia-Fruitós E, Villaverde A.

Submitted to Journal of Nanobiotechnology

In Manuscript 1, we have demonstrated that, contrary to what many claim, *L. lactis* is able to produce biologically functional protein aggregates. Owning to its potential as LPS-free biomaterial producer, we decided to explore if *L. lactis* was also able to produce soluble self-assembling protein nanoparticles. These nanostructured soluble proteins are gaining interest for medical purposes because of being easy to produce and their potential functionality.

In particular, we have produced tumor-targeted soluble self-assembling protein nanoparticles specifically designed to bind CXCR4 receptor (over-expressed in colorectal cancer cells). My group have previously observed that these soluble self-assembling protein nanoparticles, once administered *in vivo*, accumulate principally in tumor but also in metastatic foci, being a drug with a great potential for colorectal cancer. However, *E. coli* has been the only recombinant cell factory used for its production. For this reason, in this manuscript, we have studied the production of these soluble self-assembling nanoparticles in a food grade microbial cell factory more suitable for biomedical purposes.

Manuscript 4

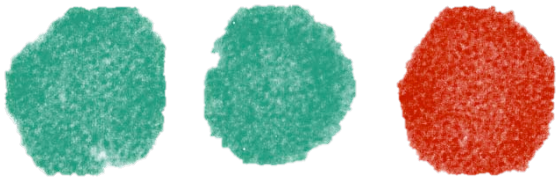
Genetic engineering of *Lactococcus lactis* towards a suitable protein production platform with biomedical interest

Cano-Garrido O, Sánchez-García L, Villaverde A, Bermúdez-Humaran L, García-Fruitós E.

Experiments carried out at the laboratory of "Interaction des bactéries commensales et probiotiques avec l'hôte" led by Prof. Bermúdez-Humarán in the 'Institut National de la Recherché Agronomique" (INRA, France)

Currently and as previously mentioned, *L. lactis* is becoming an appealing alternative to produce recombinant proteins because of being accepted as GRAS for regulatory authorities. Although many genetic and molecular tools have been developed in the last few years, there is still an important lack of awareness regarding protein folding in these Gram-positive bacteria. Thus, take a step forward in this area will allow obtaining new protocols to produce recombinant protein at high yields and with an improved conformational quality.

The research described in this manuscript mainly consists in the genetic engineering of *L. lactis* to ameliorate the yield and the conformational quality of the proteins produced in this lactic acid bacteria (LAB). Being known that chaperones and proteases are involved in conformational quality, as well as, in the aggregation phenomenon, the aim of this work is the creation of a library of mutants of those genes encoding for the main components of the protein quality control, to carefully study how these mutations affect protein solubility and conformational quality.



Discussion

The supramolecular architecture of *E. coli* IB

Recombinant proteins have an enormous potential and a wide market in both the biotechnological and pharmaceutical field, because, unlike chemical goods, they have a high versatility in design and permit the synthesis of high molecular mass products. Under overproduction conditions, cell factories suffer stress responses which are intimately associated to protein aggregation [180], becoming a major obstacle when producing soluble proteins in microbial cell factories and especially in *E. coli* [31, 181, 313]. Since IBs have been for long considered as by-products [31], several approaches have been explored to increase heterologous protein solubility and to minimize protein aggregation [228]. Such strategies are based on adjusting growth temperature, inducer concentration, promoter strength and/or co-production of molecular chaperones [314]. Unfortunately, due to the complexity of the cells' protein quality control system, these strategies often result in insignificants changes in protein solubility and protein yields [52, 315, 316].

Nevertheless, the detection of native-like secondary structures and the biological activity retained by IB proteins [317], along with their exceptional mechanical stability [219], make them suitable as immobilised biocatalysts for industrial procedures [195-197]. Besides, they also open exciting possibilities as biocompatible therapeutic agents for both human and veterinary medicine [318]. As IBs present a high membrane penetrability [261] and are not toxic neither *in vitro* [253] nor *in vivo* [202], IBs formed by therapeutic proteins can acts as nanopills for protein-based cell therapy [202]. Other advantages to point out are that IBs can be easily produced at high yields under inexpensive processes.[319]. Furthermore, any protein of interest can be produced in such format by fusing, if necessary, an aggregation tag which drives the recombinant polypeptide to IBs [193, 320, 321].

Up to now, analysis of the inner architecture suggested that IBs are formed by stereospecific protein-protein interactions [322]. Contrary to what was generally believed, IB protein disposition has some degree of internal order [199], being the polypeptide sequence key in the manner how proteins are deposited in such aggregates [202]. Moreover, FTIR and nuclear magnetic resonance (NMR) examinations have demostrated that different conformational species coexist in IBs architecture: amyloid-like cross-molecular β-sheet and native-like secondary structures [206, 323, 324].

This work reports for data that back-up the hypothesis that proteinase K-resistant fibril-like scaffold confers the mechanical stability of IBs, whilst the proteinase K-sensitive populations

are made up of biologically functional proteins (see Article 1, Table 1 and Figure 3). In more detail, after massive protein degradation, there is a dramatic decrease of the fluorescence emission (see Article 1, Figure 3 and 4 A). However, the morphology and the size of the particle are no affected (see Article 1, Figure 2 A), even though an important loss of electrodensity has been observed by cryo-TEM (see Article 1, Figure 4 B). In addition, our results experimentally confirm the cotton-like model proposed by Peternel and Komel for the inner IBs architecture [216], in which the amyloid-like scaffold acts as a kind of sponge for the native or native-like proteins filling in the spaces of the matrix (see Article 1, Figure 4 C). Interestingly, a similar slow release platform has also been described in the hormone secretion of the endocrine system, at high concentrations, hormones spontaneously aggregate forming membranous granules which can be stored for a long time at cell cytoplasm because of the stability conferred by the amyloid-like organisation. Upon signalling, functional hormones are released form the secretory granule [325]. Besides, something similar has been described too with the protein Ure2p that forms amyloid filaments in yeast; while the prion domain make up the fiber nucleus, the globular domain stick out it enlarging its size [326]. However, in this prion-oriented model, the proteinase K resistant fraction was the only composed by the prion domain. Nonetheless, our results on VP1GFP bacterial IBs present a peculiarity, the full-length and the fragmented protein are both part of the releasable fraction but also part of the scaffold (see Article 1, Figure 2 B and C). We have demonstrated, by carrying out also proteinase K kinetics with non-fused proteins (see Article 1, Figure 5), that these IBs are as well fractioned in different proteinase K sensitivity population. In other words, this behaviour does not depend on the nature of the fusion protein. In short, the conformational isoforms of the same protein compose the different IBs populations: the fibrillary supportive scaffold and the properly folded proteins filling the gaps of the matrix.

Moreover, a relevant fraction of protein is naturally released from the IBs under incubation in water-based salt solution (see Article 1, Figure 2 B), evidencing its complete solubility. Accordingly, soluble and quasi-soluble functional proteins would be the components of the proteinase K-sensitive population. This dual organization of protein within IB can be also supported by other evidences such as the similar digestion pattern between fusion and non-fusion IB forming proteins (see Article 1, Figure 5) and the release of functional proteins from IBs once uptaken by mammalian cells, when they are used as intracellular drug delivery system in the form of nanopill [202].

We have analysed if the bimodal supramolecular organization was exclusive of *E. coli* IBs. Interestingly, *L. lactis* nanoparticles described in Annex I exhibited also a fraction fully resistant to proteinase K degradation around 30-40 %, but unlike *E. coli,* just one degradable population, with a comparable half-life **(see Annex I, Figure 3)**. Furthermore, the presence of two differentiated conformational protein species has been confirmed by FTIR analysis **(see Annex I, Figure 5)**, representing these two population: one amyloid-like abundant on proteins adopting an intermolecular β-sheet conformation and a second population with protein in a native-like structure which would represent the biologically active embedded protein.

The relative quantity of biologically active and scaffold-forming proteins depend on the cell protein production activity, being likely to be regulated by the pertinent selection of strains, with mutations in protein quality control system, used for the IB biofabrication **(see Article 1, Table 1)**, but also by the protein production conditions. Interestingly, the type of IBs that had been produced in bacteria over a longer period (5h), the Hsp70 IBs, are formed by only two protein populations **(see Article 1, Figure 5)** like *L. lactis* protein aggregate nanoparticles **(see Annex I, Figure 3).** IB architectonic phenotypes are affected by the different cellular protein quality control system from either diverse microorganisms or mutant strains. Nevertheless, select the most appropriate mutant for tuning the IB organisation is still a trial-and-error process due to the complexity of the folding protein pathway.

In a time when the regard of amyloid as a toxic is changing [325, 327] and functional amyloids are seen as potential nanoparticles in nanomedicine [215, 328, 329], the controlled protein packaging of bacterial IBs is suggested as a precious tool to be used as smart nanopills for the controlled administartion of drug molecules [259].

Nanoparticle production conditions in *Lactococcus lactis*

Despite the mechanical stability of *E. coli* IBs and their potential as a drug and a nanoscale material with biomedical applications, the use of *E. coli* as recombinant cell factory for their production has important limitations for further applications: the presence of LPS in their cell membrane. The presence of endotoxins in the final product is one of the main concerns of regulatory agencies [60], especially in the case of nanoscale products which might naturally exhibit inherent toxicity [330]. Whereas industrial-scale protocols have been developed and extensively tested for conventional soluble recombinant proteins [61, 331, 332], these endotoxin removal processes are time-consuming, costly, and, in some cases, ineffective [333]. Furthermore, there are not appropriate methods for endotoxin purification of protein materials with a higher structural complexity. Given the expansion of the protein-based drug market [334], the exploration of Gram-positive microorganisms as LPS-free microbial cell factories is gaining relevance.

In the last years, the great capacity of LAB, and specially of *L. lactis,* in the field of recombinant protein production have been confirmed by its implementation as a soluble protein secretion platform, as well as an efficient alternative for the production of difficult-to-express and membrane proteins [88, 134]. Besides, they have also successfully used as live bacterial vector to deliver antigenic or therapeutic proteins to mucosal tissue in the treatment of diverse diseases such as autoimmune diseases, diabetes, cancer and viral infections. So far, these genetically modified organisms haven been tested by using animal models and they have even been proved in human clinical trials. Although, there is still a lot to be done, the results are very promising, being excellent candidates for vaccination and autoimmune treatments [86, 87].

Even though the production of homologous and heterologous proteins have been deeply studied in *L. lactis*, the conformational quality and the solubility ratio of recombinant proteins expressed in this microbial cell factory has never been explored. In Article 2 and Annex IV we provide data describing how the production conditions (growth conditions, production time, temperature and genetic cell background) have a dramatic impact not only on protein yield, as it has previously been described in LAB [335], but also on protein solubility and conformational quality. Concerning growth conditions, our data suggest that fermentative growth is the optimal situation to successfully produce recombinant proteins in *L. lactis* **(see Article 2, Figure 1)**. Although hemin-stimulated respiration has been presented as improved growth condition to enhance cell biomass and control media acidification [336], our experimental data prove that

under this condition the protein production of our GFP model protein has been clearly affected in both soluble and insoluble fractions (see Article 2, Figure 1). Our results are in concordance with previous published studies [337, 338], in which recombinant GFP producer cells were not fluorescent under aerobic conditions stimulated by hemin (see Article 2, Figure 1), indicating that this kind of induced respiration does not assist the production of active proteins.

Additionally, our results show that, although protein activity is nearly the same at 30ºC and 25ºC, a significant decreased is observed at the soluble fraction at 16 ºC (see Article 2, Table 1). Probably, because under suboptimal temperatures *L. lactis* glycolytic metabolism is affected [339]. However, the conformational quality of soluble proteins is improved at 16 ºC, while the insoluble fraction has better quality at high temperatures, as expected (see Article 2, Table 1). In recombinant protein production, it is widely acknowledged that synthesis at low temperatures increase solubility and conformational quality [340]. About production time, we observe an increase of fluorescence at longer production time, being more pronounced in the soluble fractions (see Article 2, Figure 2). Contrary to what was observed in *E. coli*, protein solubility reached 67 % versus the 10-18 % at 3 h post-induction [190]. On the other hand, the specific activity (see Article 2, Figure 5) was opposite to the protein quantity (see Article 2, Figure 3). This signify that those circumstances that benefit protein solubility are those which do not favour good conformational quality, something that seems to be generic in bacterial protein overproduction and it has been previously described in *E. coli* [315, 341].

Besides, we decided to explore the role of the genetic background in the solubility and conformational quality of proteins produced in *L. lactis*. In this context, it has been described that, in *E. coli*, when mutant strains affecting the protein quality control are used to produce recombinant proteins, diverse conformational ratios are envisaged [315]. For this reason, we planned to create a library of *L. lactis knock-out* strains lacking the main chaperones. Specifically, we designed the experiment to obtain *L. lactis* mutant strains for GroEL, Trigger factor (TF), and DnaK. Unfortunately, we were only able to get the NZ9000 DnaK$^-$ mutant (see Annex IV, Table 3). The protocol used to create these knock-out strains was based on the introduction of a non-replicative plasmid with an antibiotic resistance. The antibiotic presence in the media has an impact on *L. lactis* mutants' cell growth even when carrying the antibiotic resistance cassette (see Annex IV, Table 4). Probably, the combination of a relevant gene mutation and the antibiotic addition might affect *L. lactis knock-out* strain survival, being an antibiotic-free protocol a more adequate way to create *L. lactis knock-out.* Focusing our attention in the results obtained with *L. lactis dnaK knock-out*, we observed that, unlike in *E.*

coli DnaK⁻, bacterial growth in NZ9000 DnaK⁻ was affected, which could be interfering the protein production **(see Annex IV, Figure 7)**. Data shows lower production yields and activity in both soluble and insoluble fractions in NZ9000 DnaK⁻ compared with the wild type strain **(see Annex IV, Figure 6 and 7)**. However, unexpectedly, at 3 h post-induction, the conformational quality of our reporter protein was 3 times higher than the standard strain **(see Annex IV, Figure 8)**. Therefore, we can conclude that the growth conditions and the producer strains largely influence the quality of the recombinant protein. Their control could modulate the quality and the quantity of soluble and insoluble fractions. Therefore *L. lactis* offers a high versatility to produce endotoxin-free standard soluble protein, but also to further explore other conformational species such as protein aggregation or self-assembling proteins with interest for human and animal medicine.

Characterisation of *Lactococcus lactis* nanoparticles

Nanostructured biopolyester beads [333] and polyhydroxybutyrate (PHB) granules [342] are examples of materials produced in *L. lactis* with successful results. For instance, *L. lactis* PHB granules are purer than those produced in *E. coli*. Besides, their associated costs have been considerably lower due to the reduction of downstream purification processes. In this context, *L. lactis* appears as an exceptional option for the production of other LPS-free products such as the protein-based nanostructures, which are gaining interest in biomedicine. After making a deep characterisation of *E. coli* IBs and showing that it is possible to produce both soluble and insoluble protein in *L. lactis* as microbial cell factory, we have isolate and deeply analysed *L. lactis* protein aggregates. In this regard, the mechanical and chemical properties such as size, shape, surface charge and wettability, among others have a great importance in the determination of the nanoparticle functionality [222], allowing set the basis to explore future applications **(see Annex I)**.

Regarding protein aggregate nanoparticles, purified particles were analysed by microscopy techniques (FESEM and TEM) **(see Annex I, Figure 1 and Table 1)**. The ultrastructure shows that these nanoparticles look like IBs formed in *E. coli* [198, 261]. We have noticed that these protein-based nanoparticles are also blocks where the protein is heterogeneously distributed **(see Annex I, Figure 1 B and C)**. Interestingly, specific labelled ultrathin section reveals irregular distribution in the protein localization **(see Annex I, Figure 2)**. Cortical regions are much electrodense, in other words, richer in protein than particle core. This is apparently supported by previous studies which prove the effect of the production conditions on the concentration of the protein in a native-like conformation in IBs [209]. Hence, the heterogeneous organisation of protein in its secondary structure would be related to the process of IB formation [261]. Then, highly electrodense cortical regions could imply nanoparticles with an important amount of functional protein, especially valuable as protein drug with a slow release for advanced therapies.

It is widely known that the genetic cell background of *E. coli* cells and the production conditions considerably influence in both the nanoparticle features and functionality [222, 265], being possible to tune them during the *in vivo* fabrication. That is particularly interesting because we could easily obtain a wide spectrum of functional nanoparticles in order to choose the best for each approach. Interestingly, our data proved that this tailored process can be also performed in *L. lactis* nanoparticle generation. In particular, the appropriate combination of

protein and strain can easily customize protein aggregate nanoscale characteristic such as size, compactness, and shape (see Annex I, Table 1 and Figure 1 B and C). Moreover, their wettability can also be modulated depending on the strain chosen for its biofabrication (see Annex I, Table 1). Surprisingly, we have determined that Z potential values of *L. lactis* IBs (see Annex I, Table 1) are greater than those observed in *E. coli* IBs [222] which seems to indicate that endotoxin-free aggregated nanoparticles are more stable. Moreover, we also observed that the genetic background has a clear effect on the biological activity of the protein species forming such aggregates (see Annex I, Figure 4 A). In conclusion, our results prove that, as it was previously described in other expression systems [169], it is possible to produce LPS-free protein aggregates in *L. lactis* contrary to it was claimed before [163].

During the thesis, it has been confirmed that using *L. lactis*, as protein expression system, is not only possible to synthesise protein aggregates, but also other kind of nanostructures such as soluble self-assembling nanoparticles (see Annex III, Figure 1 E and F). Specifically, T22-GFP-H6 soluble self-assembling protein nanoparticles produced in this Gram-positive microorganism preserve the specificity to bind the CXCR4 cytokine receptors (see Annex III, Figure 2C and D) and to be internalised by an endosome mediated pathway, as it occurs with *E. coli* T22-GFP-H6 soluble self-assembling nanoparticles [265, 278]. Nevertheless, size was one of the main differences observed; in spite of presenting a similar morphology, *L. lactis* soluble self-assembling protein nanoparticles are slightly smaller than those produced in *E. coli* (20 nm versus 14 nm) (see Annex III, Figure 1). Furthermore, a lower specific fluorescence and a higher cell uptake (see Annex III, Figure 2 A) are evidences of a divergent supramolecular organisation of the T22-GFP-H6 protein units produced in *L. lactis*. That fact perfectively fits with a model generated with different *E. coli* T22-GFP-H6 soluble protein nanoparticles (see Annex III, Figure 2 A), which point out that protein organisation on biomaterial performance is dependent of the produced cell factory used [278]. In this context, we expected differences between *L. lactis* and *E. coli* soluble self-assembling nanoparticles. In particular, the quality control of both microorganisms might have different regulators of stress responses [343], and this could determine the final characteristics and biological functionality of the self-assembling protein nanoparticles produced[344, 345].

In vivo potential of *Lactococcus lactis* nanoparticles

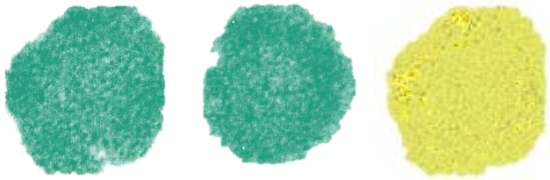
In Manuscript 1, we have proven the great potential that harbours *L. lactis* protein aggregated nanoparticles in drug delivery **(see Annex I, Figure 4 A)** and tissue engineering **(see Annex I, Figure 4 B)**, because they combine a functional activity associated to MMP forming IB materials **(see Annex I, Figure 4 B)** and their ability to enhance cell proliferation **(see Annex I, Figure 4 B).** Furthermore, in **Manuscript 3**, results evidenced the important role in medicine that *L. lactis* soluble self-assembling protein nanoparticles could have not only due to their ability to specifically target a tissue **(see Annex III, Figure 2 A and C),** but also due to their size around 20 nm which could theoretically avoid renal clearance **(see Annex III, Figure 2 A)**. Therefore, in this last part, and with the aim to test the potential of both aggregated and soluble self-assembling nanoparticles as a possible drug or drug delivery vectors for human and animal medicine, we have performed two *in vivo* experiments. A first i*n vivo* assay was done in dairy cows by MMP-9 protein aggregated nanoparticle infusion in mammary gland. On the other hand, a second *in vivo* experiment was performed in a human colorectal cancer mice model by systemic administration of T22-GFP-H6 soluble self-assembling protein nanoparticles.

Among all the MMP nanoparticles analysed, we have chosen the MMP-9 produced in the *clpP-htrA* strain because of its higher activity **(see Annex I, Figure 4 A)**. Surprisingly, our results support that the nanostructured conformation of these aggregated nanoparticles provide stability to the functional proteins which favoured the therapeutic activity. All the analysed markers were significantly stimulated at the first day post dry-off **(see Annex II, Figure 1 and 2)**, while soluble MMP-9 infusion presented values similar to the saline control. That evidences that these MMP-9 protein nanoparticles may short the mammary gland involution and regeneration period. Although these previous results are very promising, there is still a lot of work to do analysing their effect in the cow dry period. As a first step it would be interesting to as determine the optimal nanoparticle concentration, performing an in vivo assay comparing different MMP-9 protein nanoparticles concentration. In a second stage it would important to repeat the experiment with a higher number of animals to finally determine the effect of this treatment in the milk production yields.

Regarding soluble self-assembling protein nanoparticles, upon *L. lactis* T22-GFP-H6 systemic administration in a human colorectal cancer model in mice, we observe that the soluble nanomaterial was efficiently accumulated in the tumor **(see Annex III, Figure 3)**. Moreover, any protein is observed in kidney and this means that these nanoparticles present a high stability

and T22 peptide has a good performance *in vivo* escaping from the renal clearance. Nevertheless, contrary to *E. coli* T22-GFP-H6 soluble self-assembling protein nanoparticles, these food grade self-assembling proteins were also found in liver **(see Annex III, Figure 3 C)**. This slightly different biodistribution does not discard these soluble self-assembling nanoparticles as a potential drug delivery carrier in colorectal cancer, but this indicates the supramolecular organisation of the nanoparticle influences either in the targeting or passive accumulation. Interestingly, in previous works, a T22-GFP-H6 produced in a DnaK$^-$ *E. coli* mutant also presented an unexpected distribution pattern, presenting some accumulation in brain [278]. Therefore, considering the distinct role of DnaK in *L. lactis* and *E. coli* stress response [343]; we hypothesise that the chaperone has a critical role in the final destination of T22-GFP-H6 self-assembling particles at system level, becoming a promising alternative to produce food grade soluble nanoparticles but at the same time with different characteristics in order to use this which fits better with the desire application.

As a conclusion, our study reveals for first time that *L. lactis* is a powerful tool to produce and tune fully safe different kind of protein-based nanoparticles though an inexpensive and scalable process. Interestingly, these functional nanostructures (both aggregates and soluble self-assembling soluble nanoparticles) isolated from *L. lactis* show encouraging results *in vivo* experiments. Thus, this food-grade microbial cell factory is an ideal platform for the synthesis of any protein in a nanoparticle format for a wide range of applications in biomedicine.



Conclusions

- The analysis of the supramolecular organization of IBs reveals a sponge-like architecture combining a proteinase K-resistant scaffold and proteinase K-sensitive fractions. These proteinase K-sensitive fractions can spontaneously be released to the media under mild conditions.

- There is heterogeneity in the protein folding states, with three distinguishable populations in *E. coli* IBs, which are clearly influenced by the cell's genetic background.

- The proteinase K-resistant IB skeleton is responsible for the mechanical stimulation of mammalian cells during conventional cultures onto IBs decorated surfaces.

- Metabolism, growth conditions and temperature have a high impact in the modulation of recombinant protein quality in *L. lactis*, being under anaerobic fermentation and at 30ºC the optimal condition for protein aggregation.

- *L. lactis* as a microbial cell factory produces a spectrum of conformational protein forms under overexpression conditions. Moreover, fully functional IB nanoparticles and soluble self-assembling protein nanoparticles have been successfully produced and isolated.

- *L. lactis* IB nanoparticles are intracytoplasmic round deposits clearly similar to IBs produced in *E. coli* by size, shape, electrodensity and wettability. Nevertheless, *L. lactis* aggregates show higher stability. All these features are modulated by the genetic cell background of the produce strain.

- Relating to the supramolecular organization, *L. lactis* aggregated nanoparticles presents a comparable organization, presenting two differentiated populations: an amyloid-like structure and a native-like structure population.

- Three critical proteins in the cow dry period have been successfully produced in *L. lactis* as microbial cell factory in a biologically active IB nanoparticle format. In addition, theses nanoparticles are able to stimulate mammalian cell proliferation and to be internalise inside the cell.

- MMP-9 IB nanoparticles administration has a significant impact on enhancing the involution process and immune system at dry-off, in comparison with the MMP-9 soluble protein.

- Although *L. lactis* soluble self-assembling nanoparticles have a similar morphology than those produced in *E. coli* and target specifically to $CXCR4^{+}$cells, those proteins present some significant differences in the nanoscale architecture and biodistribution in colorectal cancer mice models when compared with equivalent materials produced in *E. coli*

- As for IB nanoparticles, the cell background of the producer bacteria has an impact on the characteristics of self-assembling nanoparticles, which is essential for the design of these soluble protein nanostructured for biomedicine applications.

- *L. lactis* as a platform for the production of protein nanomaterials represents an intriguing opportunity for the production of different high quality, biologically safe protein nanoparticles, ideal for human and veterinary medicine.

Annex 1: Results

Annex I

Manuscript 1

Functional protein-based nanomaterial produced in GRAS microorganism: a new platform for biotechnology

Cano-Garrido O*, Sánchez-Chardi A, Parés S, Giró I, Tatkiewicz W, Ferrer-Miralles N, Ratera I, Natalello A, Curbasi R, Veciana J, Bach A, Villaverde A, Arís A, Garcia-Fruitós E.

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Abstract

Inclusion Bodies (IBs) are protein-based nanoparticles formed in *Escherichia coli* through stereospecific aggregation processes under overexpression of recombinant proteins. During last years, it has been shown that IBs can be used as nanostructured biomaterials to stimulate mammalian cell attachment, proliferation and differentiation. In addition, these nanoparticles have also been explored as natural delivery systems for cell replacement therapies. Although the production of these protein-based nanomaterials in *E. coli* is economically viable, important safety concerns related to the presence of endotoxins in the products derived from this microorganism need to be addressed. Lactic acid bacteria (LAB) are a group of food-grade microorganisms that have been classified as safe by biologically regulatory agencies. In this context, we have demonstrated here, for the first time, the production of fully functional, IB-like protein nanoparticles in LAB that share the main physico-chemical characteristics with IBs from *E. coli*, but devoid of any harmful endotoxin contaminant. These findings reveal a new platform of protein-based safe products with high pharmaceutical interest.

Keywords: endotoxin-free, nanoparticles, functional nanomaterials, GRAS, Lactic acid bacteria

Introduction

Over the last years, *Escherichia coli* has been described as a cell factory for the production of self-assembling nanostructured and functional protein materials known as inclusion bodies (IBs) [1-4], which have been studied as stimulators of cell proliferation and differentiation [5, 6] and as natural protein delivery systems [2, 7]. However, the presence of lipopolysaccharide (LPS) in the *E. coli* outer cell membrane becomes a major obstacle in terms of therapeutic applicability of this biomaterial. LPS, also known as endotoxin, can elicit undesirable immunogenic responses [8, 9]. FDA regulations establish an endotoxin level limit of 5 EU/kg/h for pharmaceutical compounds and medical devices. Thus, all products from *E. coli*, as well as from other Gram-negative microorganisms, need to be finely purified through processes with important associated costs to ensure the removal of any pyrogenic or inflammatory contaminant inherently present in the sample [8, 10]. Nowadays, the development of a universal and effective method for endotoxin removal is a still unresolved problem [10], being particularly critical for complex structures such as protein-based nanostructured materials [9, 11].

In this context, a group of Gram-positive bacteria known as lactic acid bacteria (LAB) has been recently gaining momentum as an alternative cell factory [12-14]. These microorganisms do not have LPS and, in consequence, their derived products do not contain pyrogenic impurities [15, 16]. Indeed, they have been classified by regulatory agencies as Generally Recognized as Safe (GRAS) organisms [17]. The development of this biologically-safe production platform based on LAB clearly opens a new era in terms of therapeutic applicability, leaving behind all the important drawbacks associated to *E. coli*-derived recombinant products. So far, the possibility to produce protein-based nanostructures in these bacterial cell factories has not been explored. It is widely believed that LAB, contrary to what occurs in *E. coli*, are only able to produce soluble proteins, rendering the production of protein-based biomaterials from these GRAS microorganisms rather impossible. However, some recent studies contradict this general assumption. Lu and collaborators described the presence of highly fluorescent clusters inside *Lactococcus lactis* cytoplasm, under the overexpression of a fluorescent protein [18]. Besides, our group also observed the presence of protein deposits in *L. lactis* cytoplasm [19]. Considering all these results together it can be expected that *L. lactis* is also able to form fully safe protein-based protein clusters that could be further explored as functional nanomaterials.

Thus, the aim of this study is the isolation and detailed characterization of the main morphometric and physico-chemical properties of such a new class of protein deposits. For that, we selected *L. lactis*, the most used LAB in the field of recombinant protein production as cell factory [20, 21]. Three relevant proteins in human and veterinary medicine such as bovine metalloproteinase 9 (MMP-9) and 2 (MMP-2) and interferon gamma (IFN-γ) have been used as model proteins. The obtained data, which reveals the possibility to produce both fully functional and safe protein-based nanoparticles in LAB, offers an attractive opportunity for the production of a new generation of a type of biomaterials with a wide range of applications in biotechnology and human and animal medicine.

Materials and Methods

Bacterial strains and plasmids

L. lactis subsp. *cremoris* NZ9000 [20] and NZ9000 *clpP- htrA-* (*clpP-htrA*; Em^R) [22, 23] (kindly provided by INRA, Jouy-en-Josas, France; patent nº EP1141337B1) strains and *E. coli* MC4100 strain (StrepR) [24] were used. Three proteins from bovine (*Bos taurus*) origin were cloned in the Cm^R pNZ8148 plasmid (MoBiTech): the mature form of the interferon gamma (IFN-γ(from

Gln23 to Thr101 NM_173925) and the catalytic domain of metalloproteinase 9 (MMP-9) (from Phe107 to Pro449 NM_174744) and 2 (MMP-2) (from Tyr110 to Asp45 NM_174745). In addition, a fusion of MMP-9 with an aggregation-prone peptide (ELK16: (LELELKLK)2) was also constructed (MMP-9ELK16). All genes were C-terminally fused to a His-tag for detection and quantification purposes in western blot analysis. Gene sequences were codon optimized (Geneart). In the sequence design we added a *NcoI* restriction site at 5' followed by nucleotides CA to restore the reading frame and a *XbaI* restriction site at 3'. The digestion product was ligated into the expression plasmid pNZ8148 and ligation product was transformed by electroporation into *L. lactis* NZ9000 and *clpP-htrA* competent cells [25]. Electroporation was performed using Gene Pulser from Bio-rad fitted with 2500V, 200 Ω and 25 µF in a pre-cooled 2 cm electroporation cuvette. Following, samples were supplemented with 900 µL M17 broth with 0.5 % glucose and incubated for 2 h at 30 ºC. The electroporation mix was centrifuged for 10 min at 10,000 x *g* at 4 ºC and the pellet was resuspended in 100-200 µL of M17 media and plated. Besides, recombinant Green Fluorescent Protein (rGFP) previously described in [19, 26] were also used.

Nanoparticle production and purification

L. lactis strains containing the previously described plasmids were grown in M17 medium enriched with 0.5 % glucose at 30 ºC without shaking. *E. coli* was grown in LB rich medium at 37 ºC, 250 rpm. Nanoparticle production was induced by adding 12.5 ng/mL nisin (Sigma-Aldrich) in *L. lactis* or 1 mM IPTG in *E. coli* cultures at OD_{550nm} = 0.5. After induction, cultures were grown for 3 h. Antibiotics were used for plasmid maintenance at the following concentrations: chloramphenicol (5 µg/mL) and erythromycin (2.5 µg/mL) for *L. lactis* and ampicillin (100 µg/mL) and streptomycin (30 µg/mL) for *E. coli.*

Once produced, protein nanoparticle were purified using the purification protocol described by [27], adding at the beginning a mechanical disruption step by French Press. The protocol has been done under sterile conditions and all incubations were carried out under agitation.

The amount of recombinant proteins present in nanoparticles has been quantified by denaturing SDS-PAGE as described in [19]. Bands were identified using a commercial polyclonal serum against histidine tag (#A00186-100 Genscript) and an anti-mouse secondary antibody (#170-6516, Bio-Rad). Recombinant protein yield were estimated with a standard curve with known amounts of a GFP-H6 protein. Quantification was performed with the Quantity One software.

Field Emission Scanning Electron Microscopy (FESEM)

For nanoparticles morphometry (size and shape), microdrops of protein aggregates samples were deposited during 2 min in silicon wafers (Ted Pella Inc.), air-dried and observed in a FESEM Zeiss Merlin (Zeiss) operating at 2 kV. Micrographs of nanoparticles morphology at a nearly native state were acquired with a high resolution in-lens secondary electron (SE) detector. A quantitative analysis of size particles was performed with a total number of 474 nanoparticles using Image J software.

Transmission Electron Microscopy (TEM)

For ultrastructure, samples were fixed with aldehydes, post-fixed with osmium, dehydrated in acetone, embedded in Epon resin, and polymerized following conventional methods [28-30]. Ultrathin sections were placed on copper grids, contrasted, and observed with a TEM Jeol JEM-1400 (Jeol Ltd.) equipped with a CCD Gatan ES1000W Erlangshen camera. For MMP-2, MMP-9 and IFN-γ immunolocalization, pellets of bacterial cells and protein nanoparticles were fixed in 4 % (w/v) paraformaldehyde and 0.1 % (v/v) glutaraldehyde in PB, cryoprotected in sucrose, cryofixed in propane, dehydrated in methanol, embedded in Lowicryl HM20 resin (Polysciences Inc.), and polymerized with UV rays. Ultrathin sections placed on carbon-coated gold grids were labeled for the 3 antigens of interest using polyclonal primary antibodies (anti-MMP-2: #AV20016, Sigma-Aldrich; anti-MMP-9: #50560-RP01, Sino Biological Inc.; and anti-IFN-γ: #ab9657, Abcam, at working dilution 1:5, 1:5 and 1:2 respectively) and protein A coupled to 10 nm-gold particles (BBI Solutions), following standard methods [30, 31]. Grids were contrasted and examined with a TEM Jeol JEM-1400 at same conditions than previously described.

Z-Potential

Z-Potential characterization of each kind of protein nanoparticles was carried out using dynamic light scattering (DLS) equipment (Malvern Nanosizer). In order to avoid the electrodes from getting burned, the samples were prepared in deionized (MilliQ) water, a low ionic strength medium. Each sample was analyzed by triplicate.

Proteinase K assay

Protein nanoparticles were resuspended in PBS with protease inhibitor and sonicated to obtain a homogenous sample. Kinetic analyses were performed as described in [32]. In control

suspensions MilliQ water was added instead of Proteinase K. Sample was recovered after every measurement to maintain constant the reaction volume. The experiment was done by triplicate.

Mathematical modeling

The dynamics of nanoparticle disaggregation process is described through a mixture of decreasing exponential functions with up to three populations according to one of the following two models. If the protein of nanoparticles at time t is denoted by $y_m(t)$, a mixture without a constant population is given by

$$y_m(t) = N_0 e^{-c_1 t} + \cdots + N_k e^{-c_k t};\ \ m = 2\mathrm{k};\ k \geq 1$$

denoted with an even subscript according to the even number of terms. Otherwise, if there exist a non-disaggregated population, the nanoparticle amount is given by

$$y_m(t) = N_0 + N_1 e^{-c_1 t} + \cdots + N_k e^{-c_k t};\ \ m = 2\mathrm{k}+1;\ \ k \geq 1$$

with an odd subscript. In both cases the fit will depend on a set of m parameters P that is evaluated from a least squares fit. Only positive values of N_i and c_i are allowed. The parameter N_i is the initial protein of the i-th component and c_i, in the exponent, provides the expected life $T_i = 1/c_i$ (the half-life is $t_i = \ln 2/c_i$). The number of components in the mixture model is $C = \left[\frac{m+1}{2}\right]$ (integer part). From a data array $L = \{(x_i, t_i); i = 1, \ldots, n\}$, where x_i is the nanoparticle amount at time t_i, we minimize the $\chi^2$of the fit [29]. Among all the possible fits, as increasing *m* we choose the one satisfying … $> \chi^2_{m-1} > \chi^2_m \leq \chi^2_{m+1}$.

In the context of the disaggregation process, not all fits are admissible. Indeed, the procedure can be applied in two ways: (a) As for each strain, the experiment is repeated *q* times, there is a number of *q* arrays by strain that we average to build a single array $\bar{L} = E(L)$ (*E* means the expected value). We then determine the specific parameters *P*⃗ of the strain. (b) Alternatively, for each strain, each experiment may be fitted separately. This provides q families of parameter sets P that we average as $\bar{P} = E(P)$ in order to characterize the strain components. A meaningful fit must fulfill that the values $\bar{P}$ and *P*⃗ match within a low error margin. This is a sufficient condition to accept the fitting, otherwise the strain average values *E*(*L*) would not represent the individual experiments. In particular, this implies that the number

of components of the strain obtained from the average *E(*L) and that obtained for each individual experiment must match. The necessary condition for comparing both procedures is to determine the same number of strain components *C* from the average array and the single arrays. If both approaches provide a different number of strain components, other fittings to reconcile both procedures must be chosen among those with low χ^2, preferably among the simplest models, although they not provide the lowest value.

Metalloproteinases activity assay (zymography)

MMP enzymatic activities were determined by gel 10 % SDS-PAGE + 1 % gelatine under non-denaturing conditions. IBs were loaded onto the gel diluted 1:1 with a sample loading buffer (0.125 M Tris, 0.005 % bromophenol blue, 20 % glycerol, 4 % SDS). Then, the gel was incubated with developing buffer (50 mM Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02 % Brij 35) for 48 h at 37 ºC. Finally, it was dyed with Coomassie and discoloured until degradation bands become visible. Densitometry analyses of the bands were performed with the Image J software.

Fourier transform infrared spectroscopy (FTIR)

The protein hydrated films were measured by the infrared microscope Varian 610-IR, coupled to the Varian 670-IR spectrometer (both from Varian Australia Pty Ltd.), as reported in [33, 34]. For hydrogen/deuterium (H/D) exchange experiments, the protein film on the BaF_2 window was hydrate by adding 5 µL of a solution of 1/4 of glycerol/D_2O ratio (w/w) around the dried film. The sample was then tightly closed by a second window using a flat O-ring.

Proliferation Assay

Human skin fibroblast cells (1BR3.G) were grown at 37 ºC and 10 % CO_2 on protein nanoparticles-decorated surfaces. A total of 1 µg of rGFP based protein nanoparticles from *L. lactis* and *E. coli* were added to each well of an untreated Costar 3370 plate and incubated with 100 µL of Dulbecco's Modified Costar Medium (DMEM) without serum at 4 ºC overnight. After incubation, 1BR3.G cells were added (5,000 cells per well) in 100 µL DMEM medium with 2% fetal bovine serum (FBS). As controls, we plated cells on non-decorated wells and we also used wells decorated with nanoparticles but without cells. Plates were incubated at 37 ºC for 24, 48 and 72 h, and the MTT proliferation cell assay was performed as described [35].

Nanoparticles internalization (cytometry)

HeLa cells (60,000 cells per well) were seeded in treated 12-well plates in the presence of Minimum Essential Medium (MEM-α) medium supplemented with 10 % FBS and 2 mM Glutamax (Gibco). After incubation at 37 °C and 5 % CO2 for 24 h, the medium was removed and the cells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS). Then, 5 µg of rGFP based protein nanoparticles from *L. lactis* and *E. coli* were suspended in MEM-α containing 10 % FBS and 2 mM Glutamax and added per well. After 48 h, cell samples were treated for 15 min in 1 mg/mL trypsin and samples were analysed 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.

Nanoparticles internalization (confocal microscopy)

Hela cells (100.000 cells/mL) were seeded on MatTek culture dishes with MEM-α medium supplemented with 10 % FBS and 2 mM Glutamax (Gibco), and incubated at 37 °C and 5% CO_2. 5 µg rGFP nanoparticles were added to cells in the presence of Optipro medium (Gibco) and incubated O/N. For confocal analysis, cell membrane and nuclei were stained and stacks were obtained as described elsewhere [36].

Modification of gold substrates with mixed self-assembled monolayers (SAMs)

SAMs with different proportions of 1-undecenethiol (-CH_3 terminated) and 11- mercapto-1-undecanol (-OH terminated) were prepared by immersion of gold substrates in an ethanolic solution of the thiols with the appropriate molar ratio for 24 h as descrived elsewhere [5]. Then, the substrates were dried under a stream of N_2 getting surfaces with different wettability properties.

Wettability

The wettability of mixed thiols SAMs on gold surfaces before and after protein nanoparticles deposition was determined via static contact angle (CA) measurements using a DSA100 from KRÜSS.

Deposition of protein nanoparticles on SAM modified substrates and surface covering analysis

Substrates with mixed SAMs were immersed in the protein nanoparticles suspensions for 2 h, and rinsed with MilliQ water. Light microscopy images were obtained using an Olympus Bx51 microscope and surface coverage analysis using ImageJ 1.47

Statistical analysis

Data were analyzed using a general linear model (JMP, SAS Institute Inc.). For the analysis, strain, protein, and the interaction between strain and protein were used as fixed effects. When more than two means were compared, differences were established using the Tukey's multiple mean separation test. Data were previously transformed when necessary to achieve a normal distribution. Results are expressed as the means of non-transformed data ± standard error of mean (SEM), except otherwise stated.

Results

Ultrastructural analysis of *L. lactis* nanoparticles by electron microscopy

TEM micrographs obtained of *L. lactis* cells under overproduction conditions show the formation of intracytoplasmic round protein deposits in practically all cells **(Figure 1A)**. Furthermore, the specific immunolocalization confirms that these deposits are formed by the recombinant protein **(Figure 1A, right images)**. In a second step, once the production of these protein deposits in *L. lactis* was successfully proven, MMP-9, MMP-2 and IFN-γ nanoparticles were isolated and quantified by Western blot (data not shown). The ultrastructural morphology of the nanomaterial was analysed by FESEM and TEM **(Figure 1B and C)** and the obtained micrographs allowed us to conclude that these protein deposits clearly correspond to well-structured and compact-defined particles at the nanoscale range **(Figure 1B and C)**.

Size determination of *L. lactis* nanoparticles

Using FESEM micrographs we determined the size of the nanostructures, obtaining values ranging between 300 and 450 nm **(Table 1)**. Extreme mean values were found in NZ9000 *clpP*$^-$ *htrA*$^-$ strain (hereafter called *clpP-htrA*), with lowest mean values in IFN-γ and highest mean values in MMP-9 **(Table 1)**. The statistical analysis shows that the size of the particles is determined by the combination of the specific protein and the strain. MMP-9 round particles

(Figure 1B) were bigger in *clpP-htrA* strain than those produced in wild type strain NZ9000 (Table 1). In marked contrast, more elongated IFN-γ nanoparticles (Figure 1B) showed high variable size and shape and higher mean values in NZ9000 strain (Table 1).

Electrodensity and immunolocalization analysis of *L. lactis* nanoparticles

The internal ultrastructure reported by TEM (Figure 1C) not only strengthens the analysis performed by FESEM (Figure 1B), but also shows that the electrodensity of protein aggregates is again protein- and strain-dependent. Interestingly, the electrodensity and compactness observed by TEM (Figure 1C) perfectly correlates with the solubility of each protein (Table 1). The higher the solubility of a protein is, the less prone to form protein nanostructures it is. Among tested proteins, IFN-γ shows higher solubility and its nanoparticles present low electrodensity and a less compact ultrastructure (Figure 1C). Additionally, these particles, in marked contrast to what occurs with particles formed by prone to aggregate proteins, such as MMPs, that form highly compact, smooth surface, and round-shape nanostructures, exhibit rough surface and more variable size and shape (Figure 1B). To evaluate in more detail, the distribution of the protein forming such nanomaterial we performed an immunolocalization of purified nanoparticles using TEM. Ultrathin section of central parts of MMPs nanoparticles showed cortical electrodense parts of the nanostructure (heavily labelled), whereas central and less electrodense areas were less marked (or not marked) (Figure 2). This finding, observed both in nanoparticles inside cells (Figure 1C) and in purified ones (Figure 2), is indicative of high differences in protein concentrations in cortical and central parts of nanoparticles.

Study of the stability and supramolecular organization of *L. lactis* nanoparticles

The structure and stability of the protein forming such nanomaterials are important parameters to be dissected. Z-potential measurements showed that all nanoparticles present negatively charged surfaces with large negative values ranging from -33 to -26 mV (Table 1), being an indication of stable suspensions. To get further detail of supramolecular organization of nanoparticles produced in *L. lactis*, a time-course of nanoparticle stability was performed (Figure 3). After proteinase k incubation, the three proteins evaluated showed a population susceptible to degradation (population 1), with half-life values in the same range for all proteins (Figure 3). Besides, a fully resistant population (population 2), ranging from 33 to 40 %, was preserved. Thus, nanoparticles produced in *L. lactis* present two differentiated populations in terms of proteolytic resistance (Figure 3).

Determination of *L. lactis* nanoparticles functionality in vitro

At this point it was critical to determine the biological activity of the nanoparticles, produced and purified for the first time from *L. lactis*. Figure 4A shows that nanoparticles of relevant proteins for human and veterinary medicine produced in *L. lactis* are biologically functional. All MMPs tested have the ability to degrade the substrate in a strain and protein-dependent manner (Figure 4A). It is worth mentioning that it is also possible to control the conformational quality of the protein embedded in such nanomaterials (Figure 4A). The selection of the appropriate strain would allow increasing the specific activity of *L. lactis* nanoparticles, as it occurs with MMP-9. Besides, MMP-9 improves significantly its specific activity, just by the presence of an aggregation tag (ELK16) fused to the protein. Moreover, the statistical analysis indicates that the strain, combined with the protein used, is an important parameter to take into account in the production of such functional protein delivery agents in safe systems. Interestingly, data obtained from the FTIR analysis confirms the coexistence of intermolecular β-sheets in nanoparticles (components at around 1695 and 1627 cm^{-1}) with the presence of native-like structures (Figure 5 and Suppl Figure 1) [33, 34]. These two populations perfectly correlate with the model mentioned above (Figure 2), in which resistant (intermolecular β-sheets) and sensitive (native-like structures)-proteinase K populations are coexisting in the same nanostructure. As an example, the second derivative spectra of MMP-9 and MMP-9-ELK16 are dominated by the marker bands of intermolecular β–sheets in protein aggregates, but an important component around 1658 cm^{-1} was also present, indicating the presence of native-like α-helices and of random coil structures. The comparison of MMP-9 and MMP-9-ELK16 spectra allows concluding that MMP-9-ELK16 is characterized by a lower level of aggregation, particularly in the case of *clpP-htrA* sample, which is in agreement with a higher specific activity observed with these nanoparticles (Figure 4A).

The ability to stimulate cell proliferation is another interesting property of IBs from *E. coli*. Interestingly, surfaces decorated with the protein-based nanomaterial produced in *L. lactis* are also able to stimulate the proliferation of mammalian cells at a level similar to that achieved by the particles produced in *E. coli* (Figure 4B). Moreover, *L. lactis* nanoparticles are able to internalize inside the cells better than those produce in *E. coli* (Figure 4C and D).

Study of the hydrophilic/hydrophobic nature of *L. lactis* nanoparticles

Since it has been already described that the hydrophilicity of a surface influences its cell adhesion properties [37], we have determined the nanoparticles wettability. Thus, the

wettability of mixed self-assembled monolayers (SAMs) on gold substrates before and after being functionalized with *L. lactis* nanoparticles was determined via contact angle (CA) measurements (Figure 6). As expected, prior to nanoparticle deposition the CA decreased as the hydrophilicity of the SAM increased. An increase of 10% of the molar fraction of OH-terminated thiols implied a decrease of 6º of the contact angle of the SAM-covered substrates. However, after MMP-9 nanoparticle deposition, the reduction of the contact angle in response to a 10%-increase of the hydrophilic thiols concentration relative to the hydrophobic one was smaller (4.0º in NZ9000 and 2.1º in *clpP-htrA*). Regarding MMP-2, after deposition of both strains, the contact angle remained rather constant around 60º, which is an indication of its higher hydrophilic character. The MMP-9-ELK16 present different wettability property depending on the genetic variant it is obtained from. Thus, MMP-9-ELK16 from the wild type strain gives also a constant contact angle around 60º but the one obtained from *clpP-htrA* strain gives place to a reduction of 3º of the contact angle in response to a 10%-increase of the hydrophilic thiols concentration. After IFN-γ deposition produced in both strains, the contact angle remained rather constant at around 65°-70°. From the change of CA after nanoparticle deposition, we can conclude that the deposition of nanoparticles produced in *L. lactis* buffers the change of wettability of the substrates; or in the case of IFN-γ and MMP-2 nanoparticles, it keeps it relatively constant around a certain value.

Nanoparticle surface coverages obtained are shown in **Table 2**. From these data, it is possible to affirm that the affinity of protein nanostructures toward hydrophilic and/or hydrophobic surfaces also depends on the genetic background in which they have been produced. Thus, the interactions between these protein-based biomaterials and substrates, and the density of deposited nanoparticles can be tuned to a certain extent choosing a specific *L. lactis* strain. With MMP-2 and MMP-9-ELK16 there is a higher coverage and higher affinity of the nanoparticles towards the substrate for the NZ9000 than for the genetically modified *clpP-htrA*. Regarding the MMP-9 and the IFN-γ the potential difference between NZ9000 and the mutant strain is not clear. The surface coverage significantly varies also in response to changes of substrate wettability. For some proteins, high coverages are obtained for substrates of different wettability, which could be attributed to the presence of amphiphilic properties of the obtained nanoparticles.

Discussion

E. coli IBs have been shown to be a mechanically stable nanomaterial with interesting properties as a drug delivery system, but also as a promising biomaterial for tissue engineering. However, the use of *E. coli* as recombinant cell factory for their production has an important shortcoming. Because of its endotoxic nature due to the presence of LPS, even at low concentration, can trigger a non-desired immunogenic response. Therefore, any *E. coli*-derived product have to be necessarily further purified through a time-consuming, costly, and, in many cases, ineffective depyrogenation process [14]. For this reason, and with the goal of producing protein-based biomaterials in an endotoxin-free environment, we explored *L. lactis* as cell factory. Although *L. lactis* has already been proven to be an excellent choice for the production of other LPS-free biomaterials such as polyhydroxybutyrate (PHB), the main mechanical and chemical properties of protein-based nanostructures produced in this Gram-positive microorganism have never been studied. Thus, the present study shows for the first time an accurate analysis of a novel protein-based nanomaterial produced in a safe cell factory, which allows us to set the basis to further explore the applicability of this platform to produce biomaterials for the pharmaceutical industry.

As starting point, we successfully produced and isolated such nanomaterial from *L. lactis*. The obtained nanoparticles were carefully assessed by high resolution microscopy techniques, noticing that proteins acting as building blocks of these nanoparticles are heterogeneously distributed within the nanomaterial **(Figure 2)**. In particular, immunolocalization analysis reveals high differences in protein concentrations in cortical and central parts of nanoparticles, being cortical regions much more rich in protein than particle core. This is probably a phenomenon closely related to the activity of cell mechanisms during nanostructure formation and growth, previously observed in IB formation [30]. In addition, the ultrastructure observed by FESEM and TEM **(Figure 1 and Table 1)** also resembled that of IBs formed in *E. coli* [3, 30, 38]. Interestingly, our data proved that nanoscale features such as size, compactness and shape can be easily tailored by selecting the appropriate combination of protein and strain **(Table 1, Figure 1B and C)**. The interaction between strain and protein had also a clear effect on the biological activity of our nanoparticles **(Figure 4A)**. This finding opens a whole range of possibilities in the customization of this functional and safe biomaterial. This is particularly interesting considering that these protein-based nanomaterials can be also genetically engineered, being possible not only to define size, shape and electrodensity, but also the organization and the biological activity of the produced protein-based nanoparticles in this

safe microorganism. Considering the enzymatic activity of MMP nanoparticles (Figure 4A) and their ability to stimulate cell proliferation (Figure 4B), this biomaterial has a great potential for both drug delivery (Figure 4A) and tissue engineering purposes (Figure 4B).

It has also been described that *E. coli* IBs show a bimodal supramolecular organization of the embedded protein. In this case, *E. coli* IBs contain a fraction of protein ranging from 9 to 33% with an amyloid-like organization, which is fully resistant to proteinase K degradation acting as a nanoparticle scaffold, and two other protein populations, one of them being immediately degraded and another one sensitive to proteinase K digestion being associated with the biological activity [32]. The food-grade nanomaterial characterized in the present study exhibited a degradable population, with a half-life comparable to the populations described in *E. coli* [32], and a resistant population (Figure 3). The coexistence of these two distinguished populations was further confirmed by FTIR analysis (Figure 5). Proteins adopting an intermolecular β-sheet conformation would correspond to the proteinase-K resistant population, whereas proteinase-K sensitive population exhibited a native-like structure which contributed to its remarkable biological activity.

The determination of the chemical properties of these nanoparticles also supports that such nanoclusters have many characteristics in common with those obtained when using *E. coli* as cell factory. Depending on the genetic background chosen to produce the nanoparticles, the wettability properties of the nanostructures and thus, the wettability of surfaces modified with them, can be modulated [5]. Besides, the presence of a simple aggregation tag, such as ELK16 in MMP-9, clearly modifies the behavior of the nanostructure produced. However, Z potential values of *L. lactis*-derived nanoparticles determined in this study are higher than those observed in IBs producing in LPS enriched environment [5], indicating that, although endotoxin-free nanostructures share many characteristics with those derived from *E. coli*, they are more stable in suspension.

Considering *E. coli* limitations, this study opens a range of possibilities in terms of applications. The implementation of lactic acid bacteria as a routine cell factory for the production of functional nanomaterials would allow the development of safe production processes, avoiding at the same time complex downstream purification steps to eliminate toxic components.

Conclusions

Data presented in this study reveal for the first time, that it is possible to produce, tune and isolate endotoxin-free IB-like nanoparticles from LAB through a cost-effective and fully scalable process. All results evidence that under overexpression conditions *L. lactis* is able to produce protein nanoparticles with the same properties that those produced in *E. coli.*

Interestingly, these functional nanostructures isolated from *L. lactis* show even higher stabilities and activity that those obtained from *E. coli*. This finding allows making a qualitative step, since it opens a field of opportunities for the production of recombinant proteins in a cost-effective format using GRAS systems as cell factories. Thus, this makes LAB an ideal alternative for the synthesis of tuned protein-based endotoxin-free nanoparticles for a wide range of applications in both human and veterinary medicine. In summary, this work indicates that LAB could become the workhorse bacteria in a near future, replacing *E. coli* in this role.

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Figure

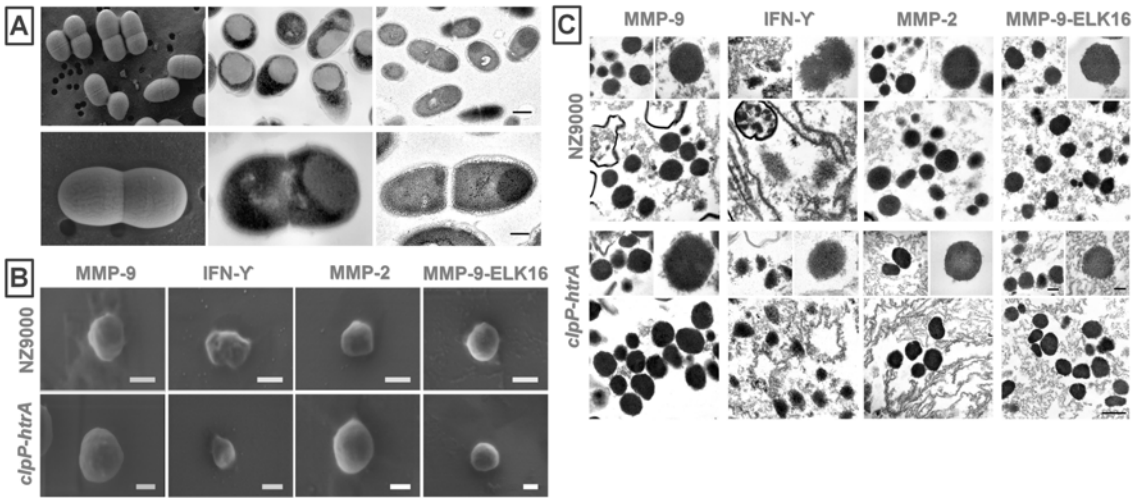


Figure 1: FESEM and TEM micrographs of *L. lactis* and purified nanoparticles. A) On the left, FESEM micrographs of *L. lactis* bacteria. In the middle, TEM sections of *L. lactis* with MMP-9 nanoparticles inside (light grey particles). On the right, TEM immunolocalization of MMP-9 nanoparticles (labeled by anti-MMP-9) in *L. lactis*. Scale bars: Upper micrographs 500 nm; lower micrographs 200 nm. **B)** FESEM micrographs of purified nanoparticles. Scale bars: 200 nm. **C)** TEM micrographs of purified nanoparticles. Scale bars: left inset 200 nm; right inset 100 nm; lower micrographs 500 nm.

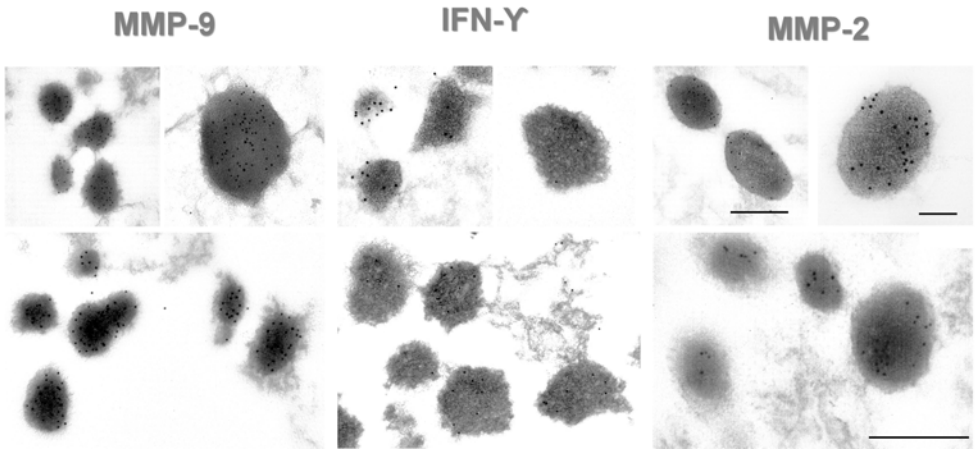
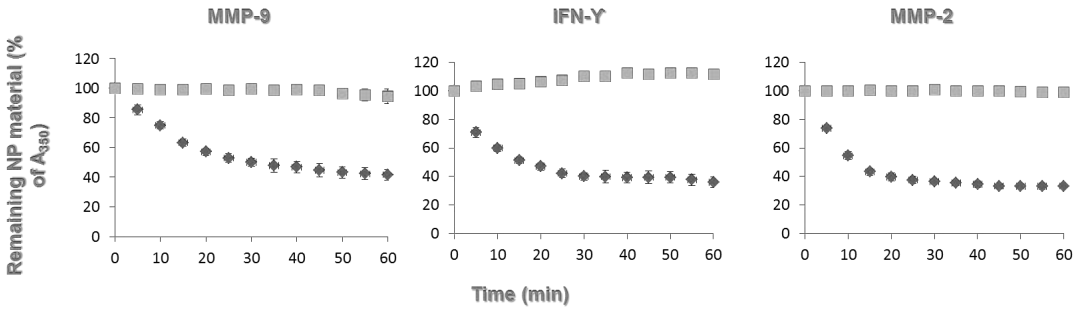


Figure 2: TEM micrographs of purified nanoparticles labelled by specific antibodies. Scale bars: Left and right inset 200 nm; lower micrographs 500 nm.



	MMP-9		IFN-Y		MMP-2	
	Percentage (%)	t_{50} (min)	Percentage (%)	t_{50} (min)	Percentage (%)	t_{50} (min)
Population 1	60.56 ± 3.81	11.47 ± 1.98	60.76 ± 4,50	6.52 ± 0.50	67.53 ± 2.80	6.16 ± 0.53
Population 2	40.18 ± 3.11	∞	38.16 ± 5.37	∞	33.24 ± 2.56	∞

Figure 3: Time-course stability of nanoparticles. MMP-9, IFN-γ and MMP-2 produced in the *clpP-htrA* during proteinase K digestion (dark grey diamonds). Controls without proteinase K (light grey squares). The standard error is represented by black lines at each time point. The values detailed in the table indicate the distribution of protein populations in the nanoparticles according to their half-life under proteinase K treatment.

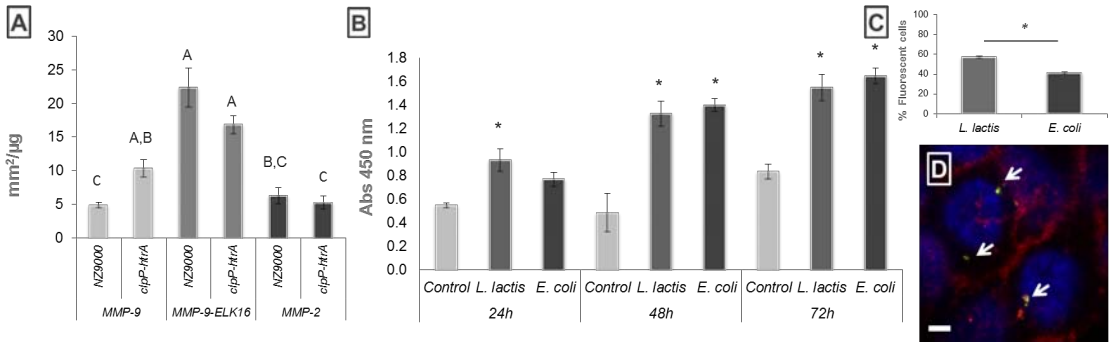


Figure 4: Functionality of *L. lactis* protein-based nanoparticles. A) Specific activity of metalloproteinases. Different letters depict differences between proteins ($P < 0.0001$) and due to the interaction between strain and protein ($P = 0.0022$). **B)** Biological effects of GFP nanoparticles on human mammalian cell cultures. **i.** Proliferation assay with 1BR3.G cells cultured on surfaces decorated with 1 µg of GFP nanoparticles at 24, 48 and 72 h. Bars with asterisk differ (24 h, $P = 0.0433$; 48 h, $P = 0.0008$; 72 h, $P = 0.0055$) from negative control. **ii.** Percentage of fluorescent HeLa cells analysed by cytometry after 24 h exposure to GFP nanoparticles. Significant differences depicted by asterisk ($P = 0.0010$). **iii.** Confocal micrographs of HeLa cells after 24 h exposure to GFP nanoparticles. Scale bar in micrograph represents 5 µm. In all cases, results are expressed as means of non-transformed data ± SE, which correspond to un-transformed data.

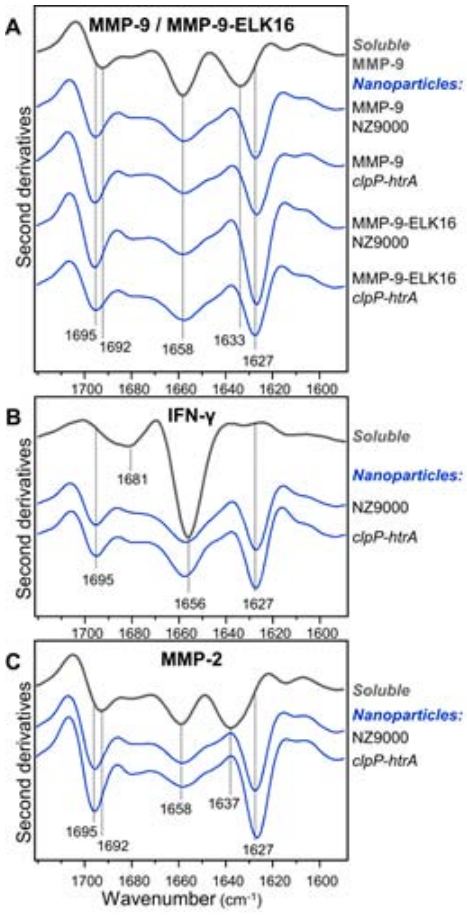


Figure 5: FTIR analysis of purified nanoparticles. Second derivatives of absorption spectra of **A)** MMP-9 and MMP-9-ELK16; the component around 1658 cm-1 can be assigned to α-helices and random coils and the two components at around 1633 cm-1 and 1692 cm-1 to the native intramolecular β-sheet structures of the protein. **B)** IFN-γ the components around 1656 and 1681 cm-1 can be assigned to the native α-helices and to turn structures of the protein, respectively. **C)** MMP-2; the component around 1637 cm-1, along with the peak at ~1692 cm-1, can be assigned to native, intramolecular β-sheets. The component around 1658 cm-1 is due to the native α-helices and to the random-coil structures of the protein. In A), B) and C) the two components at around 1627 and 1695 cm-1 are the typical marker bands of intermolecular β-sheet in protein aggregates.

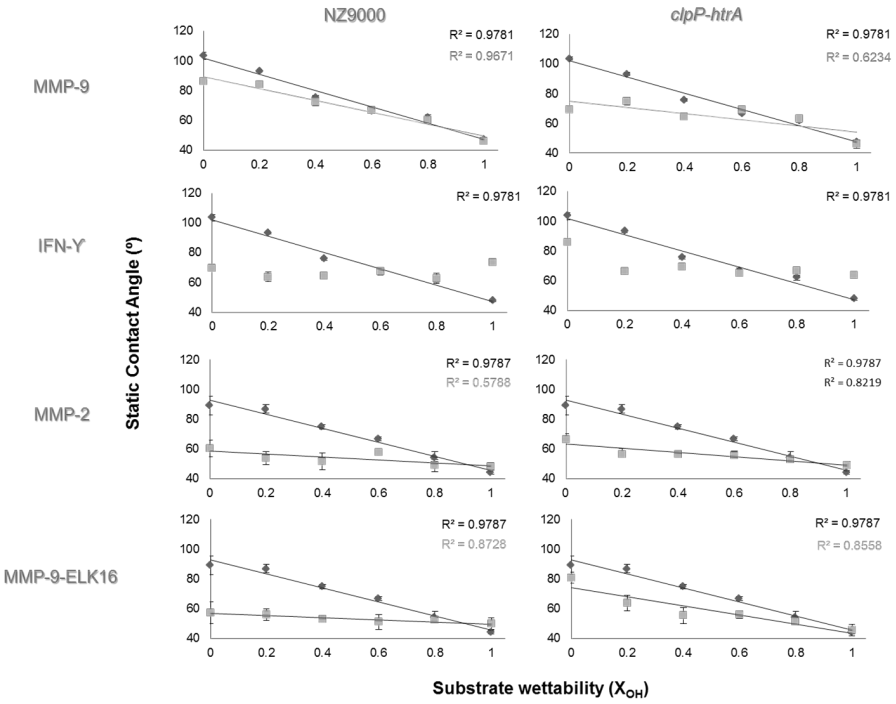


Figure 6: Contact angle measurements. Contact angles of 6 different functionalized gold surfaces, containing different molar ratio of –OH terminated (XOH) alkanethiols. Surfaces before nanoparticle deposition (dark grey diamond) and after nanoparticle deposition (light grey square).

Tables

Table 1: Nanoparticle diameter, Z-potential and aggregation propensity. Measurements (mean ± standard error, in nm) done 3 h post-induction. Significant differences between all and each productive strains are indicated by superscripts.

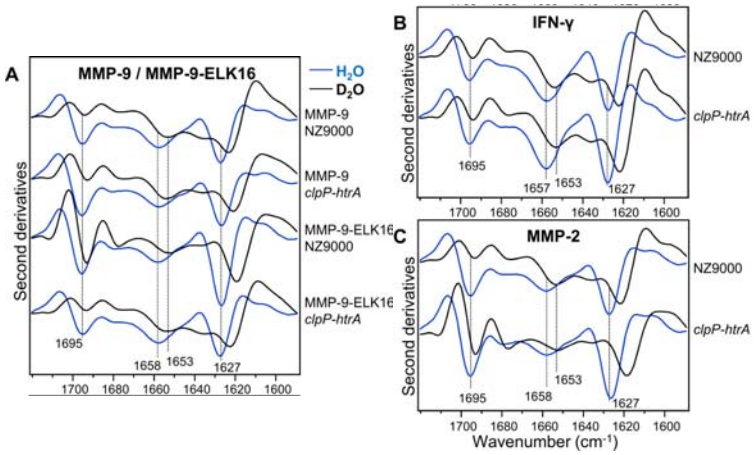
	NZ9000				*clpP-htrA*				SEM	P-VALUE		
	MMP-9	IFN-γ	MMP-2	MMP-9-ELK16	MMP-9	IFN-γ	MMP-2	MMP-9-ELK16		protein	strain	Protein x strain
Diameter	353.21^{c,d}	422.35^{a,b,c}	387.54^{b,c}	410.58^{a,b}	431.58^{a}	317.04^{d}	391.84^{a,b,c}	402.84^{a,b,c}	13.91	0.2965	0.4895	<0.0001
Z-potential	-29.6^{b,c}	-28.43^{b,c,d}	-33.83^{a}	-26.23^{c,d}	-30.77^{a,b}	-26.77^{c,d}	-27.5^{b,c,d}	-25.27^{d}	0.70	<0.0001	0.012	0.0006
Aggregation (%)	99.24^{a,b}	14.76^{a}	92.13^{a,b}	97.33^{c}	98.27^{b,c}	3.07^{b,c}	100^{a}	100^{a}	0.18	0.0121	0.9947	<0.0001

Table 2: Percentage of nanoparticle coverage on SAM functionalized gold substrates. Dependence of the nanoparticle coverage on the functionalized gold substrate wettability.

Substrate	MMP-9		IFN-γ		MMP-2		MMP9-ELK16	
(X_{OH})	NZ9000	*clpP-htrA*	NZ9000	*clpP-htrA*	NZ9000	*clpP-htrA*	NZ9000	*clpP-htrA*
0	0.14	1.54	0.41	0.47	6.31	2.74	11.44	5.32
0.2	0.22	0.89	0.40	1.31	5.61	1.74	19.36	3.46
0.4	0.23	1.66	0.68	0.4	5.26	2.75	14.73	5.70
0.6	0.58	0.61	1.91	0.23	10.38	1.03	15.02	11.24
0.8	0.20	1.56	2.17	0.29	12.05	0.81	4.26	4.27
1	0.39	0.37	1.39	0.43	15.14	3.30	13.70	1.14

Supplementary data

Supplementary Figure 1: Hydrogen/deuterium exchange of purified nanoparticles. Spectra of A) MMP-9 and MMP-9-ELK16, B) IFN-γ and C) MMP-2 are shown. Second derivatives of the absorption spectra of nanoparticles produced in NZ9000 and *clpP-htrA* strains before (blue lines) and after 3 h of H/D exchange (black lines). The downshift of the ~1657-1658 cm-1 component to ~1653 cm-1 after D2O incubation strongly indicates the presence of α-helical structures in the investigated nanoparticles. Indeed, a much higher downshift (to around 1645 cm-1 in D2O) is expected for random coil structures.



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Annex II

Manuscript 2

In vivo study to determine the potential of soluble and insoluble recombinant metalloproteinases for the optimization of the cow dry period.

Parés S*, Cano-Garrido O*, Fàbregas F, Bach A, Ferrer-Miralles N, Terré M, Villaverde A, Garcia-Fruitós E and Arís A

*Equal contribution

Experiments carried out in collaboration with the Department of Ruminant Production at the "Institut de Recerca i Tecnologia Agroalimentàries" (IRTA, Caldes de Montbui).

The objective of this study was to explore the role of the infusion of soluble MMP-9 and recombinant aggregated MMP-9 nanoparticles at dry-off.

At this point, it is important to stress that dairy cow mammary gland has 4 quarters: two rear (the biggest) and two front (the smallest) **(Figure 1)**.

In this *in vivo* study we used 12 animals (n= 48 quarters) and treatments were randomly assigned to the front or rear quarters. Thus, 24 quarters were divided in 2 treatments: soluble and protein-based nanoparticles of recombinant MMP-9, providing both the equivalent metalloproteinase activity measured by zymography. The other 24 quarters (the contralateral quarters) were infused with saline solution (negative control).

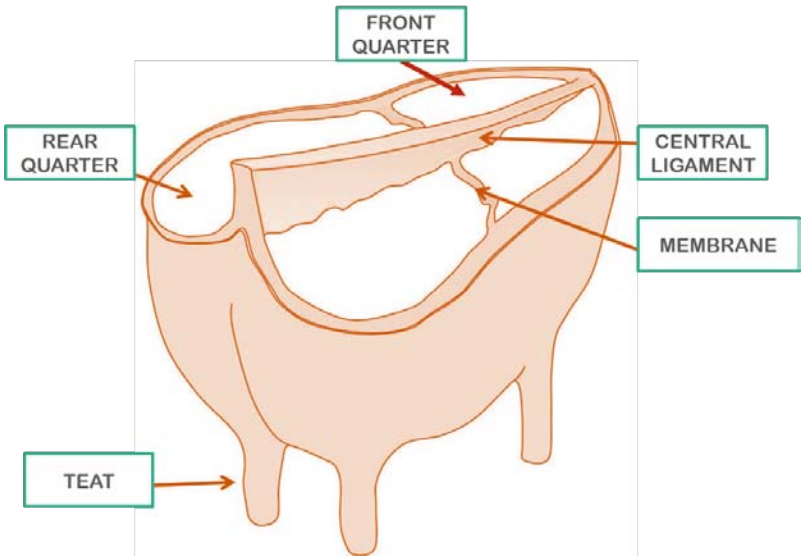

Figure 1: Dairy cow mammary gland anatomy.

Samples of mammary secretion were collected at 0, 1, 2, 3, and 7 days relative to dry-off. The mammary secretion markers analysed were:

- Somatic cells (SCC) and lactoferrin as indicators of immune system stimulation.
- MMP-9 activity as an indicator of the extracellular matrix regeneration and mammary gland involution.
- Bovine serum albumin (BSA) and Na^+/K^+ ratio infiltration from blood to milk as makers of *tight junction* disruption and tissue involution.

Both treatments (soluble MMP-9 and MMP-9 nanoparticles) increased ($P < 0.0001$) the metalloproteinase activity in mammary gland compared with controls (data not shown). Nevertheless, the nanoparticle form was exclusively able to dramatically strengthen the dry-off

stimulation by increasing immunity markers. For example, the lactoferrin concentration increases 1.8 folds ($P < 0.05$) at days 1 and 3 after dry-off and the SCC increased up to 400 fold ($P < 0.0001$) at days 1, 2, 3, 6 and 7 (Figure 2A and B). SCC are the principal effector of the immune system, not only to protect the gland from intrammamary infections, but also to phagocyte the rest of cells and milk allowing the epithelial tissue involution. With the soluble protein treatment, the SCC increase in milk secretion starts at day 4, but accordingly to the negative control, it seems that this is the normal behaviour of mammary gland during cow dry period (Figure 2B).

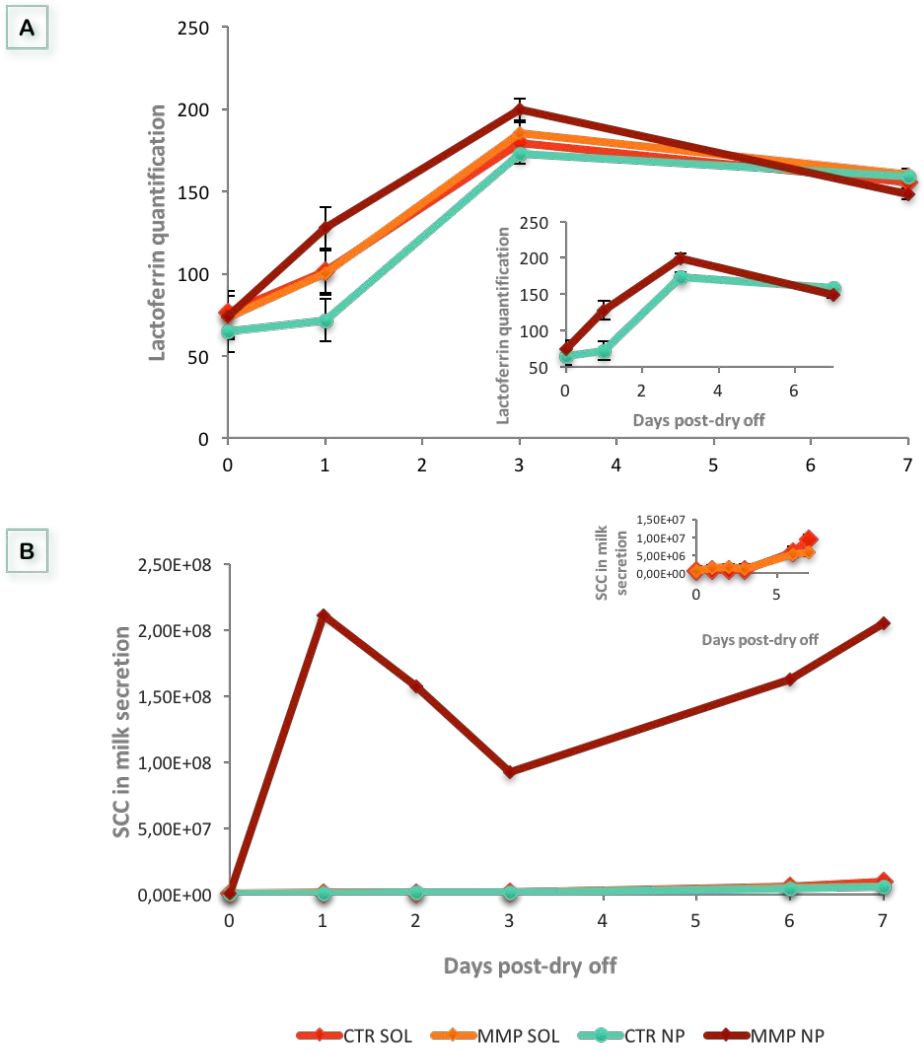

Figure 2: A. Lactoferrin quantification in milk secretion post dry-off. In the inset, there are only the recombinant MMP-9 nanoparticles treatment and its negative control **B.** Somatic cells (SCC) quantification in milk secretion post-dry off In the inset, there are only the recombinant soluble MMP-9 treatment and its negative control **CTR SOL:** Saline control soluble protein. **MMP SOL:** Recombinant soluble MMP-9 protein. **CTR NP:** Saline control insoluble protein. **MMP NP:** Recombinant MMP-9 nanoparticle.

Concerning involution markers, there is also an increase of them. When treating with MMP-9 nanoparticle, BSA concentration in mammary secretion raised up to 8 fold ($P < 0.001$) at 1, 2 and 3 d and the sodium/potassium ratio ($P < 0.001$) by 4.5 fold at day 1, 3 and 7 after dry-off, compared with controls (Figure 3A and B). However, in the case of soluble MMP-9, there was only a BSA increase from the third day and sodium/potassium ratio increase only at day 7 (Figure 3B).

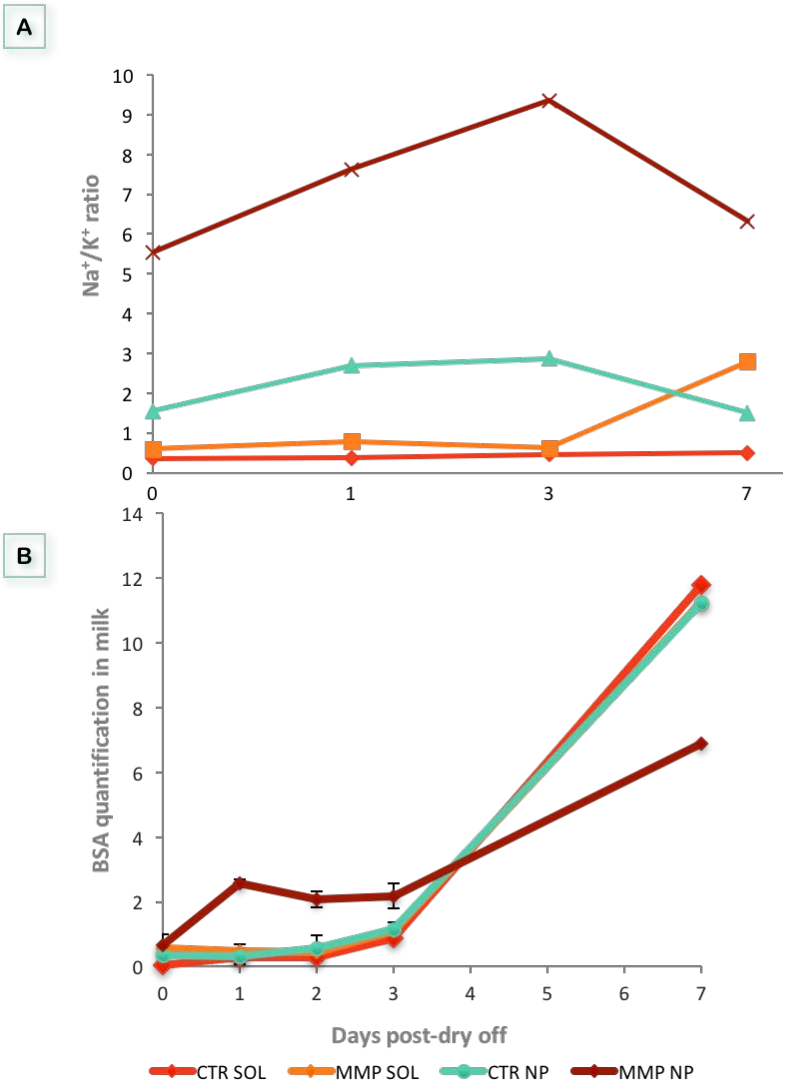

Figure 3: **A.** Sodium/potassium ratio **B.** BSA quantification in milk secretion post dry-off. **CTR SOL:** Saline control soluble protein. **MMP SOL:** Recombinant soluble MMP-9 protein.**CTR NP:** Saline control insoluble protein. **MMP NP:** Recombinant insoluble MMP-9 protein.

In a current cow dry period, neutrophils do not reach the mammary gland, in other words, the immune system do not act until seven days post dry-off [278, 282]. Nevertheless, in this work, we demonstrate that the infusion of MMP-9 nanoparticles has a significant impact on enhancing

the involution process and immune system before this seventh day, showing that MMP-9 nanoparticles administration has a clear effect shortening the cow dry period.

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Annex III

Manuscript 3

Systems-trimmed properties of CXCR4+-targeted protein nanoparticles produced in the food-grade bacterium *Lactococcus lactis*

Cano-Garrido O*, Cespedes MV, Unzueta U, Saccardo P, Roldan M, Sanchez-Chardi, Curbasi R, Vasquez E, Mangues R, García-Fruitós E, Villaverde A.

Submitted to Journal of Nanotechnology

Abstract

Background: Nanostructured protein materials produced in bacteria are gaining interest in biomedicine because of their unique combination of regulatable structure and functionalities, and the versatility of genetic design combined with easy biological fabrication. However, their applicability is compromised by the potential contamination with endotoxins from producer cells, often the Gram-negative bacterium *Escherichia coli*. *Lactococcus lactis* is an emerging bacterial factory that benefits from its Gram-positive nature (endotoxin-free) and from its food-grade denotation. By promoting efficient protein secretion and elevated solubility, *L. lactis* has been exploited as an alternative to *E. coli* for recombinant protein production and as a living vehicle for protein and DNA delivery *in vivo*. Despite these unusual values, the production of complex, smart protein materials have never been explored in this system.

Results: In this context, we have investigated the production in *L. lactis* of paradigmatic, tumor-targeted protein-only nanoparticles aimed to $CXCR4^{+}$ cancer stem cells. Productivity, material's properties, cell penetrability and *in vivo* biodistribution upon systemic administration in colorectal cancer mice models have been compared with the performance of the equivalent material produced in *E. coli*.

Conclusions: The obtained data confirms the robustness of self-assembling proteins as materials but also supports the occurrence of functional and structural variability linked to the particular cell factory used for biofabrication.

Keywords: Protein nanoparticles; tumor homing peptides; self-assembling proteins; biodistribution; colorectal cancer; recombinant proteins; protein folding; biofabrication; *Lactococcus lactis*

Background

Proteins as materials benefit from full biocompatibility, functional and structural convergence and genetic manipulability, apart from the potential to be produced by biofabrication in cell factories by fully scalable, tuneable and cost-effective processes [346]. The optimization of methodologies for protein engineering and production has allowed the approval of hundreds of protein drugs for human use by the major medicament agencies, FDA and EMA, with a tendency to engineered proteins versus plain, natural protein versions [347]. In this direction, and as a side aspect of protein drug development, many principles of protein engineering allow the production of self-assembling polypeptides as building blocks of complex oligomeric structures [348], of defined morphometries and biological and physical properties. These protein materials that include fibers, layers, ribbons, cages, particles and hydrogels, being biocompatible and highly tuneable, have vast applications in biomedicine (targeted drug delivery, local drug release, protein replacement therapies and tissue engineering, among others) [349], what demands standardized and safe production methods.

Besides the *ex vivo* chemical synthesis of short peptides, recombinant protein production comprises a rich set of procedures deeply explored for biotechnological and biopharma products. Many types of cell factories are under development to expand the versatility of biological production and to adjust the final quality of products to the increasing regulatory constraints [350]. Among them, the required absence of endotoxins for *in vivo* uses makes advisable the use of endotoxin-free cell factories [351-355]. In this context, the Gram-positive (endotoxin-free) lactic acid bacteria (LAB) represent a promising platform for protein production as they combine fast bacterial growth (versus eukaryotic platforms), easy culture with adjustable conditions and the food-grade denomination [356-358]. Genetic tools developed for *L. lactis* and related species have been designed to favour recombinant protein secretion, what in general results in high solubility and consequently addresses another of the major bottlenecks in bacterial protein production [359]. Despite its potential, the performance of this still emerging production system has not been explored yet in the production of building blocks of complex protein materials. As further protein assembling in functional supramolecular structures depends on protein conformation, and protein conformation depends on the performance of the cell's quality control system, different bacterial species might behave differently as cell factories regarding the final structure and functional quality of protein materials. To address this issue, we have produced in *L. lactis* and characterized CXCR4-targeted smart protein nanoparticles previously designed for production in *E. coli*, for a

comparison of properties at molecular, supramolecular and systemic level upon administration to model animals. These materials, formed by the self-assembling of the modular protein T22-GFP-H6 as building block, accumulate in primary tumor and metastasis in $CXCR4^+$ colorectal cancer mice models upon systemic administration. While the data obtained here indicates robust targeting and biological behaviour of the protein nanoparticles irrespective of the production system, it also reveals detectable variability in relevant structural and functional features, when comparing materials handled by alternative quality control networks from different bacterial species.

Materials and Methods

Strains, plasmids and culture conditions.

Strains and plasmids used in this study and their most relevant features are listed in Table 1. *L. lactis* (NZ9000 strain) was transformed by electroporation with pNZ8150, while *E. coli* Origami B strain was transformed by heat-shock with pET22b. Both plasmids encode, with optimized codon usage for every host (synthetized by GeneArt), the modular protein T22-GFP-H6, which when fabricated in *E. coli* (T22-GFP-$H6^{coli}$) self-assembles in regular toroid particles of about 14 nm. T22 is a cationic peptide, that apart from promoting protein-protein contacts [360], it binds specifically to the cell surface cytokine CXCR4, overexpressed in colorectal cancer and correlating with aggressiveness [361]. T22 promotes internalization of the whole nanoparticle in $CXCR4^+$ cells [362] and its intracellular accumulation in tumor and secondary metastatic foci *in vivo* [265]. *L. lactis* was cultured in M17 media (Sigma) enriched with 0.5 % glucose and *E. coli* in Lysogenic Broth (LB) media. Overnight cultures were prepared from a glycerinate to inoculate shake flasks containing 500 ml of the appropriate media and antibiotics. *L. lactis* was cultured at 30ºC without shaking and *E. coli* at 37ºC and 250 rpm, growing up to 0.5 OD_{550} units. *T22-GFP-H6* gene expression in *L. lactis* was induced by 12.5 ng/ml nisin and cultures were incubated overnight at 30ºC without shaking. In *E. coli*, gene expression was induced by IPTG at 1 mM and cultures were incubated overnight at 20ºC and 250 rpm.

Table 1: Strains and plasmids used in this study

Bacterial strains and plasmids	Relevant genotype or phenotype	Source
Lactococcus lactis subsp. *cremoris* NZ9000	*pepN::nisRnisK*	[19]
Escherichia coli Origami B	OmpT$^-$, Lon$^-$, TrxB$^-$, Gor$^-$, Strep^R, Tet^R	[20]
pNZ8150-T22-GFP-H6	Cm^R(5 µg/ml), Nisin-inducible	This work
pET22b-T22-GFP-H6	Ap^R(100 µg/ml) , IPTG-inducible	[21]

Protein purification

Both species of bacterial cells were harvested by centrifugation (5,000 g at 4 ºC, 15 min) and resuspended in wash buffer (20 mM Tris-HCl, 500 mM NaCl and 10 mM imidazole), containing an EDTA-free protease inhibitor cocktail (Roche). Afterwards, *E. coli* cells were disrupted by pressuring 2 rounds at 1,200 psi (machine pressure) and *L. lactis* cells with 3 rounds at 1,500 psi in a French press (Thermo FA-078A). Both T22-GFP-H6^{coli} and T22-GFP-H6^{lactis} proteins were purified by His-tag affinity chromatography using 1 ml HiTrap Chelating HP column (GE Healthcare) through an ÄKTA pure FPLC (GE Healthcare). Separations were made by linear gradient of elution buffer (20 mM Tris, 500 mM NaCl, and 500 mM imidazole). Purified protein fractions were dialyzed against carbonate buffer (166 mM $NaCO_3H$, pH 7.4). Protein amounts were determined by Bradford's assay [363] and analysed by denaturing SDS-PAGE (15 % acrylamide) gel electrophoresis, using a commercial polyclonal antibody against GFP (Santa Cruz Biotechnology). Protein purification was assisted by the ICTS "NANBIOSIS", more specifically by the CIBER-BBN's Protein Production Platform (http://www.nanbiosis.es/portfolio/u1-protein-production-platform-ppp/).

Electron microscopy

T22-GFP-H6 nanoparticles were examined by transmission electron microscopy (TEM) and field emission scanning electron microscopy (FESEM). For TEM, protein samples were negatively stained with uranyl acetate by conventional methods [265] and observed in a Jeol 1400 microscope operating at 80 kV and equipped with a CCD Gatan Erlangshen ES1000W camera. For FESEM, protein samples were directly deposited over silicon wafers, air dried and observed with an in-lens secondary electron detector through a Zeiss Merlin microscope operating at 2 kV.

Determination of particle size and fluorescence

Particle size was measured by two different techniques. By dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern), volume size distribution was determined at 633 nm. Using FESEM micrographs, diameters of nanoparticles were measured by ImageJ software (National Institute of Health, Bethesda, USA). GFP fluorescence emission (510 nm) was determined by fluorimetry with a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies) using an excitation wavelength of 450 nm.

Cell culture, flow cytometry and confocal microscopy

Protein internalization was analysed in $CXCR4^+$ HeLa cell cultures in 24-well plates. Briefly, the Minimum Essential Medium (MEM-α) medium supplemented with 10 % FBS and 2 mM Glutamax (Gibco). was removed and cells were washed in PBS. Then 250 µl of 500 nM T22-GFP-H6 in OptiPro medium, supplemented with L-Glutamine, were added and incubated for 2 h, at 37 ºC at 5 % CO_2 to allow cell binding and internalization. Then, harsh trypsin digestion (1 mg/ml for 15 min) was carried out to remove protein particles bound to the outer size of the cell membranes [364]. Intracellular green fluorescence was analysed by flow cytometry on a FACS-Canto system (Becton Dickinson) using a 15 mW air-cooled argon ion laser at 488 nm excitation. Fluorescence emission was measured with a D detector (530/30 nm band pass filter). For competition assays, an specific CXCR4 inhibitor AMD3100 (octahydrochloride hydrate, Sigma Aldrich) was added 1 h before T22-GFP-H6 nanoparticles addition in a 1:10 (protein : AMD3100) molar ratio. For confocal analysis, cells were grown to 100,000 cells/ml on MatTek culture dishes (MatTek Corporation) for 24 h at 37 ºC at 5 % CO_2. Then, 500 nM T22-GFP-H6 in 1 ml OptiPro medium supplemented with L-Glutamine was added, and incubated for 24 h at 37 ºC and 5 % CO_2. Before confocal observation, nuclei were labelled with 5 µg/ml Hoechst 33342 (Life Tecnologies) and plasma membranes with 2.5 µg/mL CellMask™ Deep Red (Life Tecnologies) for 10 min in the dark. Micrographs were then taken by TCS-SP5 confocal laser scanning microscopy (Leica Microsystems) using a Plan Apo 63 ×/1.4 (oil HC × PL APO lambda blue) objective. In order to localize T22-GFP-H6 nanoparticles inside cells, stacks of 40–60 sections for every 0.25 µm of cell thickness were collected and three-dimensional models were generated using Imaris software (Bitplane).

Biodistribution of T22-GFP-H6 variants in CXCR4+ colorectal cancer mice models

Five-week-old female swiss nude mice weighing 18-20 g (Charles River), maintained in specific pathogen-free conditions were used for the *in vivo* experiments. All *in vivo* experiments were performed by the ICTS "NANBIOSIS", more specifically by the CIBER-BBN's Nanotoxicology platform of IIB Sant Pau (http://www.nanbiosis.es/portfolio/u18-nanotoxicology-unit/). All procedures were approved by the Hospital de Sant Pau ethical committee in accordance with Institutional guidelines.

To generate the subcutaneous (sc) $CXCR4^+$ CRC model, aliquots of 10 mg of SP5 CXCR4+ tumor tissue from donor animals were obtained and implanted sc in the subcutis of mice by trocher system. When tumors reached 500 mm^3 approximately, mice were randomly allocated and biodistribution was performed. T22-GFP-H6 nanoparticles in carbonate buffer (166 mM $NaCO_3H$, pH 7.4) were administered intravenously at 500 µg/mouse dose. The control mice received empty buffer. Five hours post-administration, mice were euthanized and tumors and organs were extracted for *ex vivo* recording and quantifying the fluorescence emitted by each organ. GFP fluorescence signals were detected using the IVIS Spectrum equipment (Perkin Elmer). The fluorescence signal was first digitalized, displayed as a pseudocolor overlay, and expressed as radiant efficiency ([p/s/cm^2/sr]µW/cm^2). Finally, tumors and all organs were collected and fixed with 4 % formaldehyde in phosphate-buffered solution for 24 h, and then embedded in paraffin for immunohistochemical evaluation.

Histology and immunohistochemistry

Four-micrometer-thick sections were stained with hematoxylin and eosin (H&E), and a complete histopathological analysis was performed by two independent observers. IHC stains were performed on a DAKO Autostainer automated Link48 (DAKO) using standard procedures. The anti-GFP antibody (1:100, Santa Cruz Biotechnology) was used to detect nanoparticle accumulation and localization in tumors and normal tissue as described previously [265]. Representative images were taken using Cell^B software (Olympus Soft Imaging) at 200x and 400x magnifications.

Results

The modular protein T22-GFP-H6 (Figure 1 A), displaying the peptidic CXCR4 ligand T22, was produced in *L. lactis* and purified in a single peak from bacterial cell extracts. T22-GFP-$H6^{lactis}$

resulted in a polypeptide of the expected molecular mass (30.6 KDa), suffering only from mild proteolytic degradation (Figure 1 B and C). The protein material occurred in form of nanoparticles that in DLS peaked at 20.9 nm, showing also a polydisperse secondary population of around 100 nm that might correspond to soluble aggregates. Under microscopic examination, regular toroid nanoparticles were observed, similar in morphology to those formed by the same protein when produced in *E. coli* Origami B but of larger size (20 nm versus 14 nm).

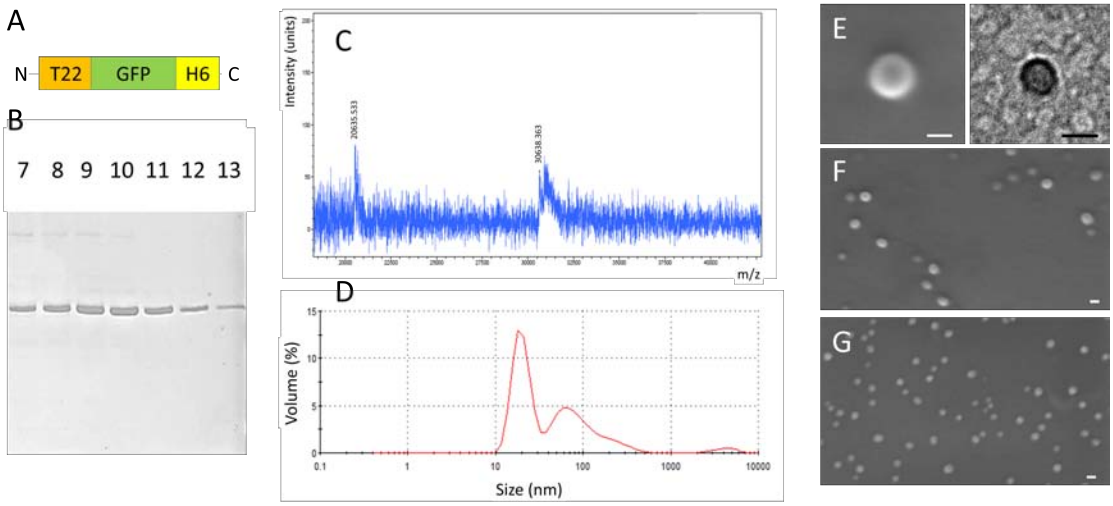

Figure 1: Nanoscale characterization of T22-GFP-H6^{lactis} nanoparticles. A) Modular scheme of T22-GFP-H6. Relative lengths of the modules are only approximate. **B)** Fractions from 7 to 13 resulting from one-step protein purification of T22-GFP-H6^{lactis} in His-tag affinity chromatography, that were pooled for further studies. **C)** Mass spectrometry analysis of T22-GFP-H6^{lactis}. **D)** Volume size distribution of nanoparticles determined by DLS. **E)** Representative micrographs of T22-GFP- H6^{lactis} nanoparticles obtained by FESEM (left) and by TEM (right). Both bars indicate 20 nm. **F)** A FESEM micrograph showing a general field is also included. **G)** A FESEM micrograph showing a general field of T22-GFP- H6coli nanoparticles. All bars size indicates 20 nm.

Regarding functionality, those particles emitted fluorescent light (as expected, because of the presence of GFP), and their specific fluorescence was determined to be 3.12 fold lower than the value obtained for T22-GFP-H6^{coli} nanoparticles produced in Origami B under comparable production conditions. Their intrinsic fluorescence was used as reporter to check the ability of the material to penetrate CXCR4^{+} cells in a receptor-dependent way, what was comparatively done regarding the protein source. While the uptake of both nanoparticle versions was efficiently inhibited by AMD3100 (octahydrochloride hydrate, Sigma) (86.7 % in the case of *E. coli* materials and 96. 6 % in the case of the food-grade nanoparticles), proving the specificity in cell entry in both cases, penetration of the material produced in *L. lactis* was surprisingly higher than that produced in *E. coli* (Figure 2 A). In previous uptake analyses of GFP-based

nanoparticles produced in diverse *E. coli* strains we observed a negative correlation between the capacity to internalize cells and the specific fluorescence of the material, indicative of alternative arrangements of the oligomers, affecting both the performance of the fluorophore and that of the cell active peptides (namely T22 and H6, as ligand and endosomal escape agents respectively).

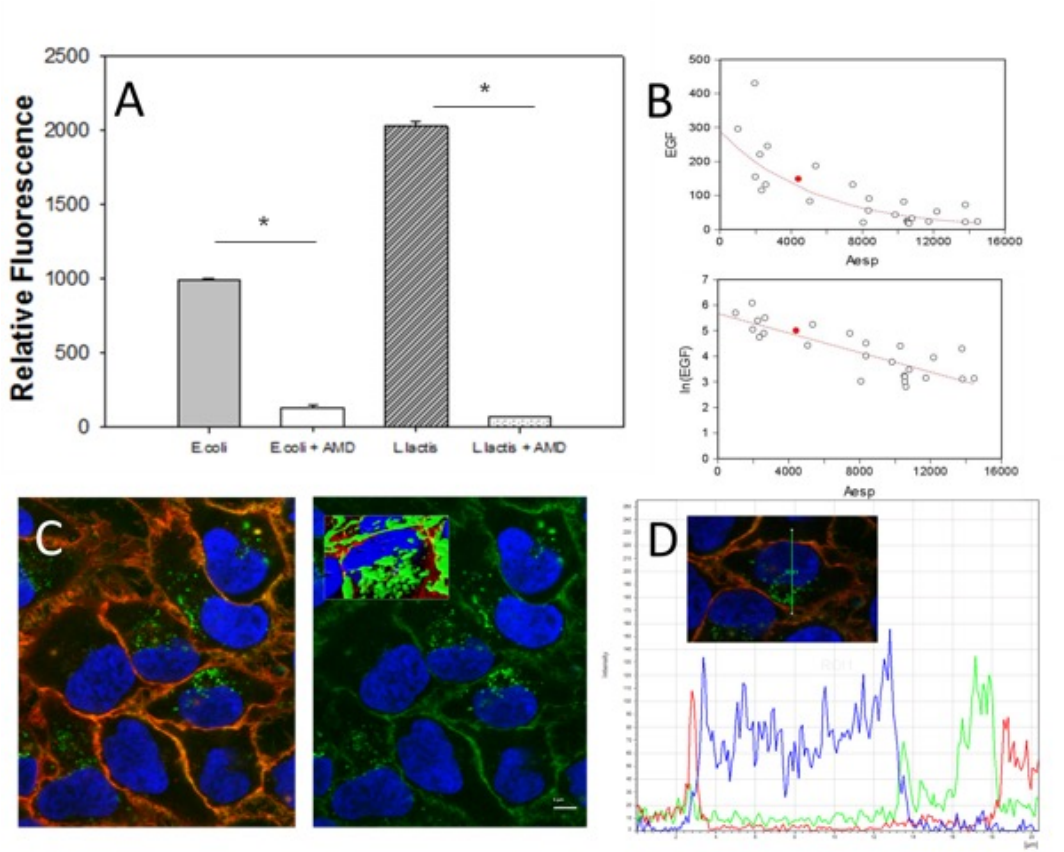

Figure 2: Cell penetrability of T22-GFP-H6^{lactis} nanoparticles into CXCR4+ HeLa cells. A) Protein amounts internalized into cells depending on the cell factory used for production, and uptake inhibition promoted by the natural CXCR4 ligand AMD3100 (AMD). Intracellular fluorescence was corrected by specific fluorescence to render values representative of protein amounts. Asterisk indicates significant differences ($p \leqslant 0.05$). **B)** Internalization efficiently represented versus specific fluorescence (as raw data, top and as logarithm, bottom) of T22-GFP-H6^{lactis} nanoparticles produced in *L. lactis* (red dots) compared with the same materials produced in several E. coli strains, as previously reported [365]. **C)** Confocal images of HeLa cells exposed to 500 nM of *L. lactis* T22-GFP-H6^{lactis} nanoparticles for 24 h. Cell membranes are labelled in red and nuclei in blue. Green signals correspond to fluorescent nanoparticles. In the inset, a 3D Imaris reconstruction shows the accumulation of the material in the cell cytoplasm. This precise intracellular localization is confirmed by a fluorescence intensity profile of a representative cell exposed to nanoparticles **D)** The high penetrability of the material from *L. lactis* combined with its low fluorescence emission fitted very precisely in the model we had formerly generated to explain such inverse relationship (Figure 2 B), supporting the concept that penetrability is linked to the precise oligomeric architecture of

the materials that can impact, in its own, via conformation, on the fluorophore performance. T22-GFP-H6^{lactis} nanoparticles accumulated in the cytoplasm of target cells in absence of any detectable toxicity (Figure 2, C,D).

The efficient and highly specific CXCR4 targeting, good penetration (Figure 2 A) and the intracellular accumulation of the nanoparticles, that remained fully fluorescent (Figure 2 C and D), prompted us to evaluate the performance of the material *in vivo*, regarding biodistribution in CXCR4^{+} colorectal cancer mice models upon systemic administration.

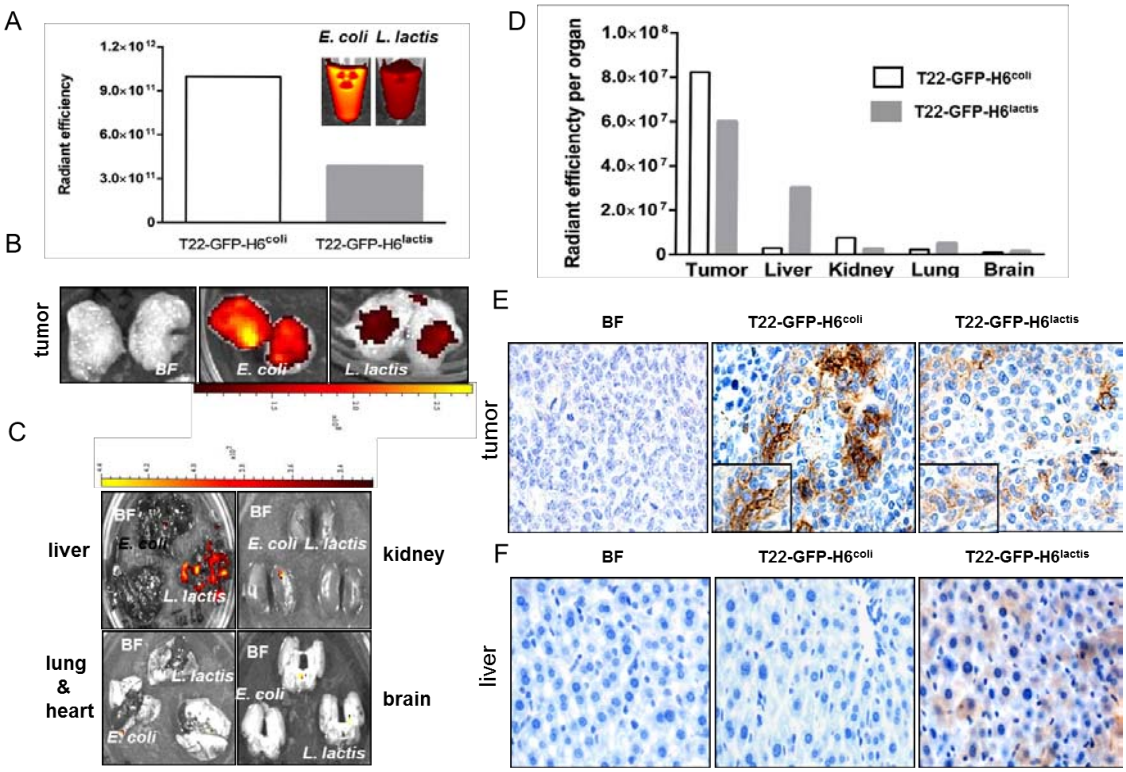

Figure 3: **Comparative biodistribution of T22-GFP-H6 nanoparticles in a CXCR4+colorectal cancer mouse model. A)** Representative fluorescence (arbitrary units) recording images and quantitation of specific GFP emission signal of each nanoparticle at the same concentration by the IVIS spectrum system. **B)** *Ex vivo* tumor fluorescence imaging (FLI) at 5 h post administration of 500 µg/mouse dose of each T22-GFP-H6 variants (nanoparticles produced in either *E. coli* or *L. lactis*). Note the enhanced green fluorescence associated with nanoparticle accumulation in tumors produced in *E. coli* as compared with *L. lactis* nanoparticles. BF is empty buffer. **C)** *Ex vivo* determination of GFP fluorescence signal in relevant organs such lung, brain, kidney and liver. No fluorescence was observed in any organ except for the liver of mice administered with T22-GFP-H6^{lactis}. **D)** Quantitation of *ex vivo* fluorescence emission (arbitrary units) in sectioned tumors and organs and expressed in Radiant efficiency. Crude fluorescence values were corrected by the specific emission of each protein for comparative purposes. **E)** Anti-GFP immunostaining showing membrane and cytosolic localization of T22-GFP-H6 variants in tumors which was absent in control animals injected with buffer (400x magnifications). **F)** The presence and location of the T22-GFP-H6 lactic variant in liver tissue sections were demonstrated by anti-GFP immunohistochemistry (400 x magnification).

In animals treated with both materials (Figure 3 A), fluorescence largely accumulated in primary tumor as expected (Figure 3 B), with background signal in non-target organs (Figure 3 C). While

this was clear in the case of T22-GFP-H6^{coli} nanoparticles those produced in *L. lactis* showed moderate but detectable accumulation in liver, that was presumed when analysing the whole organ but that was confirmed in liver sections **(Figure 3 C and F)**. Quantitative analysis of the signal, upon correction by the specific fluorescence of the variant nanoparticles confirmed a similar biodistribution pattern of both materials as well as the enhanced deposition in liver of T22-GFP-H6^{lactis} **(Figure 3 D)**, that might be due to the slightly enhanced particle size comparing with the material produced in *E. coli*.

Discussion

Regarding the biological fabrication of drugs and nanoscale materials of biomedical interest [366], endotoxin contamination is a major concern when those products are intended for human use [367], and especially in the toxicological evaluation of materials within the nanoscale that might already exhibit inherent toxicity [368]. Given the increasing trend in the approval of protein-based drugs [347] and in the line of the convenient biological fabrication of nanostructured protein materials [346], the production of endotoxin-free protein for biomedical uses is gaining relevance as an unavoidable target. Among the diversity of cell factories for protein drug production [350], bacteria offer greater opportunities for cost-effective production at industrial scale. Again, being *E. coli* the choice system for protein drugs [357, 369], endotoxin contamination is a major issue of concern. While industrial-scale protocols have been implemented and largely tested for conventional soluble recombinant proteins [351, 354, 355, 367], attention has not been paid yet to self-assembling protein materials exhibiting a higher structural complexity. In this context, endotoxin-free *E. coli* strains have been recently developed [353], and have been proved suited for the successful production of soluble protein drugs [353], but also of more complex protein materials such as inclusion bodies [370] and tumor-targeted protein nanoparticles [371]. Interestingly, both the architecture and functionalities of smart protein materials intended for *in vivo* administration, such as LDLR-, CXCR4- or CD44-targeted nanoparticles [265, 372, 373], are significantly influenced by the performance of the quality control of the producing *E. coli* cells [365, 375], which might be, itself, modulated by the particular genetics and chemical composition of the cell wall that are necessary to reach the endotoxin-free status [365].

The food-grade lactic acid bacterium *L. lactis* has emerged as a promising protein production platform [359, 376, 377], naturally devoid of endotoxins and suitable for oral and intranasal administration as a living drug (DNA and protein) delivery system [378-383]. This microorganism

has been mainly employed for the secretion, as plain soluble forms, of difficult-to-express proteins [359, 384] and for the preparation of S-layers for ordered protein display purposes [385]. Although it has been also used for the controlled preparation of nanostructured biopolyester beads [386] and hybrid polyhydroxybutyrate-protein granules [387] among a few other materials, *L. lactis* has been never explored regarding the quality and activities of complex self-assembling protein materials. That the *L. lactis* quality control might be differently acting than that of *E. coli* might be anticipated by the occurrence of different regulators of stress responses [388], potentially divergent activities of the main cytosolic chaperone (and regulator of several stress responses) DnaK [388], and alternative ways to manage protein aggregation [344,376]. Therefore, we were interested in knowing if such particular cell factory might be supportive of proper production of cell-targeted protein nanoparticles usable as *in vivo* drug vehicles for intracellular drug delivery [389]. Obtained data fully confirm the robustness of the self-assembling protein platform, as toroid T22-GFP-H6 nanoparticles are produced in *L. lactis* **(Figure 1 E and F)** that specifically bind the CXCR4 cytokine receptor **(Figure 2 A)** and efficiently internalize $CXCR4^+$ cells **(Figure 2 C and D)**, in a pathway compatible with endosome-mediated uptake. However, the slight but consistent size difference observed between T22-GFP-$H6^{lactis}$ and T22-GFP-$H6^{coli}$ **(Figure 1)**, and the lowest specific fluorescence but higher penetrability of T22-GFP-$H6^{lactis}$ **(Figure 2)** confirms that T22-GFP-H6 building blocks are distinctively organized in these bacterial cell factories. The fact that fluorescence emission and cell penetrability of T22-GFP-$H6^{lactis}$ fits in an inverse dependence model exclusively generated with T22-GFP-$H6^{coli}$ variants **(Figure 2 B)** indicates that the impact of protein conformation (or the oligomeric organization) on the material performance is irrespective of the given protein production platform used for biofabrication.

On the other hand, T22-GFP-$H6^{lactis}$ efficiently accumulates in tumor upon systemic administration **(Figure 3)**, escaping from renal clearance and proving the stability of the material and the good performance *in vivo* of the tumor homing peptide T22, as folded in *L. lactis*. However, at difference from T22-GFP-$H6^{coli}$, T22-GFP-$H6^{lactis}$ is also found; at minor extend, in liver. The variant biodistribution map, that does not preclude the potential use of T22-GFP-$H6^{lactis}$ as vehicle for drug delivery in colorectal cancer, indicates a peculiar oligomerization of the building blocks that influence targeting or promote passive accumulation, though conferring particular biomechanical properties. Interestingly, T22-GFP-$H6^{coli}$ produced in $DnaK^-$ *E. coli* mutants also show an aberrant distribution pattern, with a slight and unexpected accumulation in brain. Given the differential roles of DnaK presumed

when comparing the stress responses of *L. lactis* and *E. coli* [388], this variant distribution again suggests a critical role of the chaperone DnaK in defining the final fate of T22-GFP-H6 nanoparticles upon systemic administration.

Conclusions

L. lactis, a food-grade lactic acid bacteria specifically exploited for the biofabrication of soluble protein species has been revealed here as a good producer of complex, self-assembling protein nanoparticles. Despite the robustness of the biological platform, the nanoscale architecture and biodistribution in colorectal cancer mice models reveals significant differences when compared with materials produced in *E. coli*. This fact supports a mechanistic impact of the protein quality control network on the systems level performance of the resulting product, which it is of critical relevance for the design of emerging, protein-only, nanostructured materials in biomedicine. Opening an exciting way for the production of high quality, biologically safe protein nanoparticles with therapeutic interest, the present findings emphasize the suitability of *L. lactis* as a key player among the demanded, value-added cell factories for smart biomaterials.

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Competing interests: MVC, UU, EV, RM and AV are co-authors of a patent (WO2012095527) covering the use of T22 as an intracellular targeting agent. No other potential conflicts of interest have been identified.

Authors' Contributions section: OC performed most of the experiments regarding protein production and *in vitro* characterization, MVC performed the *in vivo* experiments and linked analyses, ASC performed the electron transmission microscopy, MR the confocal microscopy, PS parts of material's characterization, RC the numerical analysis and UU protein purification.

EV designed part of the experimental set-up, RM coordinated the *in vivo* experimental while EGF and AV conceived the whole study. All authors have participated in the preparation of the manuscript (text and figures), that has been mostly written by AV.

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Annex IV

Manuscript 4

Genetic engineering of *Lactococcus lactis* towards a suitable protein production platform with biomedical interest.

Cano-Garrido O, Sánchez-García L, Villaverde A, Bermúdez-Humaran L, Garcia-Fruitós E.

Experiments carried out at the laboratory of “Interaction des bactéries commensales et probiotiques avec l’hôte” led by Prof. Bermúdez-Humarán in the 'Institut National de la Recherché Agronomique” (INRA, France)

Objective

The aim of this investigation stage was the construction of a catalogue of *L. lactis* mutants to improve and optimize the protein production of LPS-free recombinant proteins. Specifically, the main objective of this stay was focused on the development of two-type of knock-out strains.

On the one hand, we planned to knock-out four gens involved in the protein quality control (GroEL, ClpB, Trigger factor (TF), and DnaK). In this context, it is important to point out that *E. coli* mutant strains lacking the main components of the protein quality control have shown to have different backgrounds able to produce recombinant proteins with different conformational and solubility properties. Thus, in this work, we have planned to study if this is also possible using *L. lactis* as cell factory, being a Generally Recognized as Safe (GRAS) organism with promising applications in the biotechnological and biomedical fields.

On the other hand, we wanted to create a knock-out strain for the gene *pgdA (xynD)* which should provide a lysozyme-sensitive phenotype. Regarding this point, it should be mentioned that *L. lactis* has a thick peptidoglycan cell wall that confers to the microorganism a high resistance in front of any lysis protocol. Thus, the development of this lysozyme-sensitive strain will allow us to simplify the lysis method used to isolate the proteins of interest produced in this GRAS organism.

The isolation of all these mutant strains would allow us to study and compare in detail the conformational quality, as well as the solubility, of different proteins of interest.

Materials and Methods

Bacterial strains, plasmids and *knock-out* protocol

L. lactis subsp. *cremoris* NZ9000 strain, a MG1363 derivative with the *nisK* and *niR* genes integrated in its genome, was used for gene knock-out experiments. Two different protocols were performed with the aim to isolate different knock-out mutants. Both approaches were based on single crossover integration using two different plasmids. In each plasmid a fragment from the gens of interest (*groeL*, *clpB*, *trigger factor*, *dnaK* and *pdgA)* was cloned. Integration of a plasmid via single-crossover generates two copies of the targeted gene, however, both are inactive. One deleted at the 5' end and the other deleted at the 3' end of the gene.

- Strategy 1: pRV300 suicide plasmid using the protocol described by Gosalbes et al. (Journal of Bacteriology (1993)). Regarding this protocol, it is important to note that pRV300 is a *L. lactis* non-replicative plasmid encoding for the erythromycin (Em) and the ampicillin (Ap) resistance.
- Strategy 2: pGhost plasmid using the protocol described by Maguin et al. (Journal of bacteriology (1996)). Regarding this protocol, it is important to note that pGhost 8, which is tetracycline (Tetra) resistant, and pGhost 9, which is Em resistant, are non-replicative plasmids at 37ºC.

According to the protocols described by Gosalbes et al. and Maguin et al., all primers were designed adding a *HindIII* restriction site sequence at 5' of the forward primers and an *EcoRI* restriction site sequence at 5' of the reverse primers to facilitate the cloning **(Table 1, in red)**. Moreover, three extra nucleotides in the case of the forward primers and one nucleotide in the reverse primers were added to get an optimal enzymatic restriction of the PCR fragments **(Table 1, in red and green)**.

Table 1: Primer design and characteristics.

NAME	SEQUENCE	LENGTH	TM	%GC
pgdA 5F	GTCAAGCTTGAGTCAAAAAAGAAGTGCAA	29	57.3	38
pgdA 5R	GGAATTCGCAATTCGTTCAGCCTCTT	26	58	46
DnaK F	GTCAAGCTTCTCAGCAGTCGCAGTTCTTG	29	62.9	52
DnaK R	GGAATTCCGTCGAATGTCCCACCA	24	59.1	54
GroeL F	GTCAAGCTTTGCTAGAACAGCGATGATGC	29	61.5	48
GroeL R	GGAATTCTCAGTTTGCATCCCTTTTGA	27	56.7	41
TTF F	GTCAAGCTTAAGGGTACACTCTCATTTTC	29	58.7	38
TTF R	GGAATTCTCAGTTTGCATCCCTTTTGA	24	57.4	50
ClpB F	GTCAAGCTTCGATGCAAGAAGCACTTGG	28	61.4	50
ClpB R	GGAATTCCGTCCAATGACGGGATCT	25	59.3	52
pgdA 5F	GTCAAGCTTGAGTCAAAAAAGAAGTGCAA	29	57.3	38
pgdA 5R	GGAATTCGCAATTCGTTCAGCCTCTT	26	58	46

All PCR reactions were performed using the Phusion High-Fidelity polymerase (Fermentas) in 50 µl samples. Once PCR was performed, DNA samples were purified with the Wizard® SV Gel and PCR Clean- Up System kit (Promega). Plasmids and PCR fragments were double-digested with *HindIII* and *EcoRI* enzymes for 1 h at 37 ºC. Restriction enzymes were removed by DNA

purification with the Wizard® SV Gel and PCR Clean-Up System kit. Previously, gene fragments and restricted plasmids were ligated overnight (ON) at 4 ºC. From these point there were some different according to the plasmid used.

- Strategy 1: pRV300 ligations were transformed in *E. coli* DH5α competent cells by a standard heat shock treatment. Transformation samples were plated in LB agar plates with 100 µg/mL Em and 100 µg/mL Amp and incubated at 37ºC ON. Plasmids were isolated from the positives colonies and transformed in *L. lactis* NZ9000 competent cells by electroporation (2400 V, 25 µF and 200 Ω). After electroporation samples were plated in M17B medium agar plates supplemented with 0.5 % Glucose and 5 µg/mL Em and they were incubated at 30 ºC for 48-72 h.

- Strategy 2: pGhost ligations were dialyzed in H_2O MQ for 30 min at room temperature (RT). Then, ligations were transformed in *L. lactis* NZ9000 competent cells by electroporation (2400 V, 2.5 µF and 200 Ω). Transformation samples were plated either in M17B medium agar plates supplemented with 0.5 % Glucose and 5 µg/mL Em for pGhost 9 or in M17B medium agar plates supplemented with 0.5 % Glucose and 5 µg/mL Tetra plates for pGhost 8. Plates were incubated at 30 ºC for 48-72 h. In order to favour plasmid integration, positive colonies were grown ON in M17B medium agar plates with 0.5% Glucose and the appropriate antibiotic for plasmid selection. The saturated culture was diluted in M17B medium with 0.5% glucose without antibiotics and it was incubated 150 min at 28ºC to allow exponential growth. The culture was then shifted to 37.5 ºC for 150 min to decrease plasmid copy number. Samples were diluted and plated at 37 ºC on M17 medium agar plates containing the appropriate antibiotic (to detect the integration).

Knock-out colonies were verified by PCR. Genomic DNA was purified with Wizard® Genomic DNA Purification Kit and PCR was performed using a plasmid forward primer and the gene fragment reverse primer **(Table 2)**

Table 2: Primer design and characteristics.

NAME	SEQUENCE	LENGTH	TM	%GC
pGhost5F	AACGACGGCCAGTGAGC	17	51.9	65
pRV3005F	TTGAATAGATGACTGTCTAATTC	23	48.1	30

Culture conditions and protein production

Protein production analyses were performed by using a modified Green Fluorescent Protein (mGFP) as a model protein. This mGFP was cloned in pNZ8148, a cloramphenicol resistant plasmid under the control of the nisin promoter. Protein production was carried out in the *L. lactis* wild type and mutant strains using the optimal *L. lactis* growth conditions (30 ºC, without shaking and in M17 broth medium with 0.5% glucose). The expression of the target gene was induced by nisin. At 3 and 5h post-induction 5 mL samples were taken. Moreover, during the whole production process, OD_{550} measurements were realized to control bacterial cell growth. 5 mL samples were centrifuged by 15 min at 10,000g to separate bacterial cell pellet from medium. Bacterial cell pellet was suspended in PBS supplemented with protease inhibitor cocktail. Then, cell culture samples were disrupted by sonication with the aim to separate soluble and insoluble fractions. After disruption sample was centrifuged (15 min at 15,000 g). Right after, western blot analysis were carried out in order to analyse the quality and quantity of protein from both fractions. Besides, protein fluorescent activity was tested with Cary Eclipse fluorescence spectrophotometer (Varian Inc, Palo Alto, CA) at 510 nm using an excitation wavelength of 450 nm.

Finally, a sample of bacterial cell cultures pellet from 3h and 5h post induction was also taken for optical microscopy analysis. They were harvest by centrifugation and fixed with 0.1 % formaldehyde in PBS. Fixed cells were placed on a glass slide, fixed with a slide cover and observed with a Leica TCS SP2 AOBS confocal fluorescence microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany). Photomicrographs were obtained after excitation at 488 nm and at emission wavelengths between 500 and 600 nm.

Results

All the PCR gene fragments were isolated by PCR. Nevertheless, the only constructions that we could get were pRV300-DnaK, pRV300-GroEL and pRV300-TF**.** pRV300-ClpB and pRV300-*pgdA* were not obtained, probably because restrictions enzymes weren't able to cut the PCR fragment extremes. Regarding the lack of success using the pGhost strategy, we concluded that the weak point of this strategy is ligation and transformation in *L. lactis.*

The obtained plasmids were isolated from *E. coli* DH5α and different amounts (3, 2, 1 and 500 ng) of the plasmid were transformed in *L. lactis* NZ9000 competent cells by electroporation. Once verified, just the three DnaK colonies were positive mutants **Table 3**. False positive are

usual in mutagenesis due to plates' long incubations (from 48-72 h) cause antibiotics deterioration.

Table 3: *L. lactis* pRV300 transformation positive colonies and their verification.

PLASMIDS	POSITIVES COLONIES	PCR VERIFICATION
pRV300-GroEL	1	-
pRV300-TFF	4	- - - -
pRV300-DnaK	3	+ + +

Protein production experiments were performed in the NZ9000 (wild type)/pNZ8148-VP1GFP and in the NZ9000-DnaK$^-$/pNZ8148-VP1GFP strains. At OD_{550}=0.5 bacterial cell cultures were induced with nisin and 5 mL bacterial cell cultures were taken at 3 h and 5 h post-induction. In a cell growth kinetic **Table 4** measurements show that NZ9000-DnaK$^-$ grows significantly slower than the wild type strain (p= 0.0013). Moreover, we observe that NZ9000 when carrying the plasmid is not affected, however, in the case of DnaK-, in spite of not being a significantly difference a negative tendency is observed (p=0.0313)

Table 4: Bacterial cell growth measured at OD_{550nm}.

STRAIN	μ (H^{-1})	T_D (MIN)
NZ9000	1.602	37.5
DNAK$^-$	1.316	45.6
NZ9000 pNZ8148-VP1GFP	1.588	37.8
DNAK$^-$ pNZ8148-VP1GFP	1.198	50.1

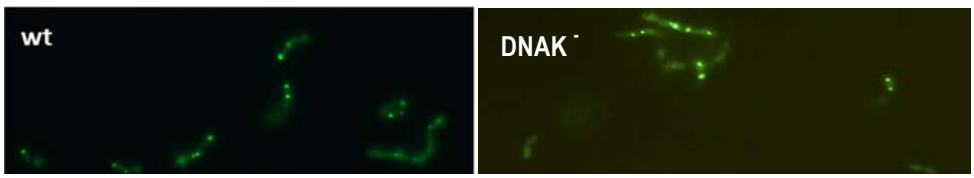

Figure 5: Fluorescence microscopy images of *L. lactis* NZ9000 (left) and *L. lactis* DnaK- (right) strains

Besides, micrographs were taken by fluorescent microscopy to verify, before continuing with the rest of analysis, that fluorescent protein has been successfully produced Figure 5. Protein activity and quantification assays determined that mGFP produced in DnaK$^-$ is less active than

the protein produced in the wild type strain in both fractions Figure 6. This result is also confirmed by fluorescence microscopy Figure 5. Moreover, as it occurs in the wild type strain, soluble and insoluble fractions at 5h are more active than at 3h. In addition, protein yield obtained in the DnaK⁻ strain is, in general, lower than in NZ9000 strain Figure 7.

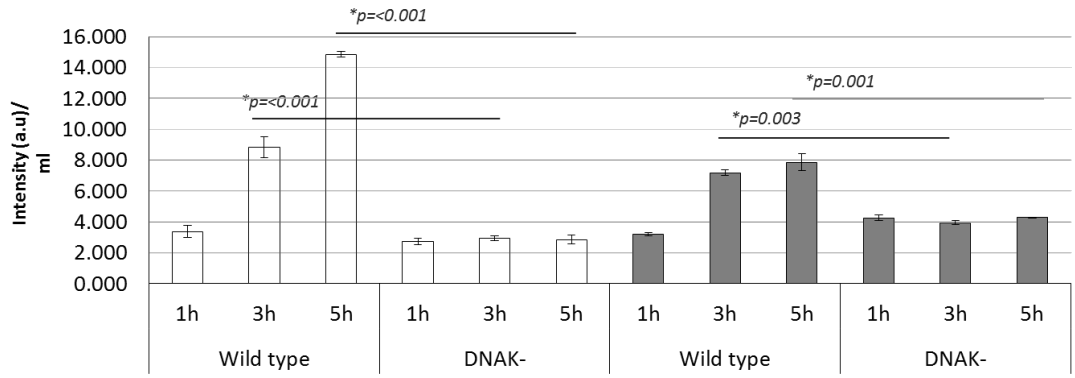

Figure 6: Fluorescence at 3 and 5 h post-induction of soluble mGFP and insoluble mGFP produced in *L. lactis* NZ9000 (white bars) and *L. lactis* NZ9000 DnaK⁻ strains (grey bars). Only significant differences between relevant data pairs are indicated.

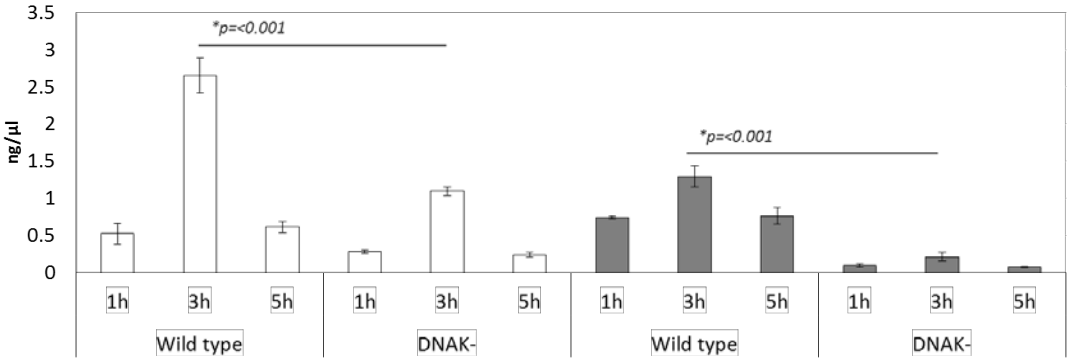

Figure 7: Yield of protein at 3 and 5 h post-induction of soluble mGFP and insoluble mGFP produced in *L. lactis* NZ9000 (white bars) and *L. lactis* NZ9000 DnaK⁻ strains (grey bars). Only significant differences between relevant data pairs are indicated.

In general terms, protein specific activity (protein conformational quality) of the protein produced in the wild type strain is higher than that produced in the strain lacking DnaK chaperone. However, the specific activity of the insoluble protein produced in DnaK⁻ strain was higher Figure 8.

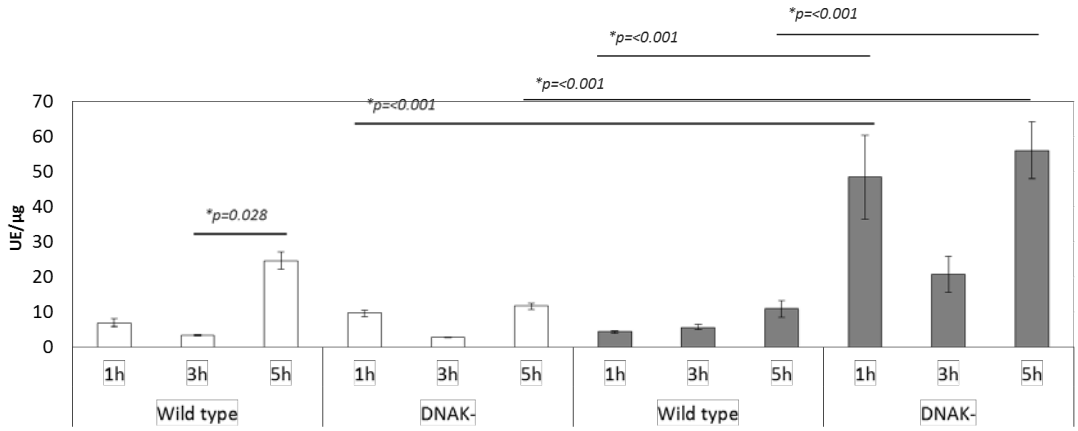

Figure 8: Specific fluorescence of soluble and insoluble mGFP produced in *L. lactis* NZ9000 (white bars) and *L. lactis* NZ9000 DnaK^{-} strains (grey bars). Only significant differences between relevant data pairs are indicated.

In conclusion, unlike as it was described before in *E. coli* ^{315}*dnaK-knock out* in *L. lactis* seems to affect the *L. lactis* growing which could be interfering in the protein production. As a result we have observed either less yield or activity in soluble and insoluble fractions in comparison with the *L. lactis* wild strain, nevertheless the specific activity of the *knock-out* is significantly higher, producing less protein but of better quality.

Annex 2: Others

Annex V

Lactic acid bacteria: reviewing the potential of a promising delivery live vector for biomedical purposes.

Cano-Garrido O*, Seras-Franzoso J, García-Fruitós E.

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MICROBIAL CELL FACTORIES

REVIEW Open Access

Lactic acid bacteria: reviewing the potential of a promising delivery live vector for biomedical purposes

Olivia Cano-Garrido[1,2,3†], Joaquin Seras-Franzoso[1,2,3†] and Elena Garcia-Fruitós[1,2,3,4*]

Abstract

Lactic acid bacteria (LAB) have a long history of safe exploitation by humans, being used for centuries in food production and preservation and as probiotic agents to promote human health. Interestingly, some species of these Gram-positive bacteria, which are generally recognized as safe organisms by the US Food and Drug Administration (FDA), are able to survive through the gastrointestinal tract (GIT), being capable to reach and colonize the intestine, where they play an important role. Besides, during the last decades, an important effort has been done for the development of tools to use LAB as microbial cell factories for the production of proteins of interest. Given the need to develop effective strategies for the delivery of prophylactic and therapeutic molecules, LAB have appeared as an appealing option for the oral, intranasal and vaginal delivery of such molecules. So far, these genetically modified organisms have been successfully used as vehicles for delivering functional proteins to mucosal tissues in the treatment of many different pathologies including GIT related pathologies, diabetes, cancer and viral infections, among others. Interestingly, the administration of such microorganisms would suppose a significant decrease in the production cost of the treatments agents since being live organisms, such vectors would be able to autonomously amplify and produce and deliver the protein of interest. In this context, this review aims to provide an overview of the use of LAB engineered as a promising alternative as well as a safety delivery platform of recombinant proteins for the treatment of a wide range of diseases.

Keywords: Lactic acid bacteria, Delivery vector, QPS, Mucosal, Therapy, Treatment

Background

Most of the existing strategies for the treatment of diseases are focused on the delivery of naked molecules with a therapeutic activity, from chemically synthesized molecules to recombinant proteins produced in diverse platforms such as bacteria, yeast, insect cells and mammalian cells, among others [1]. However, these treatments require in many cases the use of invasive administration methods such as intravenous or subcutaneous injection of the molecule of interest to reach the targeted region [2]. Moreover, soluble purified proteins and other therapeutic compounds frequently show low stability and/or poor efficiency in the organism forcing repeated administration [2], with the subsequent increase in the amount of needed pharmaceutical and the frequent derived increase in toxicity and cost of the treatment [2]. In the case of recombinant therapeutic proteins produced in microbial hosts, biosafety concerns are raised, mainly due to the possible remnants of pyrogenic or inflammatory contaminants that can trigger undesirable immunogenic responses [3]. Given the need to develop an alternative route for the administration, as well as a safety delivery platform, lactic acid bacteria (LAB) have appeared as an appealing option for the production and delivery of therapeutic molecules and antigens of interest [4]. This heterogeneous group of Gram-positive bacteria, in contrast to Gram-negative bacteria such as *E. coli*, do not contain lipopolysaccarides (LPS) attached to the cell membrane. The absence of such endotoxins avoids the generation of an anaphylactic shock

*Correspondence: elena.garcia@irta.cat
†Olivia Cano-Garrido and Joaquin Seras-Franzoso contributed equally to this work
[4] Present Address: Department of Ruminant Production, Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Torre Marimon, Caldes de Montbui, 08140 Barcelona, Spain
Full list of author information is available at the end of the article

when LAB are administered in humans [3]. In this regard, it should be noted that LAB have a long history of safe use by humans, being used for centuries in food production and preservation [5–7]. In this context, some strains have also a long record in their use as probiotic bacteria producer of metabolites and macromolecules able to maintain and promote human health [5, 8]. Then, LAB have been classified as food grade microorganisms [generally recognized as safe (GRAS) organisms by the US Food and Drug Administration (FDA)] and fulfill criteria of the qualified presumption of safety (QPS) according to the European Food Safety Authority (EFSA). Besides, it is important to stress that an exhaustive work has been done in developing different tools for the recombinant protein production using LAB as cell factories [9]. The development of these tools has made possible the development of LAB able to secrete the protein of interest to the extracellular environment, becoming a key aspect when evaluating the potential of these bacteria for mucosal targeting of therapeutic molecules [4] (Table 1). Alternatively, approaches based on protein display anchored to the bacteria cell wall have also been tested [10], being a system that, even not being as effective as secreted protein in terms of protein expression levels, gives a higher protection to the protein in front of degrading and denaturing agents (Table 1). In consequence, these microorganisms can be used for oral, intranasal or vaginal administration for protein delivery purposes, minimizing any potential side effect associated with the classical parenteral or subcutaneous administration of proteins, simultaneously reducing the dose needed.

Although LAB include microorganisms from different genus such as *Leuconostoc*, *Lactococcus*, *Lactobacillus*, *Pedicoccus* and *Streptococcus*, *Lactococcus lactis* has been the most widely used considering cloning and production of recombinant proteins [11]. *L. lactis* has been deeply characterized, being the first one whose genome was fully sequenced. In addition, it is an expression system easy to manipulate with many cloning and expression systems available. The most widely used protein expression system for *L. lactis* is the NICE (Nisin Controlled Expression) system, based on the control of a strong nisin inducible promoter (P_{nisA}), which has several advantages. The expression of the gene of interest is tightly regulated and high expression levels are achieved using a food-grade molecule (nisin) as inducer. [12]. Although several proteins with biotechnological or biomedical interest produced in *L. lactis* using inducible plasmids have been proved in both experimental models and clinical trials [13], a prior induction of protein production have been required in these cases before the administration of the recombinant bacteria. For that reason, other inducible promoters that do not require the addition of any external inducer have been developed not only for *L. lactis*, but also for other LAB such as *Lactobacillus paracasei*. These promoters are directly induced in situ, for example once bacteria suffer environmental stresses such as heat-stress (body temperature is some degrees higher than bacteria optimal growth temperature) [14] or acid-stress (because of the stomach fluids) [15], enabling the recombinant production of the protein of interest without the need for adding an external inducer. In this regard and considering that *L. paracasei* respond to stress by synthesising chaperones such as groESL [14], an Stress-Inducible Controlled Expression (SICE) system based on the groESL operon promoter has also been described [15]. The development of promoters that do not depend on the addition of external inducers have allowed to take an important qualitative leap towards the use of LAB as protein delivery vectors. In this context and aiming to take another step forward, constitutive promoters are also being extensively studied. These constitutive promoters make possible to get a maintained expression of the protein of interest over time without the need of any type of inducer. Currently it has been widely explored, being possible to find an important number of examples that have already been tested for protein delivery purposes specially with *L. lactis*, but also with *L. paracasei*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Bifidobacterium breve* and *Streptococcus gordonii* (Table 1).

Thus, the use of food grade microorganisms as recombinant protein cell factories [9] and delivery platforms at the same time, is a promising approach [6, 11]. Briefly, the administration of such microorganisms would also suppose a significant decrease in the production cost of the drugs as being live organisms, these live vectors would be able to autonomously synthesize and deliver the prophylactic or therapeutic protein of interest. Moreover, it is possible to simultaneously produce different proteins in the same bacteria [16]. Altogether has turned them into an attractive alternative not only to intravenous administration of naked recombinant proteins, but also to other classical delivery systems for mucosal targeting, such as attenuated pathogens, liposomes and microparticles [10]. Thus, here, is intended to provide an overview of the use of genetically modified food grade organisms engineered as attractive vehicles for delivering functional proteins to mucosal tissues for the treatment of a wide range of pathologies such as GIT related pathologies as well as some types of cancer and viral infections, among others (Fig. 1).

Review

Autoimmune diseases

Effective therapeutic approaches for autoimmune diseases like GIT related diseases and diabetes are urgently

Table 1 Recombinant proteins produced in LAB for biomedical purposes

LAB	Application	Recombinant protein	Expression vector	Promoter	Protein display	References
Lactococcus lactis	IDB	Anti-TNFalpha nanobodies	pTREX-derived	P1 (pH dependent)	Secreted	[22]
Lactococcus lactis	IDB	Trefoil Factors (TFF)	pTREX-derived	P1 (pH dependent)	Secreted	[23]
Lactococcus lactis	IDB	Low calcium response V (LcrV)	pNZYR-derived	P_{Usp45} (constitutive)	Secreted	[21]
Lactobacillus gasseri/ *Lactobacillus casei*	IDB	Superoxide Dismutase (SOD)	pSodA pILKS*sodA*	*sodA* native promoter	–	[39, 40]
Lactococcus lactis	IDB	IL-10	Cromosome integrated	P_{thyA} (constitutive)	Secreted	[26]
Lactococcus lactis	IDB	IL-27	pT1NX-derived	P1 (pH dependent)	Secreted	[35]
Lactococcus lactis	IBD	Murine IL-10	pLB263	P_{groESL} (Inducible)	Secreted	[15]
Lactococcus lactis/ *Lactobacillus casei*	IDB/colorectal Cancer	Catalase	pSEC:KatE/ pLEM415*mnkat*	P_{nisA} (inducible)/P_{ldh} (constitutive)	Cytoplasmatic	[24, 25]
Lactococcus lactis	Type 1 diabetes	Pro Insulin/(GAD)-65/IL-10	pT1NX-derived	P1 (pH dependent)	Secreted	[44]
Lactococcus lactis	Type 1 diabetes	HSP65-6P277	pCYT:HSP65-6P277/ pHJ: HSP65-6P277	P_{nisA} (inducible)/constitutive	Cytoplasmatic/ secreted	[43]
Lactococcus lactis	Type 1 diabetes	GAD65 and IA-2	–	–	Secreted	[46]
Lactococcus lactis	Diabetes	Single-chain insulin analog, SCI-57	pNZPnisA:uspSCI-57	P_{nisA} (inducible)	Secreted	[41]
Lactococcus lactis	Type 2 diabetes	Glucagon like peptide-1 (GLP-1)	pUBGLP-1	P1 (pH dependent)	Secreted	[42]
Lactococcus lactis	Cancer	HPV-16 E7 antigen	pLB263	P_{groESL} (inducible)	Secreted	[15]
Bifidobacterium longum	Breast cancer	Cytosine Deaminase	pBLES100-S-eCD	–	–	[57]
Bifidobacterium adolescentis	Cancer	Endostatin	pBV220-derived	P_RP_L (thermoinducible)	Cytoplasmatic	[58]
Bifidobacterium breve	Cancer treatment study tool	Luciferase	pLux MC3	P_{help} (constitutive)	Cytoplasmatic	[59]
Lactococcus lactis	Cervical cancer	HPV-16 E7	–	P_{nisA} (Inducible)	Anchored	[61]
Lactococcus lactis	Cervical cancer	HPV-16 E7	pMG36e	P32 (constitutive)	Cytoplasmatic	[62]
Lactococcus lactis/ *Lactobacillus casei*	Cervical cancer	HPV-16 E7	–	–	Anchored	[63]
Bacillus subtilis	Cervical cancer	HPV33 L1	pICHPV33L1-NS/B	Pxylose (inducible)	Intracellular	[74]
Lactobacillus paracasei	*Bacillus anthracis* infection	Antibody fragment	pAF100-derived/ pAF400-derived/ pAF900-derived	P_{apf} (constitutive)	Secreted/attached/ cell anchored	[85]
Lactobacillus paracasei	*Rotavirus* infection	Antibody fragment	pLP501-derived	P_{ldh} (constitutive)	Secreted/cell anchored	[87]
Bifidubacterium longum	Enterovirus 71 infection	VP1	pBBADs-VP1	–	–	[109]
Bifidubacterium longum	Hepatitis C infection	HCV-NS3 peptide	–	–	Cell anchored	[110]
Lactococcus lactis	Staphylococcal infection	Staphylococcal nuclease	pLB263	P_{groESL} (inducible)	Secreted	[15]
Lactobacillus acidophilus	HIV infection	Gag	pTRK1035	(Constitutive)	Cell anchored	[82]
Lactobacillus jensenii	HIV infection	two-domain CD4 (2D CD4) proteins	pOSEL144	P_{23} (constitutive)	Secreted	[69]
Lactobacillus casei	Tetanus	Tetanus toxin fragment C (TTC)	pLP401-TTFC	P amylase (inducible)	Cell anchored	[72]
Lactobacillus casei	Tetanus	Tetanus toxin fragment C (TTC)	pLP501-TTFC	P_{ldh} (constitutive)	Cell anchored	[72]
Lactobacillus plantarum	Tetanus	Tetanus toxin fragment C (TTC)	pMEC160	P_{ldh} (constitutive)	Cell anchored	[70]

Table 1 continued

LAB	Application	Recombinant protein	Expression vector	Promoter	Protein display	References
Streptococcus gordonii	Clostridium tetani infection	Tetanus toxin fragment C	pSMB158	(constitutive)	Cell anchored	[111]
Lactobacillus acidophilus	Helicobacter pylori infection	Adhesin Hp0410	pMG36e	–	Cytoplasmatic	[76]
Lactococcus lactis	Rotavirus infection	VP8	pNZ8048	P_{nisA} (inducible)	Secreted/cell anchored/cytoplasmatic	[112]
Lactococcus lactis	Malaria	$MSP\text{-}1_{19}$	pL2-PSGT	–	Cytoplasmatic	[81]
Lactococcus lactis	Peanut allergy	Ara h 2	pNZ8148	P_{nisA} (inducible)	Secreted/cell anchored/cytoplasmatic	[93]
Lactococcus lactis	Dust mite allergy	Der p2	pNZ8148	P_{nisA} (inducible)	Secreted/cell anchored/cytoplasmatic	[94]
Streptococcus gordonii	Giardia lamblia infection	cyst wall protein 2 (CWP2)	pSMB104	(constitutive)	Cell anchored	[73]
Lactobacillus zeae	Streptococcus mutants infection	ScFv protein	pLP402-scFv	–	Cell anchored	[68]
Lactococcus lactis	Streptococcus pneumoniae infection	Pneumococcal surface protein A	pTREX1	P1 (pH dependent)	Cytoplasmatic	[113]
Lactobacillus casei	SARS-associated coronavirus infection	PgsA and spike protein	pHAT	P_{HCE} (constitutive)	Cell anchored	[114]
Lactobacillus acidophilus	Chiken anemia virus	VP1	pETacmA1	–	Cell anchored	[71]
Lactococcus lactis	Avian influenza virus	hemagglutinin 1 (HA1)	pMG36e	–	Cytoplasmatic	[91]
Lactococcus lactis	Leishmania major infection	LACK LACK + IL12	pDL-PnisA	P_{nisA} (inducible)	Secreted/cell anchored/cytoplasmatic	[88]
Lactobacillus casei	Pancreatic necrosis virus (IPNV)	VP2/VP3	pG1/pG2	Pxylose (inducible)	Secreted	[89, 90]
Lactococcus lactis	Body weight control	Leptin	pSEC:lep	P_{nisA} (inducible)	–	[97]
Saccharomyces cerevisiae	Hypercalcemia	Salmon calcitonin	pAGA2-sCT	P_{GAL1} (inducible)	Cell anchored	[115]

needed and the development of oral formulation is an imperative need. Oral administration is the most preferred route since it is well accepted by patients, becoming a promising alternative for drug delivery of such autoimmune diseases.

GIT related diseases: Crohn's disease and ulcerative colitis

Inflammatory bowel disease (IBD) is an idiopathic disorder consisting in the inflammation of the GIT. It is believed that this abnormal condition is due to an uncontrolled immune response against the gut microflora [17, 18]. Although the underlying cause is still unclear [19, 20], it is known that environmental and genetic factors have an important role in these complex diseases. Crohn's disease and ulcerative colitis are included in this general IBD definition and the patients that suffer these chronic diseases usually require lifelong and costly treatments with sever side-effects. Moreover, in many cases, therapeutic agents used fail, and despite medical treatment, surgery is needed. In this sense, the use of recombinant microorganisms, that fulfill the QPS standards, overexpressing any molecule able to alleviate inflammation could be an attractive and alternative treatment, since their safe profile and administration pathway would allow an easy incorporation of the treatment to the patient's routine improving their comfort. Some strategies have been proposed using proteins such as low-calcium V antigen (LcrV) [21], anti-TNFα nanobodies [22], trefoil factors (TFF) [23], catalase [24, 25] and IL-10 [26] using *L. lactis* as microbial delivery vector (Table 1). All these proteins have been successfully produced by the *L. lactis* platform ameliorating, upon their oral administration, the inflammatory response of IBD animal models.

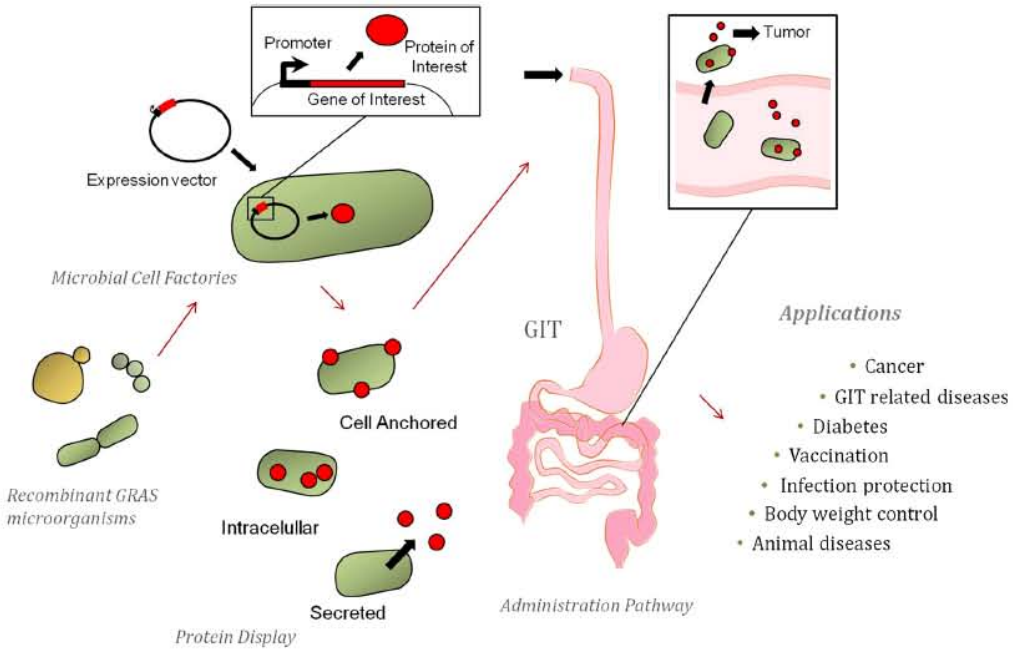

Fig. 1 Schematic representation of the use of LAB for biomedical applications using the oral administration pathway

Noteworthy, the success of these approaches is related to the delivery of the therapeutic agent at the mucosa level. A clear example is provided by the orally administered *L. lactis* secreting anti-TNFα nanobodies [22]. This nanobody secretion platform has an efficacy similar to that observed with the established therapy (Infliximab, Remicade), based on the intravenous infusion of anti-TNFα [22, 27–30]. However, contrary to what occurs by the systemic infusion, the oral administration of *L. lactis* strains secreting anti-TNFα is cost effective and lacks adverse effects [22].

LAB with potential in IBD treatment share the objective of reducing gut inflammation. Nevertheless, depending on the recombinant protein to be delivered the affected pathway differs. Thus, anti-TNFα nanobodies would reduce inflammation by neutralizing the action of the pro-inflammatory cytokine TNFα. Expression of IL-10, which is a regulatory cytokine, would decrease inflammation thanks to its antinflammatory activity [26], while the use of enzymes such as catalase would act on the inflammatory response derived from the presence of reactive oxygen species (ROS) [24, 25].

The approach that probably has been more extensively studied is the delivery of IL-10 produced in *L. lactis*. In fact, *L. lactis* secreting IL-10 has been submitted to clinical trials for the treatment of Crohn disease (ClinicalTrials.gov Identifier: NCT00729872) [23, 26, 31–34]. Nevertheless, and despite the very promising results observed in mice, the clinical trial revealed the mentioned approach inefficient (ClinicalTrials.gov Identifier: NCT00729872). This failure is thought as a consequence of a low final concentration of IL-10 in the GIT. In this regard, it has been recently proposed using IL-27, a pleiotropic cytokine, in order to get a broader response due to its immunosuppressive role as well as the capacity to induce IL-10 expression [35].

Besides, elafin, an endogenous protease inhibitor, has also been orally administered using *L. lactis* and *L. casei* as delivery vectors, observing a restoration of colon homeostasis in mice [11, 36, 37]. Elastin, which is diminished in patients with IBD, has a pleiotropic and anti-inflammatory role in healthy human gut [11, 36, 37]. Recently, two other anti-inflammatory molecules named secretory leukocyte protease inhibitor (SLPI) and the enzyme 15-lipoxygenase-1 (15-LOX-1) secreted by *L. lactis* have shown the ability to notably reduce the intestinal inflammation in mice [37, 38].

Other models used for the local display in the mice gut of therapeutic protein are *Lactobacillus gasseri* and *L. casei* both expressing superoxide dismutase (SOD) [39, 40]. As it happens with catalase, SOD action neutralizes ROS species and their derived inflammatory effect.

Diabetes

Some articles have also been published exploring the potential of food-grade bacteria for the treatment of diabetes (Table 1). In this context, Ng and collaborators proved that *L. lactis* is able to secrete an insulin analog in vitro [41], promoting the expected biological effect on target adipocytes. Some years later, Agarwal et al. have described a successful in vivo assay with rats based on the oral delivery of glucagon like peptide-1 (GLP-1) using again *L. lactis* as delivery platform [42]. Briefly, GLP-1 has emerged as a promising therapeutic peptide for type 2 diabetes treatment, being a compound that is synthesized by the GIT for the maintenance of glucose homeostasis. Up to now, GLP-1 has been administered through injection, being necessary one administration at least once a week. Interestingly, Agarwal et al. have observed that once recombinant *L. lactis* secreting GLP-1 is orally administered in rats, a reduction in blood glucose levels and an important increase in insulin take place [42].

Nowadays, it is widely accepted that current treatments based on insulin replacement for type 2 diabetes have important weak aspects [42]. On the one hand, the autoimmune response that impairs β-cells in pancreas is not inhibited [42]. On the other hand, insulin injection cannot prevent important associated complications to diabetes. Therefore, different therapeutic approaches based on immunotherapies are also being explored, being the use of antigen-based immunotherapies the most promising for this autoimmune disease. A 24 amino-acid peptide derived from human HSP60 has demonstrated to be a convenient alternative for the modulation of the immunological attack on β-cells in mouse [43]. This peptide was successfully orally administered using recombinant *L. lactis* as delivery carrier, having a clear effect on the improvement of glucose tolerance and in the reduction of insulinitis and hyperglycemia [43]. In addition, another study has been recently published describing the administration of a *L. lactis* strain delivering antigens such as pro-insulin or glutamic acid decarboxylase in combination with IL-10 and anti-CD3 as an appealing method to improve the induction of antigen-specific tolerance for the treatment of the type 1 diabetes [23, 44, 45]. Another study has been recently followed the oral administration of *L. lactis* secreting two major auto-antigens of type 1 diabetes, named glutamic acid decarboxylase (GAD65) and tyrosine phosphatase-like protein ICA512 (IA-2) in mouse models [23, 46]. In this study, modified versions of GAD65 and IA-2 have been successfully used in combination with human IL-10 cytokine [23, 46].

Cancer

Cancer has a huge relevance in human health due to its growing incidence in developed countries. The strategies for effective cancer treatment under study are countless and the use of LAB has recently appeared in this field.

Cancer development and progression have been broadly related with chronic inflammation processes produced by external factors such as infection, radiation, unbalanced diet, obesity, tobacco or the exposure to other environmental pollutants [47]. Thus, in principle, any strategy aimed to treat chronic inflammation could produce also a positive outcome in cancer occurrence. In this regard, the use of LAB organisms for colorectal cancer prevention has been explored using mainly colorectal cancer murine models [48]. Some examples of the strains used with this purpose are *Bifidobacterium lactis* [49], *L. casei strain Shirota* [50], *B. longum BB536* [51], *Lactobacillus acidophilus Delvo Pro LA-1* [52], *Lactobacillus rhamnosus GG* [53] *or Propionibacterium freudenreichii* [54] but many others rendered similar results, showing a significant decrease in cancer development. It is important to note although the significant number of LAB showing promise in in vitro and in animal models that no conclusive studies have been carried out in humans.

On the other hand, interesting studies regarding biodistribution of the microbial vectors in mice models illustrates the capacity of some food grade species to reach solid tumors, where they are able to accumulate and proliferate after intravenous administration [55, 56]. This behavior has been related with the hypoxic environment exhibited in the tumors. In such atmosphere anaerobic bacteria selectively grow [55, 56]. This capacity has been exploited in *B. longum* to propose an anti-breast cancer strategy based on the recombinant production of cytosine deaminase in the solid tumor after the intravenous administration of the microbial vector. The enzyme combined with the administration of the 5-fluorocytosine (5FC) would result in a locally high concentration of the reaction product, 5-fluorouracyl (5FU) [57]. Another example using *Bifidobacterium adolescentis* expressing a recombinant endostatin showed how this safe vector was able to selectively inhibit angiogenesis and tumor growth in tumor mice models after its intravenous administration [58]. These studies reinforce the capability of these microorganisms' genera, classified according to QPS standards, as potential drug delivery systems for cancer treatment. The protein secreted by the live vector will be more stable than those naked soluble proteins intravenously administered. However, as previously mentioned, the intravenous injection of LAB have important adverse effects. Thus, the delivery of such live vectors at mucosal level would be much more appropriate. In this context, the potential use as orally administered drug delivery vector with a natural selectivity for solid tumors has also been explored (Table 1). Recombinant *B. breve* orally administered in mice are able to effectively translocate

the GIT and colonize solid tumors at the same levels than intravenously administered ones [59]. Interestingly, the crossing of the GIT by the recombinant *B. breve,* involving an increased permeability of the GIT epithelia didn't promote the crossing of potentially pathogenic bacteria present in the regular gut flora [59].

Besides, LAB can also be orally administered taking advantage of their natural niche in the body to develop prophylactic strategies against colon cancer without the need of the GIT translocation. In this sense a recombinant *L. lactis* producing catalase has shown a protective effect in chemically induced colon cancer in mice models [60]. Tumor cells are characterized by an increased production of ROS such as hydrogen peroxide (H_2O_2), that actively participate in enhancing tumour invasion and proliferation. Thus, the administration of *L. lactis* producing catalase, an enzyme with an antioxidant activity, decreases H_2O_2 levels and, consequently, reduces colonic damage and inflammation [60]. Interestingly, since the oxidative stress associated to an increase of ROS levels is also characteristic of gastrointestinal pathologies, the approach developed in this study can be used also as a therapy for the treatment of IBD. Recently, some articles have been published using the administration of *L. lactis* expressing human papillomavirus E7 oncoprotein (HPV-16 E7) for the treatment of cervical cancer. In one of these articles, E7 protein has been produced by a secretion SICE plasmid and administrated to mice with tumours. Results show that administration of recombinant bacteria provokes a slightly diminution of tumour volume and an antigen-specific immune response [15]. In other studies these food-grade bacteria have been administered in mice via intranasal expressing HPV-16 E7 anchored to its surface [61, 62]. In one case, E7 protein has been combined with calreticulin-E7 administration inducing >80 % of tumour suppression in mice [61]. In a second approach, recombinant lactococci have been tested for the simultaneous delivery of E7 protein and murine interleukin-12 (IL-12) DNA [62], observing that this new strategy combining the delivery of both the therapeutic molecules and antigens has a high potential. Finally, E7 protein effect has also been investigated using *L. casei* as mucosal delivery vector in mice [63].

Infectious diseases

Historically vaccines have been based on attenuated pathogenic microorganisms [64, 65]. However, this approach has three important drawbacks: (a) difficulties on the construction of stable attenuated mutants; (b) presence of residual virulence in attenuated pathogens; (c) risk of genetic reassortment between the vaccine strain and the wild type. Besides, although pure antigens have also been used for vaccination purposes, these molecules have a low or non-existing immune response, especially because their rapid degradation and their poor adsorption in vivo [66]. Thus, aiming to find an alternative strategy, non-pathogenic LAB have also been explored as mucosal vaccines. Many approaches have been proposed in order to produce and present different antigens [23, 67]. Most of them have been developed in *L. lactis,* but also there are works using other LAB such as *Lactobacillus zeae* [68], *Lactobacillus jensenii* [69], *L. plantarum* [70], *L. acidophilus* [71], *L. casei* [72], *Streptococcus gordinii* [73], *and Bacillus subtilis* [74] (Table 1). It should be noted that several *Lactobacillus* species have been exploited in this field, being some of them able to attach and colonise the gastrointestinal mucosa, being acid-resistant and biletolerant [75]. Besides, the presence of these bacteria may naturally inhibit the pathogenic colonization of pathogenic microorganisms such as *Helicobacter pylori* [76]. However, although *Lactobacillus* seems to be one of the best candidates for immunization purposes, protein expression levels achieved are still lower than those obtained with *L. Lactis* [66, 77]. Since low protein yields cannot induce an immune response strong enough to trigger protection against infective agents, an optimal antigen presentation is required. That is possible with a sudden high concentration of the antigen as such obtained with inducible approaches [67]. Nevertheless, given that it is common to find either insolubility or toxicity of some recombinant proteins during overexpression, constitutive plasmids can be a good alternative [78]. The use of constitutive plasmids is a much safer approach, being not necessary to add any external inducer to get the desired amount of protein. The use of constitutive plasmids is exemplified by the expression of SspA and SspB antigens from *S. gordonii*, a major colonizer of oral hard and soft tissues, on the cell surface of *L. lactis* using P1 promoter. Both antigens were successfully expressed and anchored in the cell wall and their in vitro ability to adhere to *S. gordonii* surface was proven [79]. Another example is the *slpA* constitutive promoter based on S-layer protein. The very strong expression signal of S-layer has been proven for the secretion of β-lactamase using *L. lactis*, *L. brevis*, *L. plantarum*, *L. gasseri* and *L. casei* [80] and of Merozoite surface protein 1 (MSP1) from *Plasmodium falciparum* using *L. lactis* [81]. Interestingly, this last study proves the potential of recombinant *L. lactis* as an effective oral vaccination alternative against malaria [81]. In vivo studies with MSP1 antigen show its capacity to confer protection to the vaccinated animals [81]. Furthermore, the combination of the antigen with adjuvant molecules have been studied also aiming to achieve an increased efficiency of these vaccines. An example is the coexpression of HIV-1 with the flagellin (FliC) of *Salmonella enterica*

in *L. acidophilus*. The results show that Gag (antigen against HIV-1) and FliC coexpression promotes Gag-specific IgA-producing cells at the local mucosa [82]. Another study has been reported aiming to develop a vaccine against the chicken anemia virus (CAV) [71]. VP1 antigen produced in *E. coli* was fused to the binding domain of AcmA, the major autolysin of *L. lactis* cell wall, at N-terminal aiming to enhance the *Lactobacillus* immunization ability. In this case, the authors observed that the fusion protein remains on the cell surface at least 5 days and that the oral administration induced a moderate immune response in chicken [4, 71].

Bifidobacterium is another appealing food-grade vector. It is abundant in human gut, well recognised as probiotic and with the ability to activate Th1 cell-mediated immune responses without antigen presentation [83]. Because of its biletolerance, a regulated promoter based on upstream sequence of *bet A* (a bile-inducible transporter gene) has been recently developed in order to control gene expression specifically in the intestinal tract [84]. However, it should be noted that *Bifidobacterium* has a strict anaerobic metabolism making harder the experimentation with it.

A part from IBD, diabetes and cancer, other diseases have been targeted using food grade bacteria. Among them, interesting examples can be found in the control of microbial infections (Table 1). Regarding microbial infections neutralizing antibody fragments expressed in *L. paracasei* and *L. acidphilus* have been shown able to provide protection against *Bacillus anthracis* [85, 86] and *L. paracasei* against rotavirus [87] in mice. These studies open possibilities of generating alternative *Lactobacilli* producing antibodies against other infectious diseases affecting the GIT.

Besides, it is important to note that LAB and other organisms classified as QPS by EFSA are useful mucosal delivery vectors to treat not only human diseases but also animal diseases. Just as an example, LAB have been used to combat Leishmaniasis [88]. Moreover, *Lactobacilli* have also been used to design live vaccines to combat a wide range of diseases such as pancreatic necrosis virus (IPNV, a pathogen that infects wild and cultured salmonids) [89, 90], a highly pathogenic avian influenza (HPAI) [91], and porcine epidemic diarrhea virus (PEDV) [92].

Allergic and other diseases

Some studies have combined the bacterial effect with an expressed antigen either for food hypersensitivity or aero-allergens. The administration of *L. lactis* displaying the recombinant allergen intracellularly, in the extracellular space or cell wall-anchored show its capacity to modulate the Th2-based specific antibody responses, in the case of the allergen Ara h 2 against peanut allergic [93] and the Der p2 allergen against the dust mite allergy [94]. In the second study, the authors also observed a diminution in the cellular infiltration and inflammatory response.

It has been demonstrated that obesity and gut microbiota composition strongly correlates [95, 96]. In addition, some LAB such as *Lactobacillus* and *Bifidobacterium* are able to improve obesity in both murine models and humans [96]. In this context, obesity has also been targeted in mice models by the delivery of recombinant leptin using LAB. Leptin is a hormone with a crucial role in body weight control. In this case, the capability of this protein to carry out its action was improved by the intranasal administration of engineered *L. lactis* secreting the hormone observing a significant loss in body weight as well as a reduction in food intake in the treated animals [97].

Another example is the administration of cytokines-secreting LAB as prophylaxis therapy, being the delivery of IL-10 for inflammatory bowel diseases [98] and of IL-12 for asthma [99] just a couple of examples [100].

Finally it is necessary to stress out that although most of the applications referred to in this revision are envisaged using LAB, also eukaryotic expression systems with a safe profile can be found in the literature. This possibility would result of interest in the cases of therapeutic proteins requiring complex post-translational modifications to became fully functional [101]. In this regard, prokaryotic cell factories would lack the machinery to perform the required processing of the recombinant protein [101]. A nice example is found in recombinant *Saccharomyces cerevisiae*. This yeast has been proposed as a vehicle to secrete proteins or peptides with a therapeutic effect in the gut [102]. In this sense, an orally recombinant *S. cerevisiae* displaying a salmon recombinant calcitonin on the yeast surface prompted a decrease in calcium levels in hypercalcemic rats after oral administration.

Adverse side-effects of LAB

In the previous sections we have been focusing the attention on the positive effect of LAB as live vectors for protein delivery. However, it is important to stress that LAB are genetically modified organisms (GMOs). GMOs are widely accepted and well stablish in food industry. However, important regulatory concerns need to be addressed for its use as therapeutic vechicles. Specifically, LAB are based on expression systems carrying antibiotic resistance genes as a selection marker [103]. It has been described that these live vectors could transfer its antibiotic resistances to intestinal microbiota. Although this is a really rare event that has not been reported in this field, it is an important issue to be considered. The application of LAB as live vectors opens a broad and interesting

field of possibilities, but regulatory measures have to be considered to ensure the safety of the used strains. Up to now, some alternative and innovative selection markers have already been developed, being some of them successfully tested and positively evaluated by several health authorities [23, 34, 103]. However, these alternative and safe selection markers need to be further explored to finally ensure the real possibility of using such strains for the problems listed above.

Besides, it should be noted that some adverse effects of LAB have been reported [104–106]. This indicates that, despite the positive therapeutic effects of these microorganisms and the low number of adverse effects registered, they are not completely safe. This information should be contextualized, since the adverse effects were observed in high risk groups such as critically ill and/or immune-compromised patients, critically sick infants, and postoperative and hospitalized patients [104]. Sepsis, fungemia and GI ischemia are the main harmful effects of LAB described [104]. Briefly, in this vulnerable populations LAB could interfere with the microflora giving rise to opportunistic infections and finally to bacterimia, fungimia or other medical complications. In addition, there are strong evidences proving that LAB, when used as probiotics, have anti-inflammatory effects. However, many reports also describe pro-inflammatory effects caused by such group of bacteria [107, 108]. This means that probiotic effect of LAB is strain dependent, being a factor to be considered for the choice of host strains for therapeutic applications.

In general terms, one can conclude that the safety of LAB is widely supported by the long tradition of use of such microbes. This safety record leads us to conclude that risk–benefit ratio in the prevention and treatment of multiple disease states is overall really high, being the studies reporting adverse effects scarce. Nonetheless, risks and benefits should be carefully considered in each situation, especially on those health-compromised patients. Besides, considering that adverse events are poorly documented, an accurate safety report including pathogenicity, infectivity, virulence and toxicity would help the scientific community taking decisions and more solid conclusions [105].

Conclusions

The irruption of nanotechnology and other innovative approaches have allowed the development of alternatives to the classical medicine aiming to overcome its inherent limitations. In this regard, the exploitation of LAB as recombinant probiotics expressing any protein of interest has strongly burst them as a promising alternative for the treatment of a wide range of diseases. The mucosal, needle-free, administration of therapeutic molecules of interest gives an added value to LAB. Besides, it has been shown that the application of these recombinant probiotic bacteria via intranasal, oral or genital would have a dual effect: a direct effect designed for the treatment of a specific disease through the expression of a recombinant protein, combined with the indirect and general effect that some of these safe bacteria have in health. Additionally, the administration of such live delivery vectors is easier and relatively inexpensive compared to injectable treatments, being a large-scale production affordable [66]. Interestingly, up to now the use of LAB has been successfully tested for a wide range of medical applications, mainly using animal models, being the treatment of autoimmune diseases the most intensively investigated. These food-grade bacteria have also been proposed as excellent candidates for vaccination. There is still a lot to be done and a long way to reach the market, but all the articles published up to now let us suggest LAB as a promising delivery vector for a vast range of biomedical applications.

The next step will be the detailed study of all factors that could become an important bottleneck in the future implementation of LAB as effective live vectors delivering proteins of interest in situ. Important safety and regulatory issues still need to be addressed in depth, but some significant steps have already been done in this context. Small trials using auxotroph strains have been positively evaluated for the treatment of patients with Chron's disease [26, 34]. Other aspects such as an extensive study of real cost and efficiency are still outstanding questions that need to be answered.

Authors' contributions

All authors have contributed to this review from their complementing areas of expertise. All authors read and approved the final manuscript.

Author details

[1] Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, Bellaterra, 08193 Cerdanyola del Vallès, Spain. [2] Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, 08193 Cerdanyola del Vallès, Spain. [3] CIBER de Bioingenieria, Biomateriales y Nanomedicina (CIBER-BBN), Bellaterra, 08193 Cerdanyola del Vallès, Spain. [4] Present Address: Department of Ruminant Production, Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Torre Marimon, Caldes de Montbui, 08140 Barcelona, Spain.

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Compliance with ethical guidelines

Competing interests

The authors declare that they have no competing interests.

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Annex VI

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

Rueda F, Cano-Garrido O, Mamat U, Wilke K, Seras-Franzoso J, Garcia-Fruitós E, Villaverde A.

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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

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

Fabián Rueda · Olivia Cano-Garrido · Uwe Mamat · Kathleen Wilke · Joaquin Seras-Franzoso · Elena García-Fruitós · Antonio Villaverde

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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.

F. Rueda · O. Cano-Garrido · J. Seras-Franzoso · E. García-Fruitós · A. Villaverde
Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, Bellaterra, 08193 Cerdanyola del Vallès, Spain

F. Rueda · O. Cano-Garrido · J. Seras-Franzoso · E. García-Fruitós · A. Villaverde
Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, 08193 Cerdanyola del Vallès, Spain

F. Rueda · O. Cano-Garrido · J. Seras-Franzoso · E. García-Fruitós · A. Villaverde (✉)
CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Bellaterra, 08193 Cerdanyola del Vallès, Spain
e-mail: antoni.villaverde@uab.es

U. Mamat · K. Wilke
Division of Structural Biochemistry, Research Center Borstel, Leibniz-Center for Medicine and Biosciences, 23845 Borstel, Germany

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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 (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; Liovic et al. 2012) and also mechanical and biological stability (Diez-Gil et al. 2010; Singh and Panda 2005; Margreiter et al. 2008; 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. 2012) 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 from *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 focused on the development of optimized cell-disruption methods for the isolation of these submicron

particles lacking bacterial cells (Rodriguez-Carmona et al. 2010; Peternel and Komel 2010; Peternel and Komel 2011). 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, KPM22 L11-derivative (Mamat et al. 2008), endotoxin-free strain KPM335 (*msbA52*, Δ*gutQ*, Δ*kdsD*, Δ*lpxL*, Δ*lpxM*, Δ*pagP*, Δ*lpxP*, Δ*eptA*, *frr181*). Details of KPM335 construction will be given elsewhere. All these strains were transformed with pTrc99a-VP1GFP (Ap^R) encoding the fusion protein VP1GFP (GenBank accession number KM242650), an aggregation-prone fluorescent protein previously used as a reporter to study IB formation and consisting on the VP1 capsid protein of foot-and-mouth disease virus fused to the amino terminus of EGFP (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 37 °C 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 and 0.4 at 37 °C, 25 ml of aliquots of the cultures were centrifuged (4,000×*g*) at 4 °C 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 −80 °C.

Electrocompetent cells

KMP335 cells were grown overnight at 37 °C and 250 rpm and diluted 1/50 in 80 ml of LB. The cells were grown to an OD_{550} between 0.2 and 0.4, placed on ice for 20 min and sedimented by centrifugation (3,100×*g*, 4 °C, 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 42 °C for 45 s. The mixtures were then immediately placed on ice for 30 s, 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 37 °C. Since transformation of KPM335 by heat shock was not very efficient, we decided to transform electrocompetent KPM335 cells by electroporation in a pre-chilled 0.2-cm gap electroporation cuvette using 200 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 ms. Immediately after the pulse, 800 μl of LB medium were added, and the mixture was incubated at 37 °C for 1 h. The cells were then 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 (37, 25, and 16 °C). 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 for MC4100) and incubated at 37 °C 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 3 h at 37 °C or overnight at 25 and 16 °C. At the indicated times, 20 ml of each culture were harvested by centrifugation (10,000×*g*, 4 °C, 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 s (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*, 4 °C, 15 min), and both the IB pellets and the supernatants were stored at −20 °C.

IB purification

Overnight cultures of MC4100, BW32070, and KPM335 grown at 37 °C and 250 rpm were used as a 1/100 inoculum in 500 ml of LB, and VP1GFP IBs were produced at 37 °C. After production, lysozyme (1 μg/ml), phenylmethylsulfonyl fluoride (0.4 mM), and EDTA-free protease inhibitor cocktail (cOmplete EDTA-free, Roche Diagnostics, Indianapolis, USA) were added, and cultures were incubated for 2 h at 37 °C and 250 rpm. Mixtures were stored at −80 °C. 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 −80 °C. 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 4 °C for 1 h. After incubation, 1 mM $MgSO_4$ and DNase (1 μg/ml) were added (Roche Diagnostics, Indianapolis, USA). The samples were incubated at 37 °C for 1 h with shaking and centrifuged (15,000×*g*, 4 °C, 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 (Roche Diagnostics, Indianapolis, USA). One hundred microliters of each sample were plated on LB-agar without antibiotics, and the plates were incubated at 37 °C overnight. The samples were centrifuged again under the same conditions as described above. The pellets were resuspended in PBS, and 100 μl of aliquots were plated to monitor bacterial viability. Finally, the resuspended pellets containing the IBs were separated into 5 ml of aliquots and sedimented again by centrifugation (15,000×*g*, 4 °C, 15 min). The supernatants were discarded, and the pellets were stored at −80 °C.

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 98 °C for 5 min, whereas IBs were incubated for 40 min. For western blot analyses, protein samples were electrotransferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, England) using 0.2 M glycine, 25 mM Tris, and 20 % methanol (*v*/*v*) 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 1/2,000 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 4×3 (4 strains×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
α_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×*g* and 4 °C for 10 min. Pellets were resuspended in 1 ml of 0.1 % formaldehyde in PBS. Images were examined at ×100 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 4 °C 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, Tokyo, Japan).

Cell proliferation assay

Human skin fibroblast cells (1BR3.G) were grown at 37 °C 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 4 °C overnight. After incubation, the medium was replaced, and nonattached 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 °C for 24, 48, and 72 h, and the MTT proliferation cell assay was performed using the Non-Radioactive 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 (PerkinElmer) 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 Minimum Essential Medium (MEM-α) medium supplemented with 10 % FBS and 2 mM Glutamax (Gibco, Rockville MD). After incubation at 37 °C 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 48 h, 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 pTrc99a-VP1GFP plasmid were grown aerobically in LB medium containing 100 μg/ml ampicillin at 37, 25, and 16 °C. MC4100 was included as a reference as all previous studies on the biomaterial properties of IBs performed by our group used IBs obtained in this strain or in isogenic derivatives. As shown in Fig. 1a, the optical densities at 550 nm (OD_{550}) of all cultures following induction of recombinant gene expression for 3 h 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. To confirm this issue, growth of KPM335 under protein production conditions at 37 °C was fully monitored (Fig. 1b), confirming the longer duplication time of KPM335 cells.

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 37 °C (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 16 °C. 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 37 °C (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 the insoluble protein is organized as inclusion bodies (IBs). IBs, as submicron protein particles, are gaining relevance in

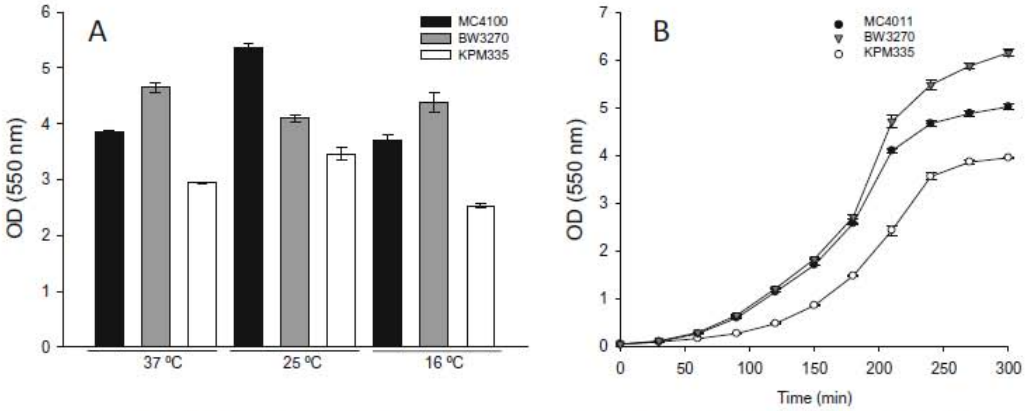

Fig. 1 Optical densities at 550 nm (OD_{550}) of the cell suspensions of *E. coli* strains MC4100/pTrc99a-VP1GFP, BW30270/pTrc99a-VP1GFP, and KPM335/pTrc99a-VP1GFP 3 h postinduction of *VP1GFP* gene expression at different temperatures (37, 25, and 16 °C) (**a**). Continuous growth monitoring of MC4100/pTrc99a-VP1GFP, BW30270/pTrc99a-VP1GFP, and KPM335/pTrc99a-VP1GFP cultures at 37 °C (**b**). IPTG was added to each culture when reaching OD_{550}=0.5

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 (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 (Fig. 4a and b) 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

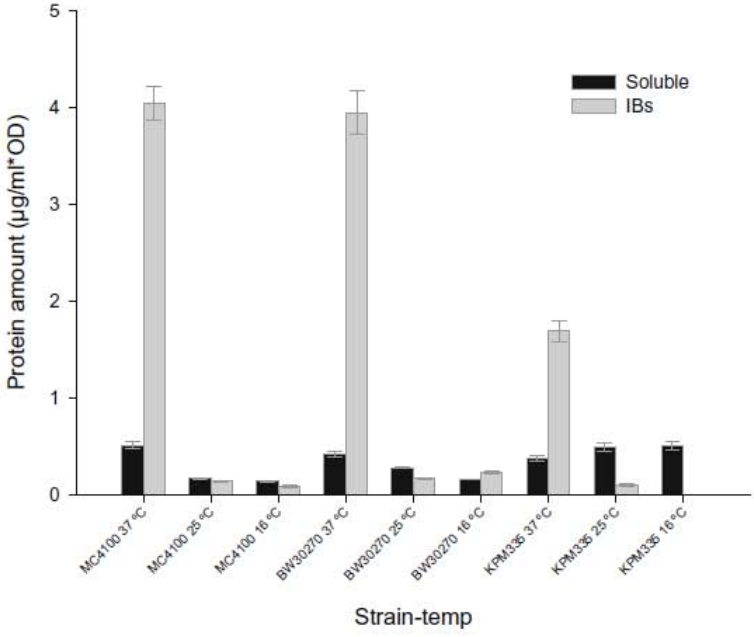

Fig. 2 Amount of soluble and insoluble VP1GFP produced in *E. coli* strains MC4100/pTrc99a-VP1GFP, BW30270/pTrc99a-VP1GFP, and KPM335/pTrc99a-VP1GFP 3 h postinduction of *VP1GFP* gene expression at different temperatures

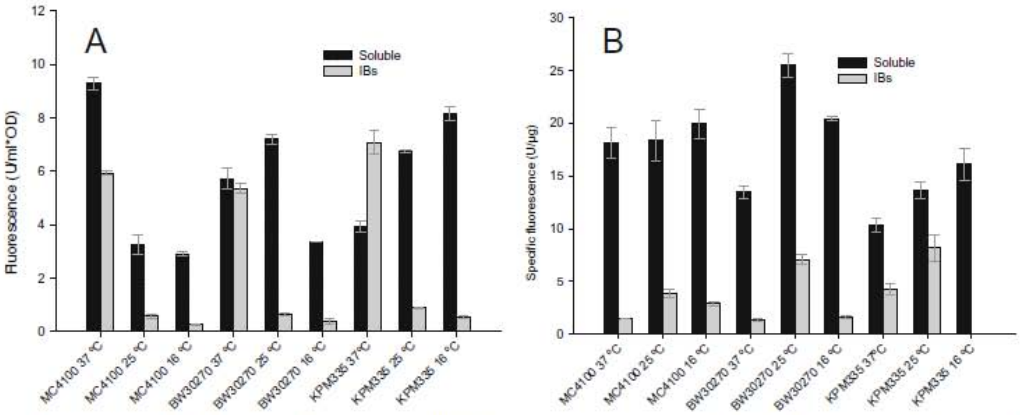

Fig. 3 Green fluorescence of soluble and insoluble fractions in VP1GFP-producing *E. coli strains* MC4100, BW30270, and KPM335 (**a**) and specific fluorescence of soluble and insoluble VP1GFP produced in MC4100, BW30270, and KPM335 (**b**) at different temperatures

from each other (Fig. 5) and similar to IBs isolated from other *E. coli* strains (Garcia-Fruitos et al. 2010).

The endotoxic activity of IBs produced in *E. coli* strains MC4100, BW30270, and KPM335 was investigated using the

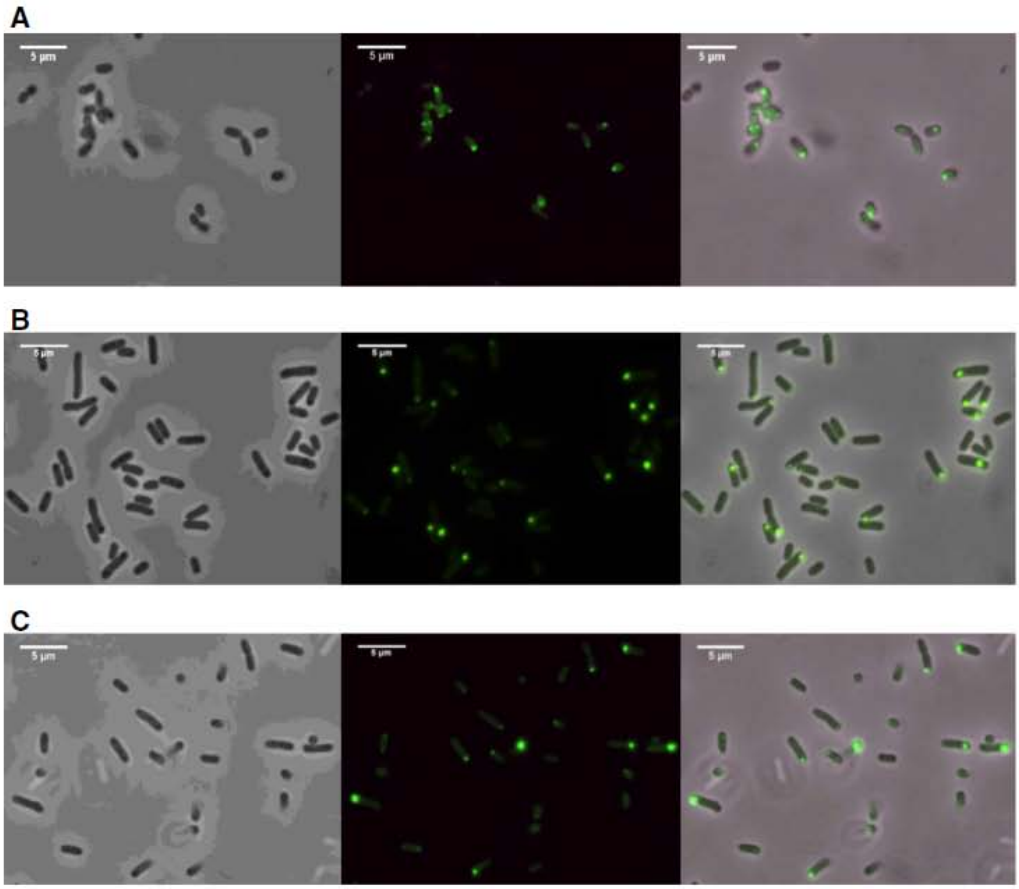

Fig. 4 Light microscopy images of VP1GFP-producing *E. coli* strains MC4100 (**a**), BW30270 (**b**), and KPM335 (**c**) 3 h postinduction. *Left* to *right*: phase contrast microscopy at 100 X magnification; fluorescence microscopy at ×100 magnification; merged images using Image J software

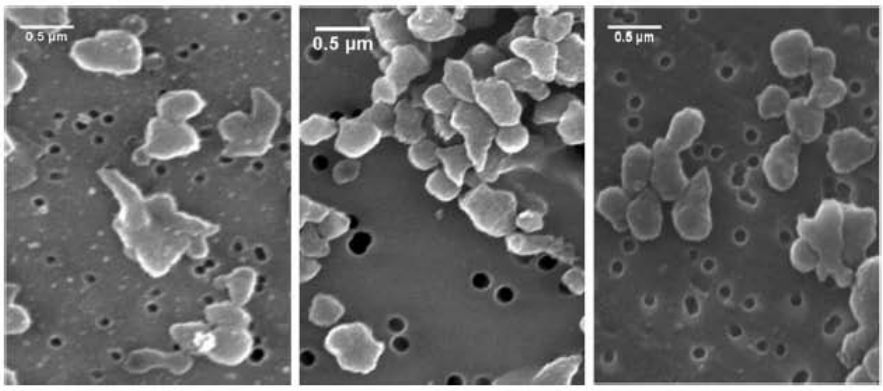

Fig. 5 Scanning electron micrographs of VP1GFP IBs isolated from *E. coli* strains MC4100 (*left*), BW30270 (*middle*), and KPM335 (*right*)

HEK-Blue™ hTLR4 LPS detection assay. As shown in Fig. 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. 2014; 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 et al. 2013b). The comparative analysis of cell proliferation on IB-decorated surfaces revealed similar properties of all tested IBs (Fig. 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),

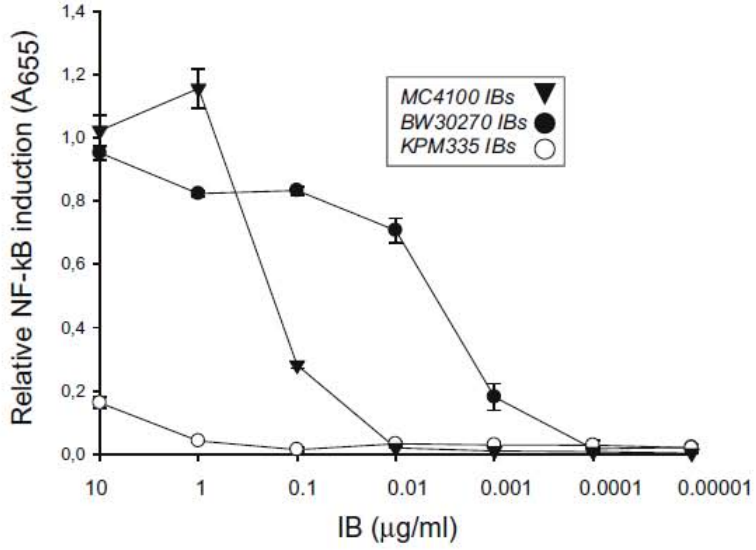

Fig. 6 Stimulation of the hTLR4 signaling pathway by IBs produced in *E. coli* strains MC4100, BW30270, and KPM335. Relative NF-κB induction was measured following stimulation of HEK-Blue hTLR4 cells with tenfold serial dilutions of IBs

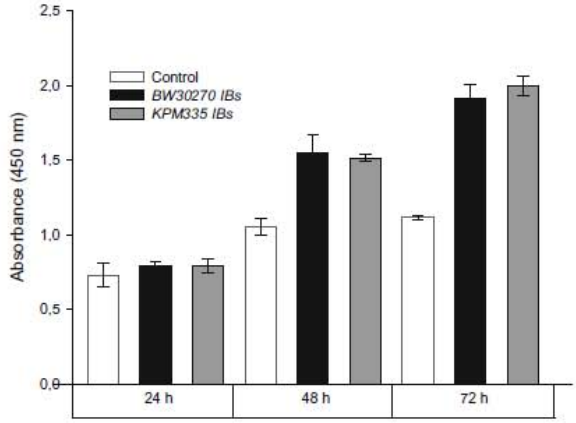

Fig. 7 Proliferation of 1BR3.G cells on matrices formed by IBs from *E. coli* strains MC4100, BW30270, and KPM335 at 37 °C monitored at several times after seeding

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 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 et al. 2014; 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-

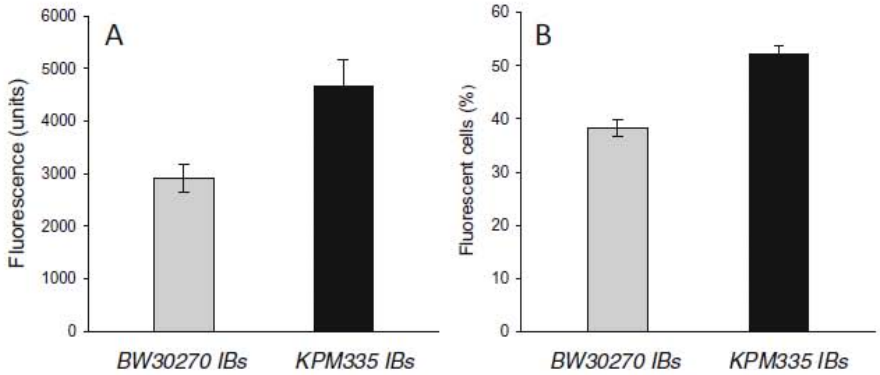

Fig. 8 Internalization of IBs by HeLa cells. The HeLa cells were incubated with 5 μg of VP1GFP IBs from *E. coli* strains BW30270 and KPM335. Internalization of IBs was monitored by measuring the intensity of fluorescence inside the cells (**a**) and the fraction of fluorescent cells (**b**)

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. 2014) 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 nonbacterial 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 et al. 2013a; Seras-Franzoso et al. 2013b), 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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Annex VII

Bacterial inclusion body purification.

Seras-Franzoso J, Peternel S, Cano-Garrido O, Villaverde A, García-Fruitós E.

Methods in Molecular Biology 2015. 1258:293-305.

Chapter 16

Bacterial Inclusion Body Purification

Joaquin Seras-Franzoso, Spela Peternel, Olivia Cano-Garrido, Antonio Villaverde, and Elena García-Fruitós

Abstract

Purification of bacterial inclusion bodies (IBs) is gaining importance due to the raising of novel applications for this type of submicron particulate protein clusters, with potential uses in the biomedical field among others. Here, we present two optimized methods to purify IBs adapting classical procedures to the material nature as well as the requirements of its final application.

Key words Bacterial inclusion body, Purification, Nanoparticles, Bacterial cell free, Cell disruption

1 Introduction

Bacterial IBs have been regarded for many years as inert waste by-products of the recombinant protein production process and therefore either straightforward discarded or isolated in order to resolubilize and refold the aggregated protein. However, recent studies have shown high levels of molecular organization as well as significant extents of biologically active polypeptides within these protein nanoparticles [1, 2] (Fig. 1). This change in the perception of IB structure prompted the appearance of new applications, becoming bacterial IBs a final product itself with potential uses in industry, biomedicine, or diagnostics [3]. In this regard, bacterial IBs have shown their ability to act as naturally immobilized biocatalysts [4–7] or stimulate mammalian cell response in terms of adhesion, proliferation, or differentiation when these protein nanoparticles are used as topographical modifiers of cell culture interfaces [8–10].

So far, IB purification protocols consisted basically of mechanical, chemical, or enzymatic cell disruption methods followed by series of washing steps exhibiting high recovery yields (up to 95 %). However, disregarding removing other impurities such as viable bacteria or bacterial debris, that typically co-sediment with

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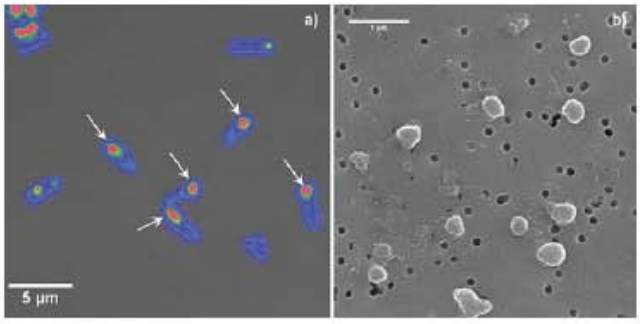

Fig. 1 Inclusion bodies. (**a**) Confocal image of *E. coli* cells producing mGFP IBs, pointed by *white arrows*. (**b**) Scanning Electron Microscopy micrograph of purified mGFP

insoluble bacterial products, make the use of IBs in biological interfaces incompatible [11]. Here, we present two general protocols focused on obtaining cell-free IBs ready to be used in mammalian cell cultures or as biocatalysts. One of the described procedures is a general protocol aiming to disrupt bacterial cells forming IBs without compromising the protein conformational quality of the nanoparticle, by means of an enzymatic attack directly performed in the bacterial cell culture, to increase the efficiency of the reaction. This treatment, in turn, is followed by series of freeze/thaw cycles. The combination of both enzymatic digestion and soft mechanical disruption allows also to completely remove viable bacteria from the samples, being the presence of bacterial contaminants monitored along the process by seeding small sample volumes on LB plates. This monitoring is crucial to tailor the number of freeze/thaw cycles required in each case to obtain bacterial cell-free biomaterial. Besides, extensive washing steps with mild detergents and further enzymatic treatments are performed to improve the purity of IBs. As a result of the whole procedure, active, bacterial cell-free submicron protein particles are obtained.

On the other hand, a second procedure aiming to specifically recover IBs composed from proteins that are prepared at low cultivating temperatures is detailed. Considering that these IBs are extremely soluble in mild detergents [2, 12–14], chemical cell disruption is not appropriate for the isolation of such nanoparticles. Nonetheless, mechanical disruption alone can also compromise the structure or the quality of protein trapped inside these IBs. Thus, the process for isolation of whole IBs with functional proteins produced at low cultivating temperatures, which merges together two mechanical processes, was designed. Firstly bacterial cells are exposed to several freeze/thaw cycles where soft mechanical forces facilitate bacterial cell walls to crack. This is then followed by homogenization, where high pressure enables total cell disruption. Extensive washing in buffers that does not compromise

IB structure facilitate IB purification. During this IB isolation process, viable bacteria are removed from the sample, and IBs can be used as whole nanoparticles as well as for further downstream protein isolation [2, 13].

2 Materials

Prepare all solutions and buffers using ultrapure H_2O at RT and store the stocks at the indicated temperature. Magnetic stirrers and beakers are required. All buffers are filtrated (0.22 µm) before storage.

2.1 IB Purification Protocol

1. EDTA-free Protease Inhibitor Cocktail Tablet.
2. 100 mM PMSF: Dissolve 0.0174 g phenylmethylsulfonyl in 1 mL 2-Propanol. Leave in 500 µL aliquots in Screw-Cap microcentrifuge tubes and store them at −20 °C (*see* **Note 1**).
3. 0.5 mg/mL lysozyme: Dissolve 0.5 mg lysozyme in 1 mL water. Store aliquots at −20 °C (*see* **Note 2**).
4. Triton X-100.
5. Nonidet P-40.
6. 1 M $MgSO_4$: Dissolve 123.24 g $MgSO_4$ in 100 mL water. Autoclave the solution and store at room temperature (RT).
7. 1 mg/mL DNAse I: Dissolve 1 mg in 1 mL water. Store aliquots at −20 °C.
8. Lysis buffer + 0.5 % Triton X-100: 20 mM Tris–HCl, 320 mM NaCl, 2 mM $MgCl_2$, 5 mM dithiothreitol (DTT), EDTA-free Protease Inhibitor Cocktail tablet, pH 7.5. Dissolve 0.1 g $MgCl_2$, 0.385 g DTT in 25 mL 1 M Tris–HCl, pH 8, and 6.25 mL 4 M NaCl. Add an EDTA-free Protease Inhibitor Cocktail Tablet dissolved in 50 mL water. Adjust volume until 500 mL and pH at 7.5. Add 0.5 mL Triton X-100. Finally, filter the solution and store it at RT.
9. PBS buffer 1×: Prepared from PBS buffer 10×: 25 mM $Na_2HPO_4{\cdot}2H_2O$, 1.5 M NaCl, 75 mM $NaH_2PO_4{\cdot}H_2O$. Dissolve 13.35 g $Na_2HPO_4{\cdot}2H_2O$, 81.18 g NaCl, and 3.45 g $NaH_2PO_4{\cdot}H_2O$ in 1 L water. Adjust at pH 7.4. Store at RT.
10. LB plates: Dissolve 10 g NaCl, 10 g tryptone, and 5 g yeast extract in 1 L water. Add 15 g agar at the bottle and autoclave it. Plate the solution. Store at 4 °C.

2.2 Purification Protocol of IBs Produced at Low Temperatures

1. PBS buffer 1×: Prepared from PBS buffer 10×: 25 mM $Na_2HPO_4{\cdot}2H_2O$, 1.5 M NaCl, 75 mM $NaH_2PO_4{\cdot}H_2O$. Adjust at pH 7.4. Store at RT.
2. Buffer B50/30; 50 mM Tris–HCl buffer with 30 mM NaCl, prepared from 1 M Tris–HCl, pH 8.0. Adjust pH at RT with

the addition of concentrated (32 %) HCl. Add water to 1 L. For 50 mM Tris–HCl buffer with 30 mM NaCl, dissolve 50 mL of 1 M Tris–HCl, pH 8.0, and 1.75 g NaCl in 1 L water. Store at 4 °C.

3. LB plates: Dissolve 10 g NaCl, 10 g tryptone, and 5 g yeast extract in 1 L water. Add 15 g agar at the bottle and autoclave it. Plate the solution. Store at 4 °C.

2.3 IBs Quantification by Western Blot

1. Solution B: Dissolve 0.4 g sodium dodecyl sulfate (SDS) and 18.2 g Tris base in 100 mL water. Adjust at pH 8.8. Store at 4 °C.
2. 10 % ammonium persulfate: Dissolve 1 g ammonium sulfate in 10 mL water. Store 1 mL aliquots at −20 °C.
3. Solution C: Dissolve 0.4 g sodium dodecyl sulfate (SDS) and 6 g Tris base in 100 mL water. Adjust at pH 6.8. Store at 4 °C.
4. Denaturing buffer (Laemmli 4×): 1.28 g of Tris base, 8 mL of glycerol, 1.6 g of sodium dodecyl sulfate (SDS), 4 mL of β-mercaptoethanol, and 9.6 g of urea. Store at RT.
5. Electrophoresis buffer SDS-free (10×): Dissolve 144 g glycine and 30.3 g Tris-HCl in 1 L water. Store at RT.
6. 10 % SDS: Dissolve 50 g in 500 mL water. Store at RT.
7. Electrophoresis buffer: Dilute 100 mL electrophoresis buffer SDS-free (10×) and 10 mL 10 % SDS in water up to 1 L. Store at 4 °C (*see* **Note 3**).
8. Transference buffer: Dilute 100 mL electrophoresis buffer SDS-free (10×) and 200 mL methanol in water up to 1 L. Store at −20 °C (*see* **Note 4**).
9. Blocking solution: Dissolve 2 g skimmed milk in 40 mL PBS 1×.
10. 40 % Acrylamide/Bis Solution, 37. 5:1.

3 Methods

3.1 General IB Purification Protocol (Fig. 2)

Carry out all procedures in a laminar flow hood;

1. Add into the bacterial cell culture EDTA-free Protease Inhibitor Cocktail (1 tablet/500 mL culture) (*see* **Note 5**), PMSF (0.4 mM), and lysozyme (1 μg/mL) (*see* **Note 2**). Incubate 2 h at 250 rpm and 37 °C. That step can be performed in the same shake flask in which the bacterial cell culture has been performed.
2. Fill a beaker with the bacterial cell culture. Freeze at −80 °C O/N (*see* **Note 6**).
3. Thaw the culture at RT (*see* **Note 7**). Add Triton X-100 (0.4 mL/100 mL sample). Incubate 1 h under gentle agitation at RT (*see* **Notes 8** and **9**).

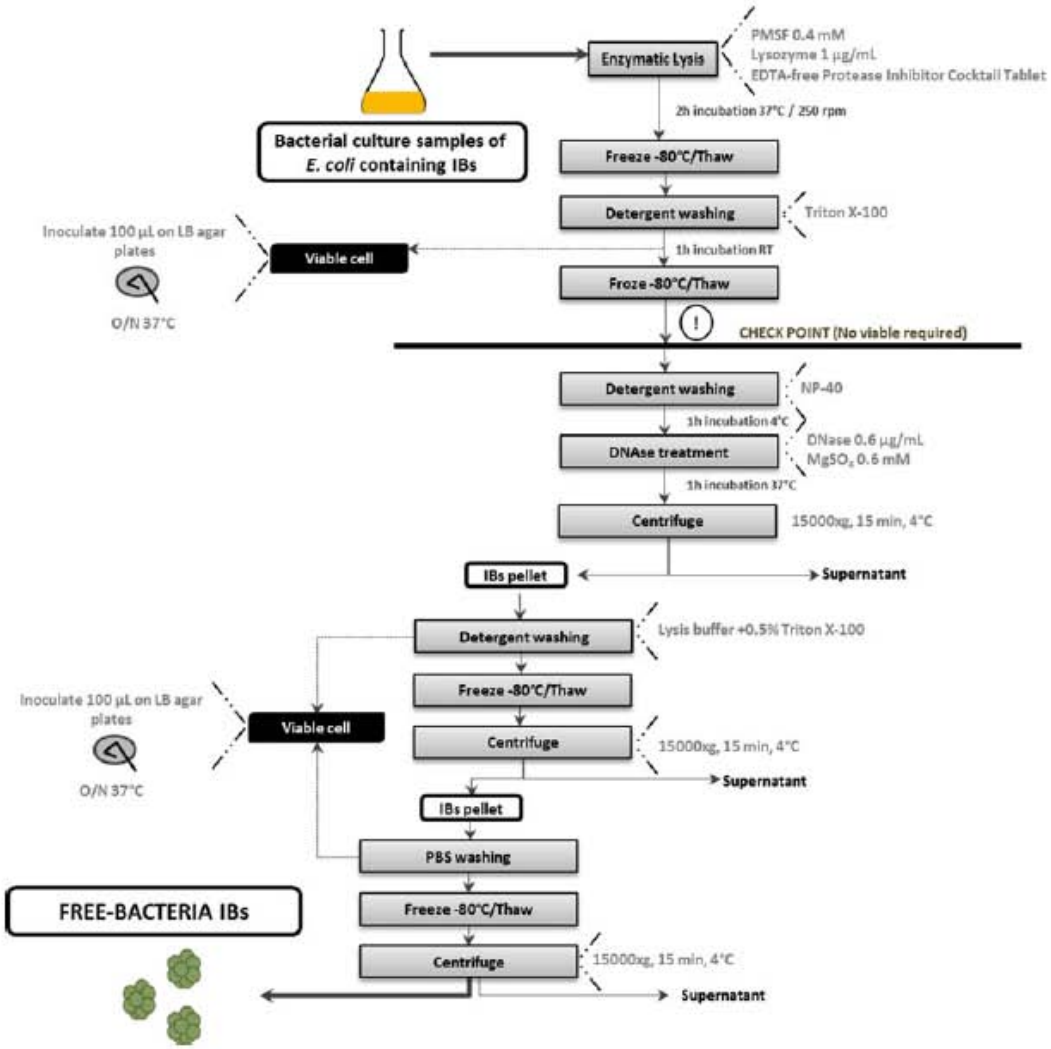

Fig. 2 IB purification protocol: enzymatic lysis, detergent washing treatment, and repeated freeze and thaw rounds

4. Test bacterial contamination by inoculating 100 µL on LB plates. Incubate the plates O/N at 37 °C. Freeze the IB sample at −80 °C O/N.
5. Thaw the IB sample at RT (*see* **Note 7**). Check the contamination level by counting bacterial colonies on the LB plates. Repeat the freeze/thaw process until obtaining no viable on LB plate.
6. Once obtained a free viable bacteria IB sample, add Nonidet P-40 (25 µL/100 mL sample) and incubate 1 h under gentle agitation at 4 °C (*see* **Notes 8** and **9**).
7. Add $MgSO_4$ (60 µL/100 mL sample) and DNAse I (60 µL/100 mL sample). Incubate 1 h under gentle agitation at 37 °C.

8. Harvest the IBs at 15,000 × *g* for 15 min at 4 °C. Remove the supernatant and resuspend the pellet with lysis buffer + Triton X-100 (5 mL/100 mL initial sample).
9. Inoculate 100 µL on LB plates and leave O/N at 37 °C. Freeze the sample at −80 °C O/N.
10. Thaw the sample at RT (*see* **Note 7**). Harvest at 15,000 × *g* for 15 min at 4 °C. Remove the supernatant and resuspend the pellet with PBS buffer (5 mL/100 mL initial sample).
11. Inoculate 100 µL on LB plates and leave O/N at 37 °C. Freeze the sample at −80 °C O/N.
12. Thaw the sample at RT. Harvest at 15,000 × *g* for 15 min at 4 °C. Remove the supernatant and do aliquots. Store the aliquots at −80 °C.
13. In order to consider the pellets free of bacterial cells, plates from **steps 9** and **11** must be without any colony. Moreover, if IBs pellets will be used in mammalian cell cultures, bacterial contamination must be further tested in mammalian cell medium (**step 14**).
14. Resuspend an IB pellet (**step 12**) in 1 mL of mammalian cell medium. Add 200 µL of this solution, as well as its dilutions 1:10, 1:100, and 1:1,000, to a 96-well plate in triplicate. Leave at least 2–3 days at the mammalian cell culture conditions. If there is no contamination, the IB stock can be validated as sterile.

3.2 Purification Protocol of IBs Produced at Low Temperatures (Fig. 3)

The process can be performed in the single centrifuge tube (*see* **Note 10**):

1. After the protein production, harvest the bacterial cells at 5,000 × *g* for 5 min at 4 °C. Remove the supernatant (medium) and wash the pellet twice in PBS or B50/30 buffer (*see* **Note 11**). Discharge supernatant. The pellet can be stored at −80 °C (*see* **Note 12**).
2. Resuspend the bacterial pellet in the selected buffer (PBS or B50/30); the volume of the suspension can be from ¼ to ½ of the original culture volume. Freeze the suspension for 2 h at −80 °C (*see* **Note 13**).
3. Thaw the culture on ice. This freeze/thaw cycle should be repeated at least three times.
4. Harvest the bacterial cells at 5,000 × *g* for 5 min at 4 °C. Remove the supernatant.
5. Resuspend the cells in the selected buffer (PBS or B50/30) and keep the suspension on ice (*see* **Note 10**).
6. Bacterial cells are disrupted in high-pressure homogenizer (e.g., EmulsiFlex-C5, Avestin) at operating pressure 75–100 MPa (*see* **Note 14**).

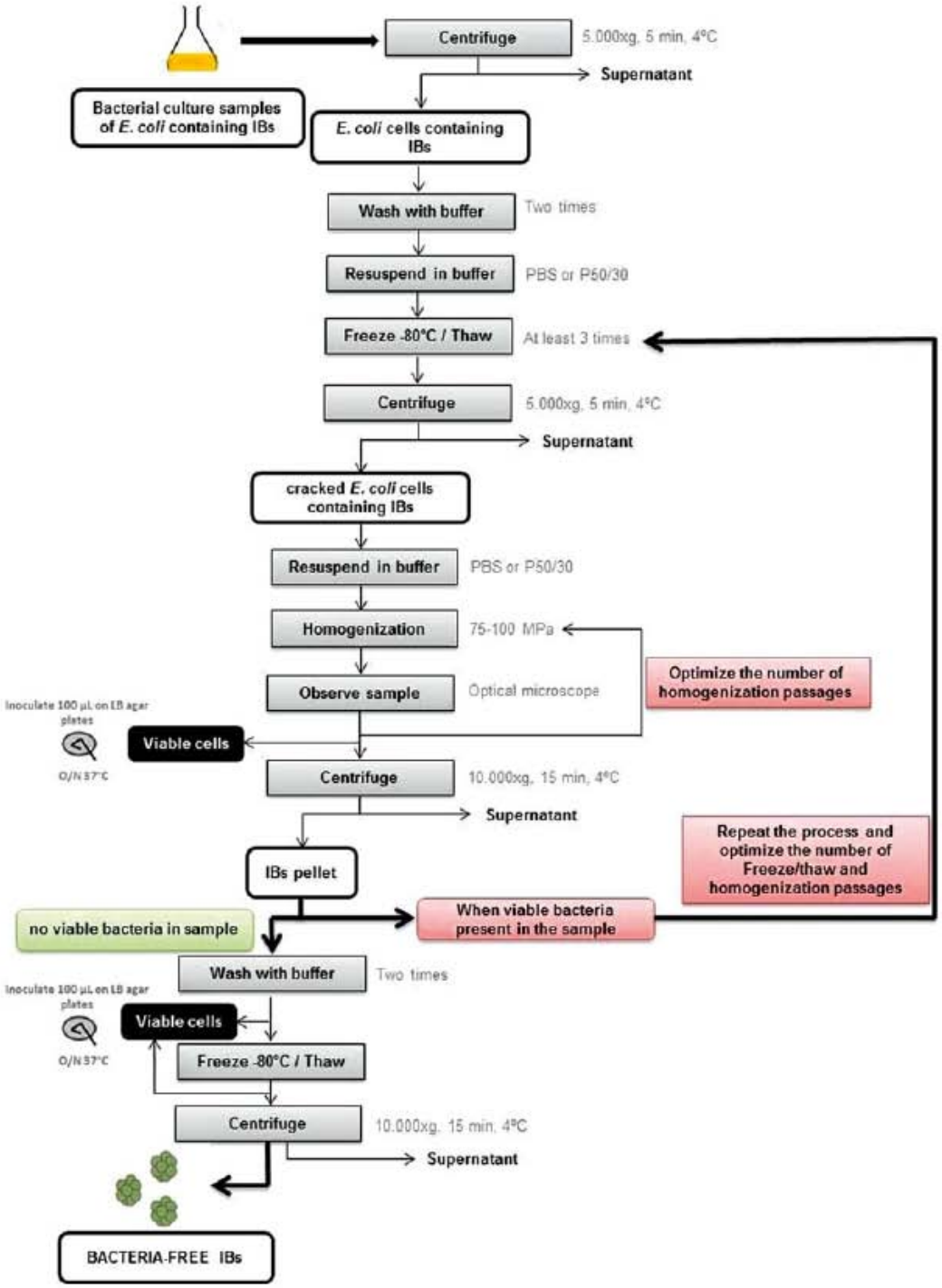

Fig. 3 IB purification protocol at low temperatures: homogenization step and freeze and thaw steps

7. Observe the sample under the optical microscope to assess the effectiveness of cell disruption. Depending on the host strain and the nature of the produced protein, together with the efficiency of previous freeze/thaw cycles, the number of passages has to be optimized (*see* **Note 15**).
8. Test bacterial contamination by inoculating 100 µL on LB plates. Incubate the plates O/N at 37 °C.
9. Harvest IBs at 10,000×*g* for 5 min at 4 °C. Discharge supernatant.
10. Freeze the IB sample at −80 °C O/N.
11. Check the contamination level by counting bacterial colonies on the LB plates. Repeat the freeze/thaw process in combination of homogenization until no viable on LB plate is obtained .
12. Once obtained a viable bacteria-free IB sample, wash the IB pellet twice with the selected buffer. Thoroughly resuspend the IB pellet in the buffer with gentle agitation at RT (10 min) for better washing efficacy.
13. Test bacterial contamination by inoculating 100 µL on LB plates. Incubate the plates O/N at 37 °C.
14. Harvest at 10,000×*g* for 15 min at 4 °C. Remove the supernatant and resuspend the pellet with PBS or P50/30 buffer (5 mL/100 mL initial sample).
15. Aliquotate the pellet. Harvest the IBs at 10,000×*g* for 15 min at 4 °C. Remove the supernatant.
16. Store the aliquots at −80 °C.
17. In order to consider the pellets free of bacterial cells, plates from **steps 11** and **13** must be without any colony. Moreover, if IB pellets will be used in mammalian cell cultures, bacterial contamination must be further tested in mammalian cell medium.
18. Resuspend an IB pellet (**step 12**) in 1 mL of mammalian cell medium. Add 200 µL of this solution, as well as its dilutions 1:10, 1:100, and 1:1,000, to a 96-well plate in triplicate (Fig. 4). Leave at least 2–3 days at the mammalian cell culture conditions. If there is no contamination, the IB stock can be validated as sterile.

3.3 IB Quantification by Western Blot

3.3.1 Acrylamide Gels Preparation (1 Gel)

1. First of all prepare the running gel (10 % acrylamide) (*see* **Note 16**). Mix 4.93 mL MQ H_2O, 2.5 mL solution B, 2.5 mL acrylamide, 60 µL ammonium persulfate (APS) 10 %, and 8 µL TEMED. Immediately, pour 7 mL solution between both glass plates. To make the top of the separating gel be horizontal, fill in 2-Propanol into the gap until the side.
2. Once gelated, remove the 2-Propanol by decantation. Dry remnants of 2-Propanol with filter paper.

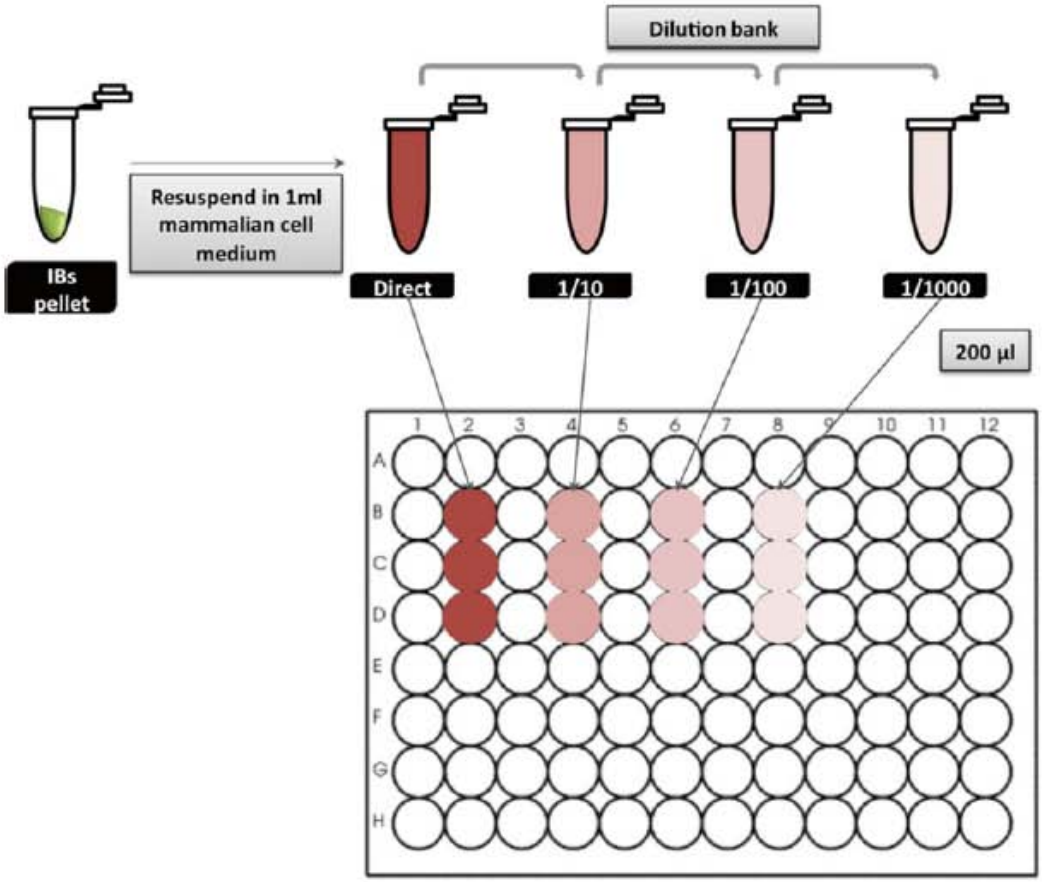

Fig. 4 IB sterility protocol performed in mammalian cell medium

3. Prepare the stacking gel (3.5 % acrylamide). Mix 4.124 mL MQ H_2O, 1.575 mL solution C, 0.7 mL acrylamide, 70 µL 10 % ammonium persulfate (APS), and 7 µL TEMED. Immediately add the stacking gel solution until the side and insert the well-forming comb.

3.3.2 Sample Preparation and Blotting

1. Resuspend IBs in denaturing buffer (Laemmli 4×) at appropriate ratios to obtain a 1× denaturing buffer concentration and boil the samples for 45 min (*see* **Note 17**). Concurrently, prepare a calibration curve using diluted series of protein samples of known concentration (*see* **Note 18**).
2. Place the acrylamide gels in the tank and fill in the cold phoresis buffer (*see* **Note 3**).
3. Take out the comb and load the sample onto, the volume of sample to load will depend on the well size. Load also a protein marker.

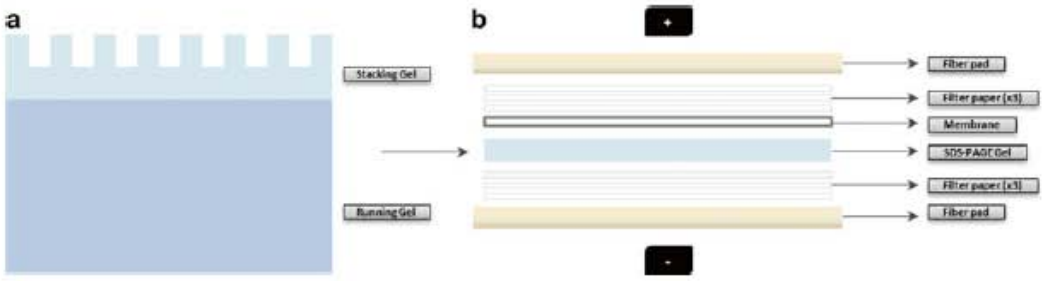

Fig. 5 SDS-PAGE analysis. (a) Acrylamide gel sketch: stacking gel at the top (*light blue*) and running gel at the bottom (*dark blue*). (b) Western blot transference preparation of the cassette (Color figure online)

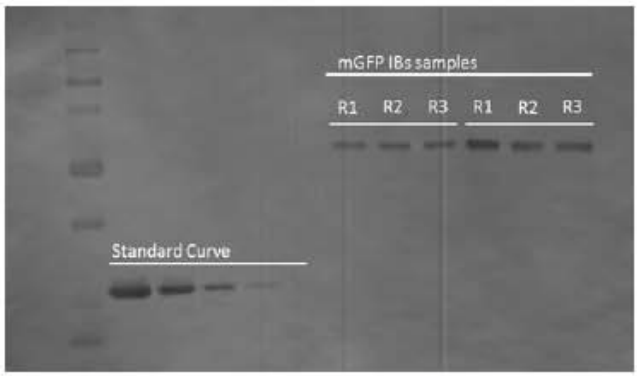

Fig. 6 Western Blot Quantification of mGFP IBs. Soluble GFP was used as standard curve. Lanes from 1 to 5 correspond to 500, 250, 125, 75, and 37.5 ng of protein, respectively. Bands from lanes 6 to 9 correspond to 10 μL of IB sample in triplicate. Bands from 10 to 12 correspond to 20 μL of IB sample in triplicate

4. Set an appropriate voltage and run the electrophoresis (*see* **Note 19**).
5. Once the electrophoresis is finished, prepare the western blot transference. Cut the nitrocellulose membrane and filter papers (six per gel). Equilibrate gels and membranes in transfer buffer.
6. Place the membrane and the gel in the cassette forming a sandwich as it is represented in the image (Fig. 5).
7. Place the cassette in the transfer cell filled with transfer buffer and the cooler.
8. Run the transference for 1 h at 100 V.

3.3.3 Immunodetection (Fig. 6)

1. Incubate the membrane O/N in blocking solution.
2. Dilute the primary antibody in the blocking solution at the suitable concentration. Incubate the membrane for 2 h at RT.
3. Wash the membrane with PBS 1× and twice with PBS-Tween for 15 min.

4. Dilute the secondary antibody in PBS 1× at the suitable concentration. Incubate the membrane for 1 h at RT.
5. Wash the membrane with PBS 1× and twice with PBS-Tween for 15 min.
6. Finally perform the detection with chosen method (colorimetry, chemiluminescence, etc.).

4 Notes

1. PMSF aliquots stored at −20 °C tend to precipitate, but once at RT PMSF becomes soluble again. Make sure that PMSF is completely solubilized.
2. A high lysozyme concentration not always helps to improve *E. coli* lysis, since at high concentrations this enzyme tends to aggregate and remain a contaminant present at the final IB purification step.
3. It should be prepared before the sample preparation and stored at 4 °C to be cold enough during the protein electrophoresis.
4. It should be prepared before running the SDS-PAGE and stored at −20 °C to be cold enough during the protein transference.
5. Add the suitable volume of EDTA-free Protease Inhibitor Cocktail Tablet suspended in sterile H_2O.
6. Make sure the beaker can be frozen at −80 °C.
7. So as to thaw faster the sample, it can be left in warm water.
8. Gentle agitation can be performed with a magnetic stirrer.
9. Warm up at 37 °C Triton X-100 and NP40 detergents prior to use in order to reduce their viscosity and facilitate their pipetting.
10. Keep the samples on cold (ice or a suitable substitute) through the isolation process.
11. The buffer should be chosen regarding the stability of the protein inside IBs.
12. Make sure the centrifugation tube can be frozen at −80 °C.
13. When proteins inside IBs are sensitive to proteolysis, the EDTA-free Protease Inhibitor Cocktail Tablet (1 tablet/500 mL) can be added to the buffer.
14. During homogenization the sample chamber as well as the sample-collecting tube should be kept on cold (ice or a suitable substitute) through the isolation process when the homogenizer is not cooled or located in the cold room.

15. The number of freeze/thaw cycles and number of homogenization passages has to be optimized for every single case, while the level of stress for the host organism varies depending on the protein produced, on expression vectors used, as well as on the different genetic backgrounds of different *E. coli* strains. Therefore, even with the same bacterial strain and very similar proteins produced in similar vectors, the number of homogenization passages needed to totally disrupt bacterial cell was found to be different [13].
16. Depending on the protein weight, a different acrylamide percentage should be used.
17. Aggregated protein need longer boiling times than soluble protein.
18. A quantified protein which can be detected with the same antibodies as the sample is necessary. A soluble protein can be used as calibration curve.
19. Generally 60 V for the stacking gel and then change to 100 V for the running gel.

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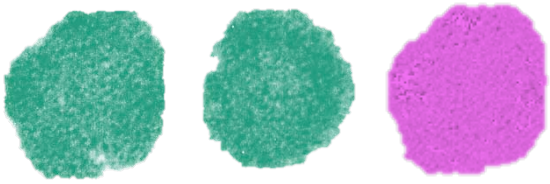

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Departament de Genètica i de Microbiologia

Facultat de Biociències

Universitat Autònoma de Barcelona

Institut de Biotecnologia i de Biomedicina

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Olivia Cano Garrido

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